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# SYMPOSIUM ON GEL FILTRATION

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## INTRODUCTION

Gel filtration, or better gel chromatography, has already become an instrument of great importance, although introduced into laboratory practice only a few years ago, because it has broadened what could be defined as the spectrum of the applications of chromatography.

Gel chromatography is based on the principle of the separation of substances according to their molecular weight, which is quite different from the principles of absorption, partition or ion exchange. Because of this it can be used not only to separate molecules from macromolecules, but especially for the separation of macromolecules of different weights, thus opening up a very interesting field in the study of proteins, enzymes and macromolecular interactions.

These problems are of great importance in the fields of biology, biochemistry and medicine. Because of the interest that these methods have in the basic and applied sciences in the field of public health, the Istituto Superiore di Sanità considered it as opportune to promote this meeting, so that, through the demonstration of new techniques and a discussion of original work, an ever increasing group of scientists could become acquainted with the application of these methods.

The solution of many practical problems and the approach to new research will undoubtedly be rendered easier in the future through the various applications of these methods.

I would like to express my deep gratitude to Prof. TH. WIELAND and to Dr. JOHANSON, Dr. BENNICH and Dr. FISCHER, who have so kindly accepted our invitation to come here and talk about their work and discuss it with us. I would also like to thank Dr. MICHAEL LEDERER for his collaboration in the organization of this meeting.

G. B. MARINI-BETTÒLO

# SOME RECENT DEVELOPMENTS IN GEL CHROMATOGRAPHY, WITH SPECIAL REFERENCE TO THIN LAYERS

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The term "gel chromatography" is not yet very familiar. Originally one spoke of "gel filtration" without indicating that the method is in fact a chromatographic technique and that its efficiency rests upon the principle of chromatography. The principle of all chromatographic separations, on a column consisting of a two-phase system, is the difference in the accessibility to substances of the stationary phase. Substances of lower affinity will travel faster than those implicated to a greater degree. To the classical principles of adsorption and of partition, the penetrability of a gel has now been added. Smaller molecules, which more readily find access to this phase, will move more slowly than larger molecules.

Nowadays many different gels are in use. There are gels with hydrophilic properties, Sephadex probably being the best known. Sephadex is a dextran of bacterial origin cross-linked by 1,3-glyceryl ether bridges. The different types from G-10 to G-200 differ in their degree of cross-linking. The greater the cross-linking, the smaller is, of course, the average diameter of the mesh or pores and, simultaneously, the capacity for swelling and their accessibility for higher molecular weight substances decreases. The same is true for another hydrophilic preparation, a cross-linked polyacrylamide, originally prepared by HJERTÉN[1] and manufactured in the U.S.A. (Biogel). Gels of agarose, a polysaccharide made up of alternating L- and D-galactose residues, are cross-linked only by hydrogen bridges and are therefore suitable for the separation of extremely high molecular weight particles[2]. Of the lipophilic gels, only Styragel is commercially available. It consists of a copolymerizate of styrene and divinylbenzene and is used in a chromatographic device for determining the molecular weight distribution of synthetic polymers (Waters Assoc.). In our laboratory beads of a copolymer of methacrylate and glycol bis(methacrylate) have been studied since 1962; this will be discussed later.

Finally Sephadex LH (lipophilic–hydrophilic) is mentioned. It has both properties and can be used in aqueous and in organic solvent systems. In Fig. 1, a separation in alcohol of polyethyleneglycols $(—O—CH_2—CH_2—)_n$ of different molecular sizes is shown.

This paper is a report on some of the applications of the Sephadex gels mainly regarding our special field of research, amino acids, peptides and proteins. Gel chromatographic separation of low molecular weight peptides can be carried out in water on the strongly cross-linked Sephadex G-10. Fig. 2 shows the different elution volumes with water ($V_e$) of a di-, tri-, and tetra-peptide registered by an automatic ultraviolet recorder.

By plotting $V_e/V_0$ (reduced elution volumes) against log $M$ (molecular weight),

a straight line is obtained. The same holds for $\sqrt[3]{K_d}$ *versus* $\sqrt[2]{M}$. ($K_d$ is defined in very much the same way as the distribution coefficient in partition chromatography; $K_d$ is directly proportional to $V_e/V_0$.) Both of these linear correlations are also valid for molecular weights of proteins and their rates of movement. A compilation was obtained by DETERMANN AND MICHEL[3], of the elution volumes of 26 proteins, from data from several laboratories. Plots of the reduced elution volumes against log $M$ resulted in straight lines with different slopes for all types of Sephadex investigated.

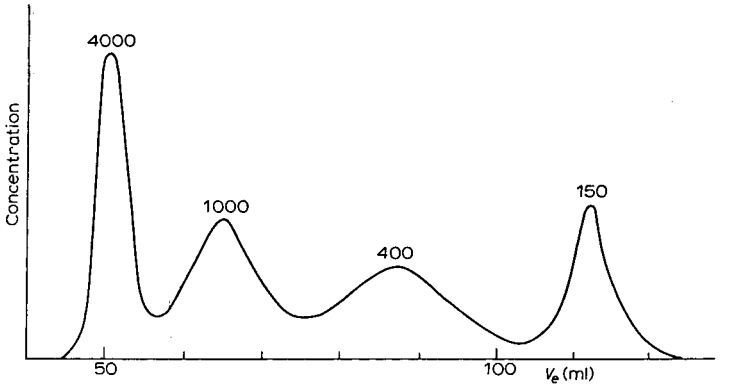


Fig. 1. Elution volumes of polyethyleneglycols of different sizes from Sephadex LH with ethyl alcohol.

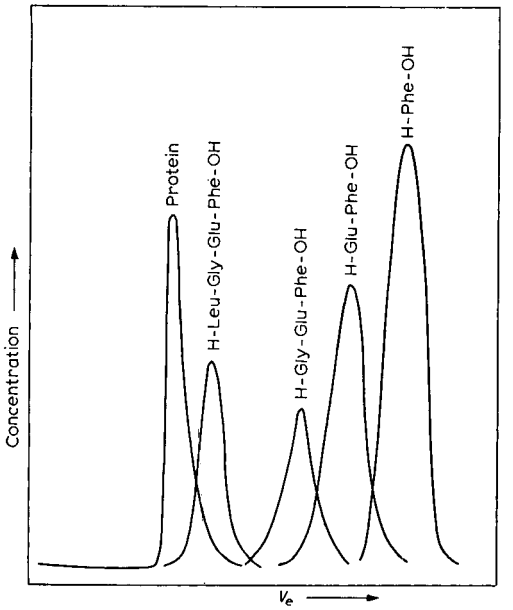


Fig. 2. Chromatographic behaviour of an amino acid, three peptides and a protein on Sephadex G-10 in water.

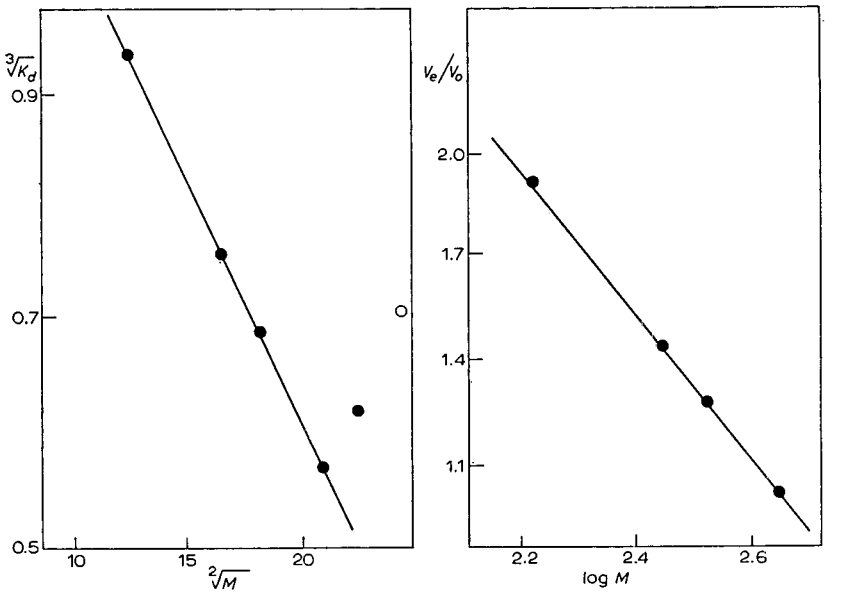


Fig. 3. Plot of *M versus* elution volumes in two different expressions.

The numerical expressions are shown in Table I. To determine the molecular weight of a protein, it is therefore only necessary to establish its $V_e/V_0$ and to insert its value into the appropriate equation.

With enzymes, it is not necessary to isolate the pure substances before chromatography as their appearance in effluent can be traced by virtue of their catalytic action. Elution volumes were found to be independent of accompanying proteins, as *e.g.* AURICCHIO AND BRUNI[4] have shown. A further application in the enzyme field is that of examining the reversible binding of several substances to proteins. Thus, as an example, the affinity of the hydrogenated pyridinium nucleotide (NADH) to lactic acid dehydrogenase (LDH) could be measured according to PFLEIDERER AND AURICCHIO[5]. The protein was run in a NADH containing buffer on Sephadex G-50. The LDH–NADH complex was visible in the eluent as a positive peak followed by a negative peak corresponding to the deficient, protein-bound NADH (see Fig. 4).

TABLE I

EQUATIONS OF THE STANDARD CURVES OF FIG. 3

| | |
|---|---|
| G-200 | $\log M = 6.698 - 0.987\ (V_e/V_0)$ |
| G-100 | $\log M = 5.941 - 0.847\ (V_e/V_0)$ |
| G-75 | $\log M = 5.624 - 0.752\ (V_e/V_0)$ |
| G-50 | $\log M = 5.415 - 0.864\ (V_e/V_0)$ |

## THIN-LAYER TECHNIQUES

By 1962, it had been found that a suspension of particles of Sephadex G-25 in water could be spread over clean glass plates to form solid layers which could be

used in thin-layer chromatography (TLC) of the ascending type[6]. Later on, Sephadex was manufactured in bead form. These particles do not adhere and cohere sufficiently for vertical layers. They can, however, be used on inclined plates in a descending technique like that described below for proteins. One of the first applications of TLC was in peptide chemistry in the investigation of the so-called plastein reaction.

Plasteins are formed by the action of pepsin at pH 4 on concentrated solutions of proper oligopeptides[7]. Tyr–Leu–Gly–Glu–Phe being a particularly reactive "monomer"[8]. The enzyme catalyses the condensation by splitting off water between the constituents. After TLC on Sephadex G-25, the plastein appeared as a mixture of several polymers, whose resolution was possible by column chromatography on Sephadex G-25 (Fig. 5). The component with highest molecular weight was the pentamer which contained 25 amino acids[9].

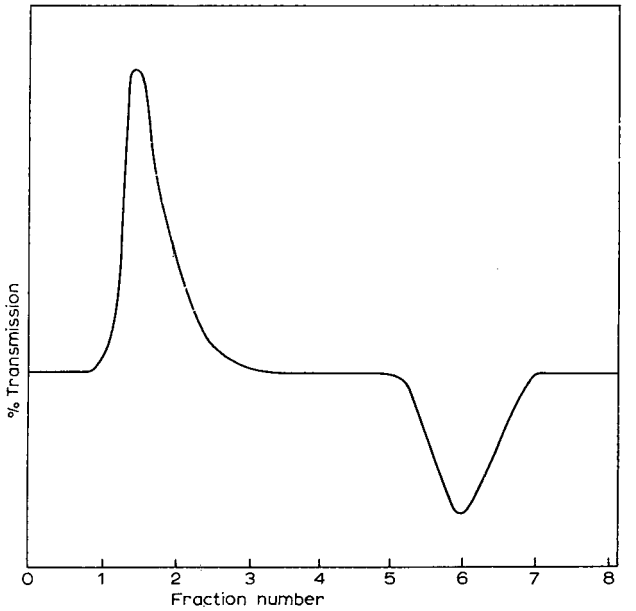


Fig. 4. Elution profile (253 m$\mu$) of LDH after passage through a Sephadex gel column being equilibrated with 0.7 m$M$ NADH.

Thin layers of the large-pore types G-100 and G-200, suitable for separations of proteins, have been employed later by different workers[10–12]. They can only be used in a descending technique. In these cases the flow rate can be regulated by inclining the plate at different angles. The chromatograms of Fig. 6 show a series of proteins which have been run at different angles.

Localization of the protein spots has been made by printing onto a filter paper and staining the prints with the usual dyestuffs or, better, coupling with diazotized sulfanilic acid (Pauly reaction). In TLC, a linear correlation also holds between $R$-values (which may be referred to cytochrome c) and log $M$, but there are considerable differences between various gel batches. By using reference proteins it is, however, easily possible to determine molecular weights by the TLC method. In the case of

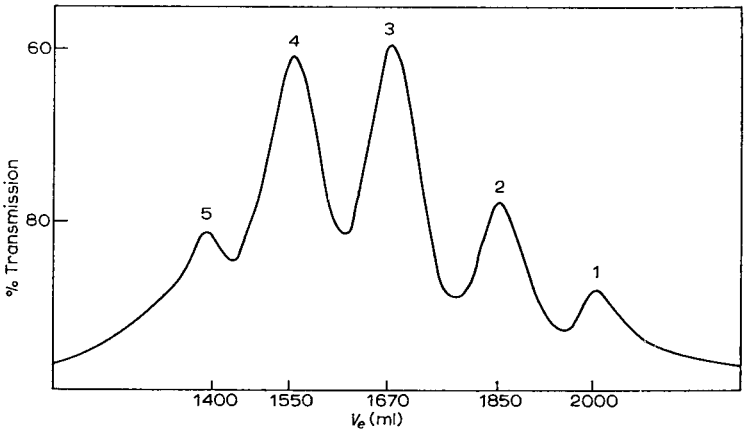


Fig. 5. Resolution of a "plastein" mixture by chromatography on Sephadex G-25. 1 = Pentapeptide; 2 = decapeptide, etc.

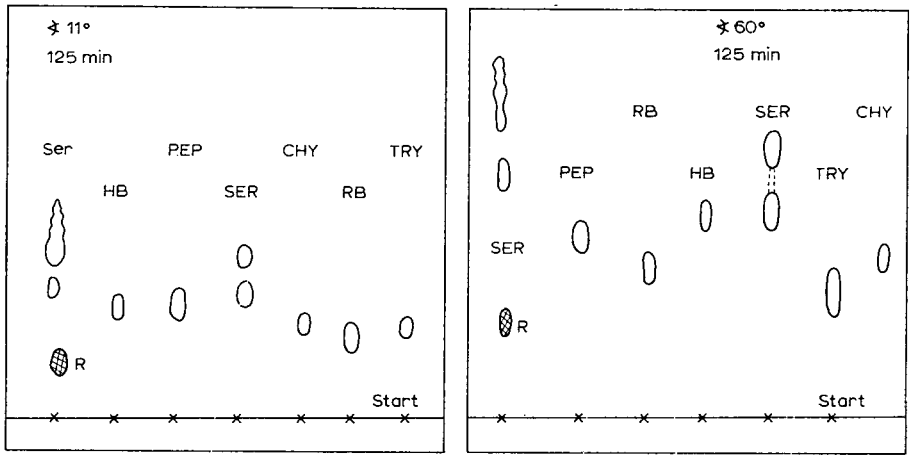


Fig. 6. $R_F$ values of several proteins in descending thin-layer chromatography at two different inclination angles. SER = Serum proteins, HB = hemoglobin, PEP = pepsin, CHY = chymotrypsin, RB = ribonuclease, TRY = trypsin; R = reference compound (cytochrome c).

enzymes, visualization is much more sensitive when an appropriate optical method is used. Thus, LDH can be detected in minimal amounts by spraying a print with NADH containing pyruvate buffer, whose fluorescence is quenched in the presence of traces of enzyme. In studies with isozymes of LDH it was found that the heart muscle type ($H_4$) travels distinctly faster than the skeletal muscle type ($M_4$) although both of them have the same molecular weight in the ultracentrifuge. Perhaps dissociation into protomers (subunits), which occurs more readily in $M_4$, plays a role in this phenomenon.

## LIPOPHILIC GELS

We tried some years ago[13] to transfer simply our experience gained with hydrophilic gels to a system of water-insoluble monomer (methyl methacrylate) and cross-linker (glycol bismethacrylate). The gels obtained in such experiments were able to separate partially very inhomogenous mixtures of low molecular weight polystyrenes. The resolving efficiency clearly depended on the degree of cross-linking, the weaker gel (0.25 % cross-linked) having more accessibility for bigger molecules, as compared with the strong gel (1 % cross-linked), a smaller part of the separation mixture being excluded. The applicability, however, was only for resolving molecules up to molecular weight 4000. We, therefore, tried to reduce the amount of cross-linking agent to get bigger "pores", but gels made up in this way proved too soft for handling in a chromatographic column. The experiments of MOORE[14] were then published, in which he made use of the fact that gel formation of polystyrene/divinylbenzene in suitable solvent systems gives a rather rigid, although large-pore gel. In our system pentanol was added in various amounts to the toluene solution of monomers before polymerization. Thus we also obtained relatively solid gels with better accessibility for larger molecules. Fig. 7 shows the fractionation of polystyrenes in tetrahydrofuran up to molecular weights of about 20,000.

Higher molecular weight polymers were also excluded from this gel. Further improvement in pore size was achieved by a method which we have called "inclusion polymerization"[15]. Here the cross-linking polymerization is conducted in the presence of large amounts (ratio to monomer 1:1) of a solid powder. The size of the granules is variable; the filler is removed afterwards by dissolving. In Fig. 8 the resolving power of a gel which has been prepared from the former monomers in the presence of sodium carbonate particles of 250 $\mu$ diameter is demonstrated. Here distinct differences exist in elution volumes of high polymer polystyrenes up to molecular weights of about half a million. The properties of several of these macroporous gels are summarized in Table II.

The principle of inclusion polymerization was also applied to the preparation of hydrophilic gels[16]. A gel prepared from acrylamide plus methylene bis(acrylamide) in the presence of powdered $CaCO_3$ (the inorganic substance was extracted by treating with 50 % acetic acid) allowed the resolution of the several components of "dextran blue" (mol.wt. $2 \times 10^6$) with the result demonstrated in Fig. 9.

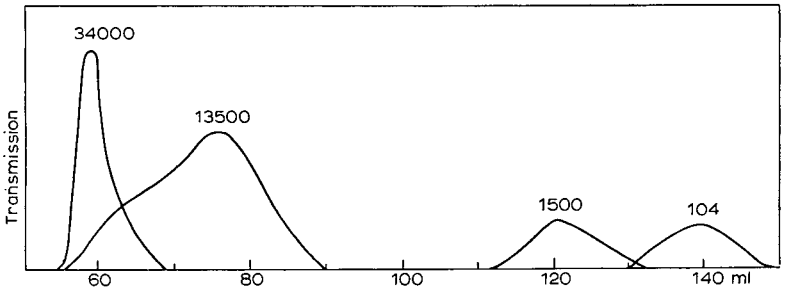


Fig. 7. Elution volumes of polystyrenes of different molecular weight from a methacrylate gel.

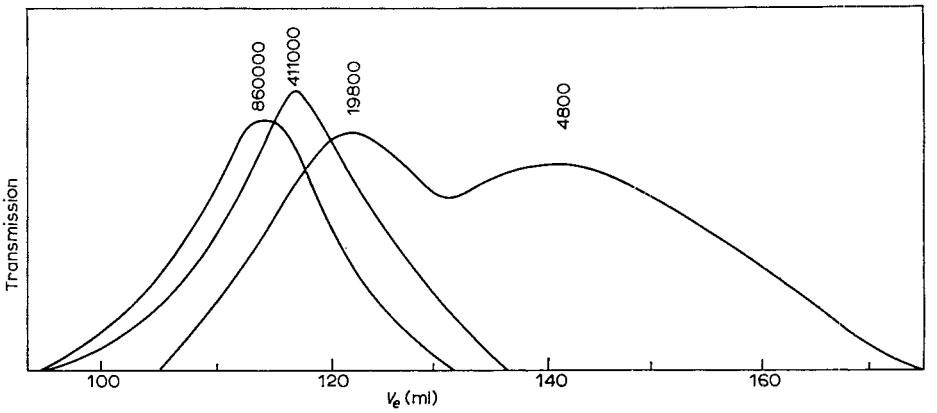


Fig. 8. Chromatography of high molecular weight polystyrenes on a gel obtained by inclusion polymerization.

TABLE II

SOME PROPERTIES OF SEVERAL MACROPOROUS GELS

Pore sizes have been adjusted by inclusion polymerization of methyl methacrylate and glycol bis-methacrylate (cross-linking agent, CA) in the presence of various substances which are eluted afterwards by appropriate solvents. I.S. = included substance.

| *Gel No.* | *Included substance* | *Particle size* ($\mu$) | *Ratio I.S.: monomer* | *% CA in polymer* | *Swelling factor* | *Minimal molecular weight of polystyrene excluded* ($\times 10^3$) |
|---|---|---|---|---|---|---|
| O | None | — | — | 1 | 7 | 5 |
| D1 | Dextrose | $<50$ | 1:1 | 1 | 6.5 | 20 |
| P1 | Polystyrene | mean molecular weight $\sim 3 \cdot 10^5$ | 1:2 | 1 | 7 | 130 |
| C1 | $CaCO_3$ | 10–20 | 1:1 | 1 | 7 | 145 |
| C2 | same | 10–20 | 1:1 | 5 | 3 | 42 |
| C4 | same | 1–5 | 1:1 | 2 | 5 | 28 |
| C5 | same | 10–20 | 2.5:1 | 1 | 7 | 150 |
| S1 | $Na_2CO_3$ | 100–125 | 1:1 | 1 | 7 | 300 |
| S2 | same | 200–250 | 1:1 | 1 | 7 | *ca.* 500 |

SEPARATIONS DUE TO ADDITIONAL INTERACTIONS

In addition to the graduated accessibility of substances to the liquid part of a gel, the classical principles of chromatography can also be developed to maximal resolving power by the nature of the gels. It is well known that Sephadex has a definite adsorbing strength for aromatic systems. In Fig. 10 the differences in elution volumes, of several amino acids, in salt-free aqueous solution, are shown.

Glutamic acid moves even faster than the neutral glycine; repulsion of the two negatively charged carboxylated groups by equally charged centers of the gel seems to prevent diffusion into the pores here. Of the three aromatic amino acids the indole system is most strongly adsorbed. This is also the reason for the widespread applica-

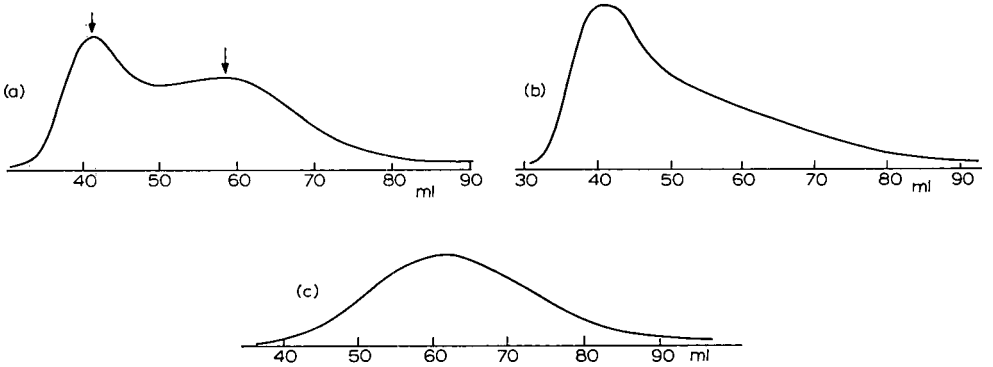


Fig. 9. Chromatographic behaviour of dextran blue on a column of macroporous polyacrylamide gel. (a) Dextran blue; (b) rechromatography of fractions of 30–40 ml; (c) fraction of 60–80 ml.

bility of Sephadex in the chromatographic resolution of the numerous constituents of the green toxic mushroom *Amanita phalloides*. The water-soluble fraction is divided into rather homogeneous fractions by chromatography on Sephadex G-50 in water as a solvent[17]. Fig. 11 shows clearly the very effective separation *e.g.* of phalloidin from α-amanitin, the two peaks before and after the 18.3 liter mark.

Adsorption chromatography is also very effective on lipophilic gels in apolar solvents. The pattern of peaks in Fig. 12 shows, impressively, the resolving ability of a methacrylate gel for nearly all pigments of spinach eluted with benzene[18].

Finally the usefulness of gels as supporting media in partition chromatography is mentioned. By adding an organic solvent to the gel–water system a change will take place whereby the hydrophilic gel phase will contain more water than the mobile phase. The same holds for partition chromatography in general, but with gels, particularly in the large-pore bead form, the interface between both the phases is much wider than in previously used materials. Thus, not only the resolving power, but also

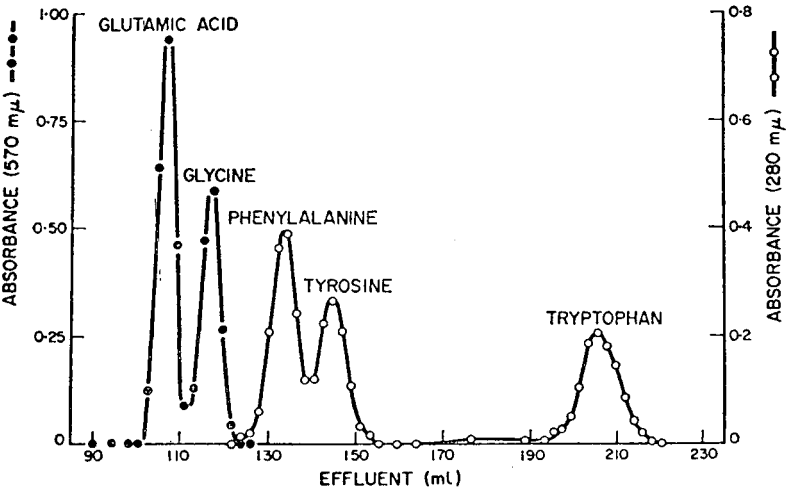


Fig. 10. Adsorption chromatography of amino acids on Sephadex G-25 in salt-free water. (After J. PORATH, *Biochim. Biophys. Acta*, 39 (1960) 193.)

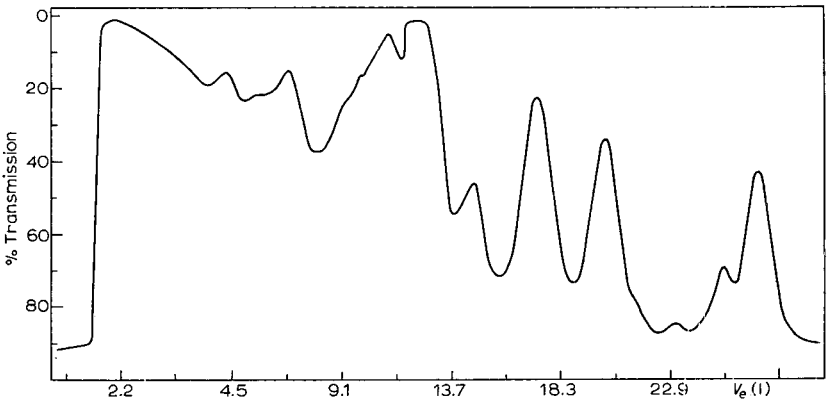


Fig. 11. Adsorption chromatography of hydrophilic components of *Amanita phalloides* on Sephadex G-50 in aqueous solution.

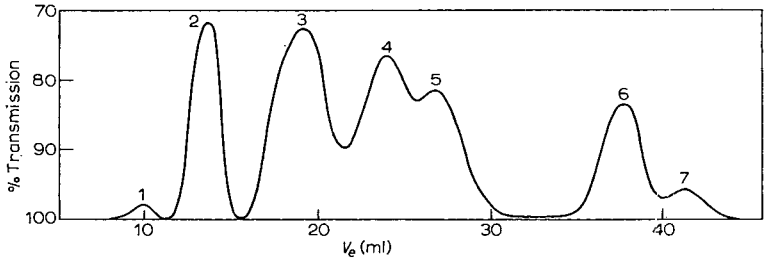


Fig. 12. Adsorption chromatography of leaf pigments on a methacrylate column in benzene (according to ref. 18).

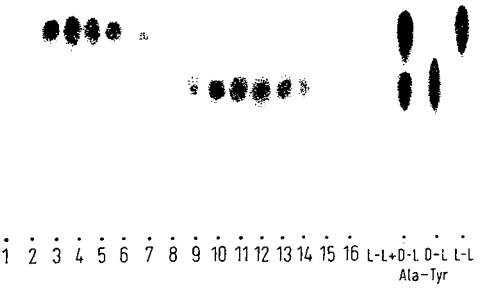


Fig. 13. Separation of L- and D-alanyl-L-tyrosine on a column of Sephadex G-50 with pyridine–water (1 : 1 moles) as an eluent. Left hand side, ninhydrin reaction of eluted fractions; right hand side, reference substances as analyzed by thin-layer chromatography on cellulose powder in the same solvent.

the capacity of Sephadex columns is surprisingly high. Fig. 13 shows the separation of two dipeptides, which differ only in the configuration of one amino acid, *viz.*, L-alanyl-L-tyrosine (left) and D-alanyl-L-tyrosine (right). In this case, 0.2 g of a 1:1 mixture could be resolved quantitatively, 0.5 g almost totally, on Sephadex G-50 in pyridine–water (1:1 moles) on a column of 1 m length and only 1.3 cm width, *i.e.* only 25 g of supporting medium[19].

Partition chromatography on Sephadex in butanone–water mixtures also plays an important role in the isolation of the lipophilic constituents of *A. phalloides* in the authors' laboratory[17].

## CONCLUSION

It is realized that we are offending against the settled nomenclature by gathering together all these chromatographic principles under the term gel chromatography. At least in the range of low molecular substances, however, a strict discrimination between the factors governing the mechanisms of separation is impossible. Therefore it seemed plausible to maintain the term until a better name for the subject under consideration has been found.

## SUMMARY

A review is given on some applications of different gels in chromatographic procedures: separations of amino acids, peptides, proteins—here especially on thin layers of Sephadex. Lipophilic gels are useful in separating polystyrenes; large-porous gels have been obtained by inclusion of different particles during polymerization and eluting the particles out of the gel. Sephadex and methacrylate gels are also suitable carriers for adsorption or partition chromatography.

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# ADSORPTION PHENOMENA ON SEPHADEX®

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Although it was the molecular sieving properties of the dextran gels which first received the greatest attention, other interactions were also soon observed. In 1960, PORATH[1] and GELOTTE[2] described a number of observations where substances showed a behaviour differing widely from what might be expected from their molecular size, *i.e.* their behaviour could not be interpreted as only a restricted diffusion into and through the gel phase caused by steric hindrance.

In general there are only two ways in which a solute can depart from its "true" elution volume, *viz.* either by appearing earlier or by appearing later. Substances which appear later are retarded either by adsorption or by electrostatic interaction, whereas an early elution is caused primarily by ion exclusion or sometimes by complex formation or aggregation.

The electrostatic interactions (including ion exclusion) are due to the fact that the cross-linked dextran chains contain a few terminal carboxylic groups. With the more tightly cross-linked Sephadex types, G-50, G-25, G-15 and G-10, the effect of the fixed charged groups may be particularly noticeable when the eluant is deionized water, and the gel will act as a weak cation exchanger with very low capacity. Thus small amounts of cations will be adsorbed, and anions may be completely excluded, especially when very small samples are applied to the gel bed. For a heavy sample load, as in the desalting of high polymer solutions, the effect will be observed as a front tailing of the salt zone. This means that a complete desalting is not possible on these gels. In most cases it is of minor importance since the relative amount of salt eluted together with the high polymer material is low. To effect complete desalting it is recommended that a solution of a volatile salt is used as eluent and afterwards the volatile salt is removed by lyophilization.

These electrostatic interactions were first discovered in the early dextran gels which contained much higher concentrations of fixed negatively charged groups (carboxylic groups) relative to the present gels. MIRANDA and collaborators[3] thus utilized the ion exchange properties of Sephadex G-25 and G-50 obtained with deionized water for the reversible retention of low molecular weight basic proteins such as toxins of scorpions, ribonuclease and lysozyme. GLAZER AND WELLNER[4] studied the binding capacity of Sephadex G-50 in distilled water for lysozyme, ribonuclease and serum albumin.

As this ion exchange effect is completely eliminated by the addition, to the medium or to the sample, of small amounts of an electrolyte, it is of minor importance and of little interest in most gel filtration experiments. Nevertheless one has to bear it in mind when interpreting elution data at low ionic strength.

Here, the term "adsorption" is meant to describe any kind of non-electrostatic

interaction between a solute and the dextran gel matrix which for the solute causes an anomalously high $K_d$.

The $K_d$ value is defined as $V_e - V_0/V_i$. $V_e$ is the elution volume of the substance, $V_0$ is the void volume, and $V_i$ is the inner volume, *i.e.*, the sum of the internal aqueous volumes of the gel grains. $V_i$ can be calculated from $V_i = g \cdot W_r$, where $g$ is the dry weight of the gel present in the column and $W_r$ is its water regain in grams per gram.

Thus a molecule which is completely excluded has a $K_d$ of zero, while a low molecular weight solute which can diffuse freely into and through the grains should have a $K_d$ value of about 1. In reality this is not the whole truth because part of the inner volume, $V_i$, is water of hydration which is firmly bound to the polysaccharide framework in the gel grains and is inaccessible to the solute molecules. In Sephadex G-25 a $K_d$ value of about 0.8, and in Sephadex G-10 a $K_d$ value of about 0.75, indicate therefore a non-restricted diffusion in the gel column. When exact values of $V_i$ are desired, one probably should use tritiated water as recommended by MARSDEN[5] in a recent paper, where he critically examines this method.

Among the adsorption phenomena, the affinity of the dextran gel matrix for aromatic and pseudo-aromatic substances is particularly striking. However, this is not a unique property of the dextran gels. It is in accordance with the affinity characteristics found for adsorption to cellulose[6]. A planar structure and an extending system of conjugated bonds in the solutes favour adsorption, and in view of the chemical similarity between dextran gels and cellulose similar mechanisms are probable. The observations of LATHE AND RUTHVEN in 1956[7] on the behaviour of many substances on starch columns support this view.

In most cases, the adsorption isotherms on dextran gels, have been found to be linear, and the solutes are therefore eluted as symmetrical peaks. This is also valid for substances with very high $K_d$ values.

As this affinity for $\pi$-electron rich compounds to the dextran gel matrix has been shown to be the most important interaction utilized for separation, the discussion of this effect will dominate this paper.

The larger the proportion of the solute molecule that consists of a gel-interactive group, and the higher the matrix density of the Sephadex gel used, the more pronounced is the adsorption effect compared with the molecular sieving effect. Consequently it is in the fractionation of low molecular weight solutes on the most tightly cross-linked Sephadex types that the most applications are expected to be found, since the chemical differences among the small solutes are much greater than among the macro-molecules usually studied in the gels of high "water regain".

Let us consider the different effects which may influence the behaviour of a solute which has some sort of "aromatic interaction" with the Sephadex gel matrix.

First, molecular sieving is always the underlying effect and always, most important, plays a decisive role as regards the selection process implied by the terms penetration and exclusion. In fact, no useful chromatography can be accomplished with substances which are too large to penetrate the gel.

Second, when discussing the mechanism of adsorption of solutes to the gel matrix one has to distinguish between the *pure adsorptivity* which is based on the structure of the substance and *superimposed effects* such as the ionic strength and pH of the eluent, and the effect of the few carboxylic groups present.

If the solute is uncharged, and possesses no acidic or basic properties, it is

practically insensitive to changes in pH and ionic strength. It is merely influenced by the presence of substances in the medium which compete for the adsorption sites in the Sephadex gel matrix. Such substances are for example pyridine, phenol, acetic acid and urea.

If the solute is charged or if it contains an ionizeable group and/or if it is an ampholyte, drastic changes in pH will definitely strongly influence the behaviour of the solute on the column. The possible explanation for this is that either there occurs a rearrangement of the $\pi$-electrons in the molecule due to the new charged group, or that the highly charged molecule is now surrounded by a larger ionic double layer which prevents the interacting group of the gel matrix from entering the adsorption sites. A combination of these two effects is of course also possible.

In distilled water, the $K_d$ values for the acidic aromatic substances are a compromise between the two counter-balancing effects, aromatic adsorption and ion exclusion. By the addition of small amounts of an electrolyte to the distilled water the ion exclusion effect, which depends on the small amounts of fixed carboxylic groups mentioned, is eliminated and the aromatic charged solute is retarded to an extent which is determined by the degree of the ionic strength of the eluent, *i.e.* the adsorption increases with increasing salt concentration. This is caused either by an increase in the number of adsorption sites available or by an increase in the strength of the interaction due to a decrease in the size of the layer of water of hydration which prevents the solute–gel interaction.

The most successful applications of the aromatic adsorption effect have probably been performed in the peptide separation field. It has been used both for the fractionation of protein hydrolysates and for the separation of naturally occurring peptides. By suitable choice of the medium it is possible to utilize both the molecular sieve effect and the aromatic adsorption effect of the Sephadex gel in the purification of a certain peptide containing one or more aromatic amino acids. It is ideally suited as a complement to ion-exchange chromatography because the two methods differentiate on the basis of different molecular properties.

EAKER[8] has studied the behaviour of tryptic digests of oxidized ribonuclease A in long narrow Sephadex columns. He found (Fig. 1) that the peptides of the hydrolysate grouped themselves into three sharply separated zones on Sephadex G-25 in 0.2 *M* acetic acid. The first zone contained peptides with 19–22 amino acid residues, the second zone contained tetra- and hexapeptides. The third zone contained, together with di- and tripeptides, one hepta- and one decapeptide, each of the two latter containing two tyrosine residues. The purification was further pursued by ion-exchange chromatography, but a partial purification could have been obtained by a rerun on the same Sephadex column in a medium which causes a complete depreciation of the aromatic adsorption, for example in the phenol–acetic acid–water solvent mixture introduced by SYNGE and collaborators[9,10] and used by CARNEGIE[11] in his work on estimation of molecular size of peptides by gel filtration on a micro scale. STEPANOV and collaborators[12] have used 8 *M* urea for the same purpose.

MACH AND TATUM[13] studying the environmental control of amino acid substitutions in the biosynthesis of the antibiotic cyclic decapeptides tyrocidine A, B and C, in a very elegant way utilized their different contents of the aromatic amino acid tryptophan for separation on a 200 cm long column of Sephadex G-25 equilibrated with 10 % acetic acid. The conditions were worked out by RUTTENBERG[14] from whose

doctoral dissertation Fig. 2 originates. The diagrams show the elution profiles obtained when intact tyrocidine and tyrocidine hydrolysate are run on the same column in three different concentrations of acetic acid; 1 %, 10 % and 50 %. In the hydrolysate run, in 1 % acetic acid, the aromatic amino acids are well separated from each other and from the neutral amino acids which are eluted together. Intact tyrosidine is strongly retarded. Tyrocidine A lacks tryptophan, tyrocidine B contains one and tyrocidine C contains two tryptophan residues. In 10 % acetic acid they are nicely separated according to their tryptophan content, but in 50 % acetic acid they are all eluted in one peak. In 10 % acetic acid phenylalanine and tyrosine in the tyrocidine hydrolysate are pushed into the neutral group of amino acids and in 50 % they are all eluted together.

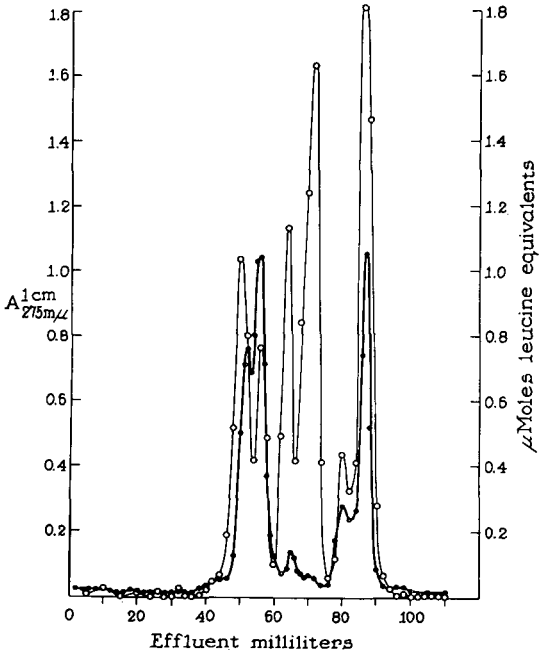


Fig. 1. Fractionation of tryptic digest of 22.6 mg of oxidized ribonuclease A on Sephadex G-25 in 0.2 *M* acetic acid. Column size: 0.9 × 150 cm; flow rate: 6.3 ml/h; fraction size: 1.0 ml. (–•–•–) Absorption at 275 mμ; (O–O–O–O–) ninhydrin analysis. (After EAKER[8]; reproduced by permission of the author and Academic Press Inc., New York).

Many other applications of aromatic adsorption can be found in the literature. It has, for example, been used to examine the polyphenolic content of turf, soil, water, wine and beer, and for separation of the flavouring matter from the colour in coffee extracts.

It is often preferable to carry out the gel filtration in strongly alkaline solution when fractionation of aromatic substances is desired. For example the estrogenic isoflavones in red clover are effectively separated on Sephadex G-25 in 0.1 *M* ammonium hydroxide (Fig. 3)[15].

Very interesting results can be obtained when advantage is taken of the difference in adsorption strength, hence the difference in migration rate of certain compounds

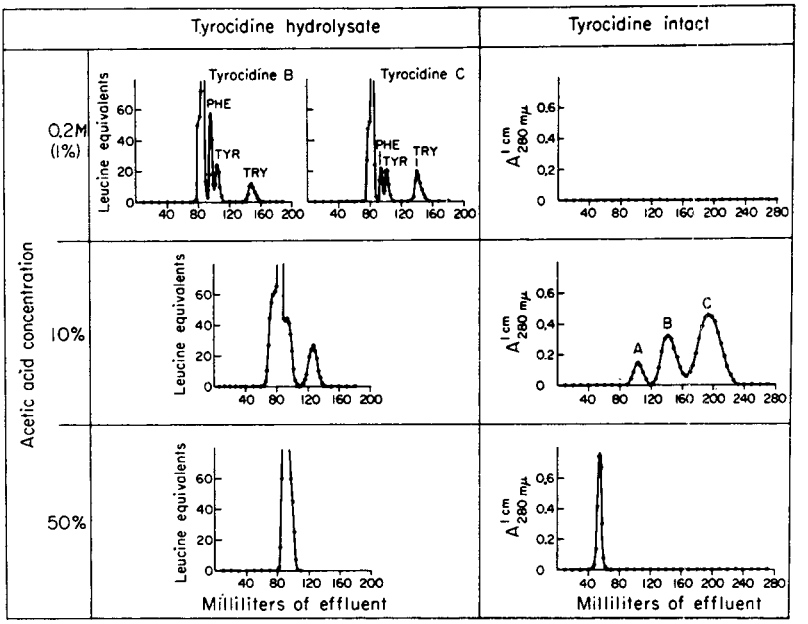


Fig. 2. Summary of results with chromatography on Sephadex G-25 in various concentrations of acetic acid of intact tyrocidine and tyrocidine hydrolysate. All experiments were performed on the same 0.9 × 150 cm column with loads from 1 to 4 $\mu$moles. 2 ml fractions were collected at a flow rate of 8 ml/h. (After RUTTENBERG[14]; reproduced by permission of the author.)

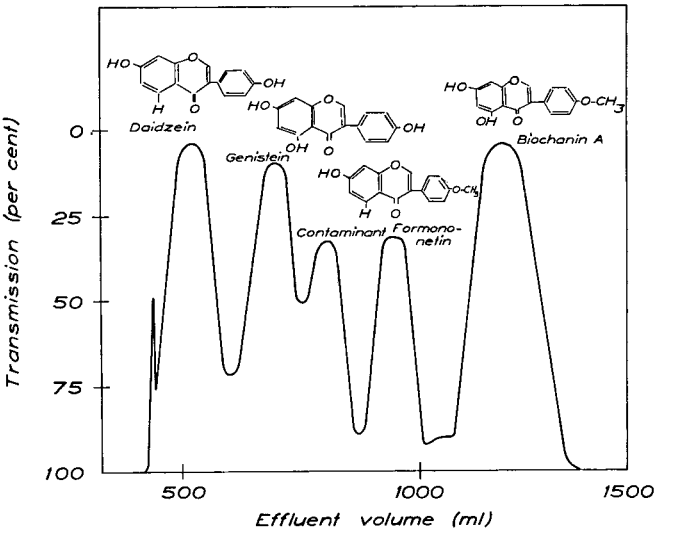


Fig. 3. Fractionation of a mixture of the estrogenic isoflavones genistein, biochanin A, formononetin and daidzein (5 mg of each) on Sephadex G-25 in 0.1 *M* ammonium hydroxide. Column size: 4.5 × 37 cm; flow rate: 1 ml per min. (After NILSSON[15]; reproduced by permission of the author.)

depending on whether they are in salt solution or in water. When a sample containing such a compound and sodium chloride is eluted with distilled water, the compound is concentrated during its passage through the column and eluted in a smaller volume than that of the original sample. This effect is only obtained if the actual compound is eluted faster than the salt in water, and is explained thus: When the compound is in the salt zone it is adsorbed and thus retarded. The salt zone moves downwards and is followed by water which accelerates the migration of the compound owing to the conditions mentioned. The final result is a concentration of the solute in the boundary between the salt and the water.

The mechanism described is probably responsible for the beautiful method for isolation of conjugated estrogens from urine described by BELING[16]. He used a column 1 cm by 50 cm with Sephadex G-25. Up to 20 ml of urine were added to the column and eluted with distilled water. The estrogens appeared in the effluent in only 3 ml and were recovered quantitatively. All the conjugated estrogens assayed for were present and most of the other materials in urine removed.

In a recent investigation, EAKER AND PORATH[17] analyzed very precisely and critically the behaviour of some low molecular weight solutes, mainly amino acids, on the very tightly cross-linked gel Sephadex G-10, under the influence of different solvent media. Their results have been presented in a large table containing more than 120 elution data tabulated in the form of $K_d$ values.

With a higher matrix density, the low molecular weight solutes are influenced more by the gel chains both as far as steric hindrance and adsorption are concerned. Thus for charged solutes the ionic double layer, and for aromatic substances a stronger adsorption, plays a greater role in G-10 than in G-25.

Some of these effects, and some of those mentioned in the introduction, are illustrated by Fig. 4 (from EAKER AND PORATH), which shows the behaviour of some amino acids on a 1 cm × 142 cm column of Sephadex G-10 equilibrated with 1 % acetic acid, with 1 % acetic acid plus 0.5 *M* sodium chloride and with 1 % acetic acid plus 2 *M* sodium chloride.

Attention is drawn to three different types of compounds, *viz.* the basic amino acids, the aromatic amino acids and urea. The basic amino acids are highly charged in the acetic acid, their ionic double layer will be very large and they are therefore almost excluded. On the addition of salt, the hydration layer will decrease and the amino acids are eluted later. Arginine is most affected by the increase in salt concentration, which may be explained by the removal of part of the water of hydration which makes it possible for the $\pi$-electrons in the guanidino group to interact with the gel matrix. This last effect may also account for the stronger retardation of the aromatic amino acids at higher ionic strengths. Urea, whose $\pi$-electrons cover its planar structure and cause it to be retarded on almost everything, is neutral and thus not very sensitive to sodium chloride.

Fig. 5 shows the behaviour of some amino acids on the same column as in the preceding series of experiments, but now the effect of pH and the addition of pyridine will be discussed. In pattern A the eluent is 1 % acetic acid, pH 2.7, in B the eluent is 1 *M* pyridine–0.03 *M* acetic acid, pH 6.7 and in C it is 0.01 *M* NaOH, pH *ca.* 12. The ordinate (as in the preceding figure) is ninhydrin colour as recorded continuously with a standard Spinco Model 120 amino acid analyzer. The flow rate through the column was 10.0 ml/h in all cases.

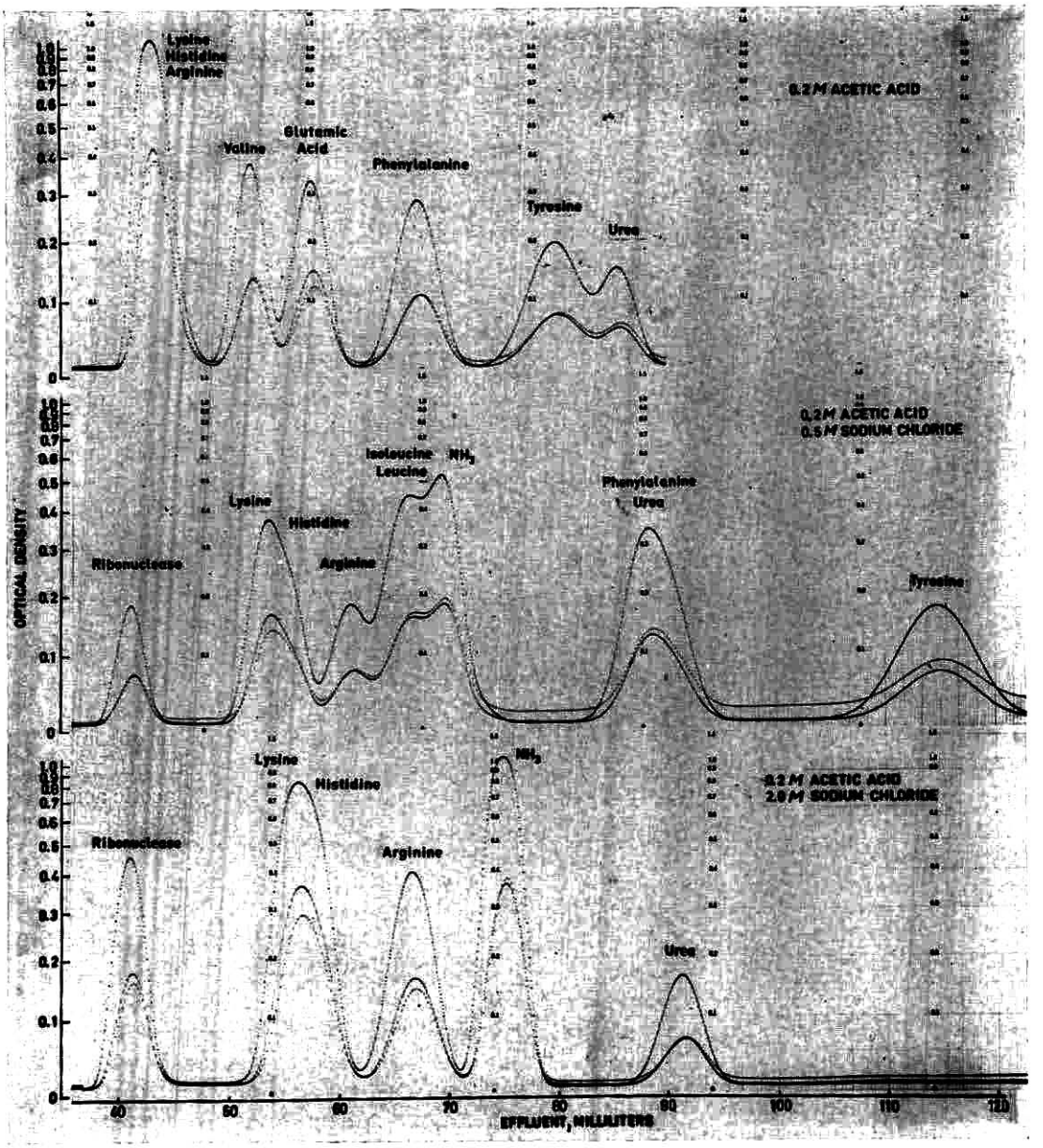


Fig. 4. Patterns showing the effect of salt on the elution behaviour of amino acids and other ninhydrin positive substances on a 1 × 142 cm column of Sephadex G-10. 0.5–2 μmoles of each solute (urea, 4–8 μmoles) in 1.0 ml. Tryptophan is out of the diagram in all cases. Tyrosine and phenylalanine are out of the diagram in the bottom chromatogram. The ordinate is ninhydrin colour as recorded continuously with a standard Spinco Model 120 amino acid analyzer. The flow rate through the column was 10.0 ml/h in all cases. (After EAKER AND PORATH[17]; reproduced by permisssion of the authors.)

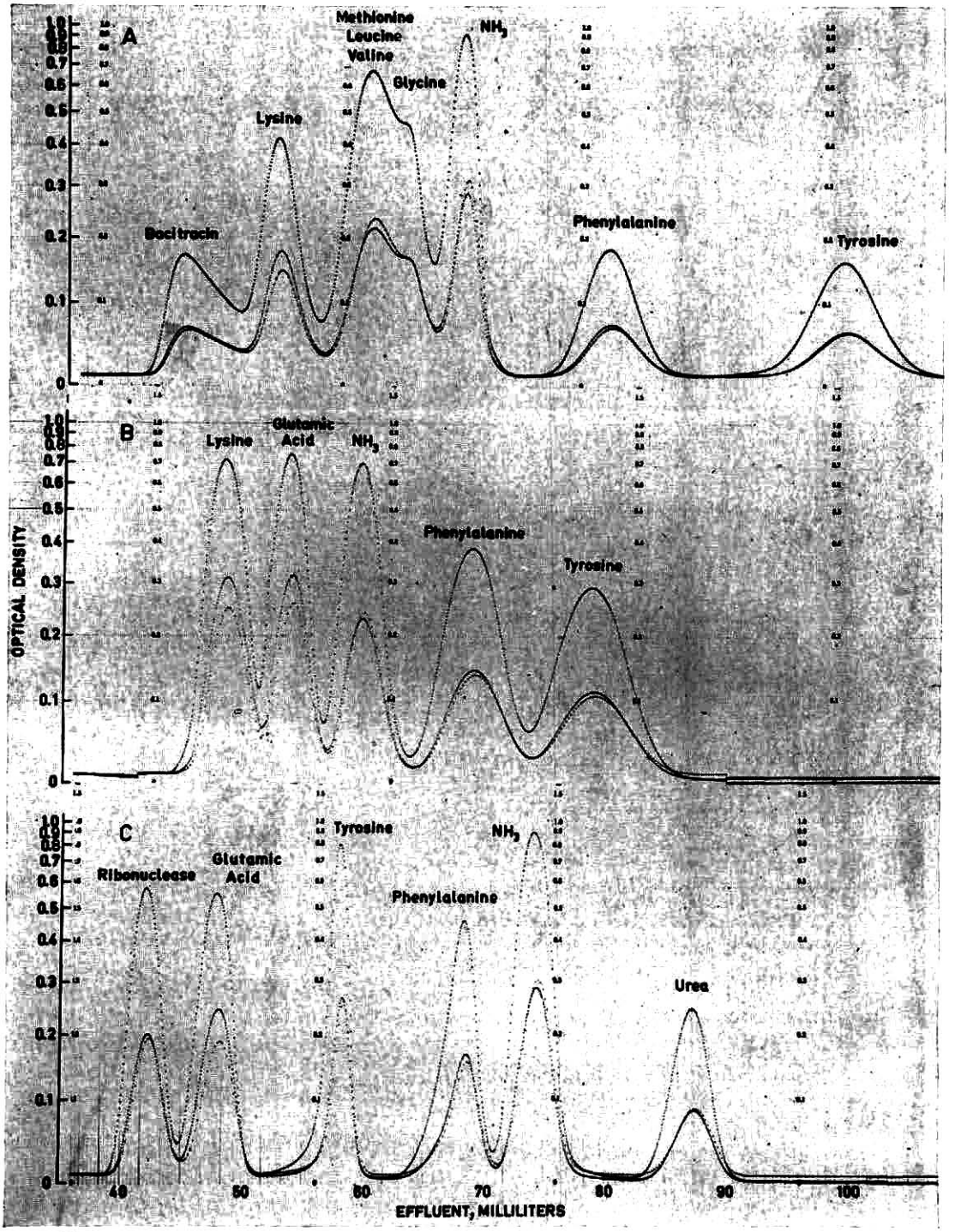


Fig. 5. Patterns obtained with amino acids and other ninhydrin positive substances on a 1 × 142 cm column of Sephadex G-10 in three different eluants. 0.5–2.0 μmoles of each solute (urea, 4–8 μmoles) in 1 ml. In pattern A the eluent is 0.2 *M* acetic acid, pH 2.7; in B the eluent is 1 *M* pyridine–0.03 *M* acetic acid, pH 6.7; and in C it is 0.01 *M* NaOH, pH *ca.* 12. Flow rate: 10.0 ml/h. Ordinate: ninhydrin colour. (EAKER AND PORATH[17]; reproduced by permission of the authors.)

The effects one can observe can be summarized as follows: Highly charged substances tend to be excluded (for the reason just mentioned). Examples: The basic amino acids are excluded at acid pH (in the absence of salt) and the acidic amino acids are excluded at alkaline pH. Apparently this is quite a powerful effect, since tyrosine is eluted well in advance of phenylalanine at alkaline pH where the phenolic group is ionized.

On addition of 1 *M* pyridine the retardation of the aromatic amino acids (which here at pH 6.7 are neutral) is markedly decreased. The explanation may be that the pyridine competes for the interaction sites in the gel.

These experiments show very well the potentialities of the new, very tightly cross-linked gel Sephadex G-10 in the low molecular weight fractionation field. EAKER[18] stresses that it is possible to separate any pair of amino acids (excepting leucine–isoleucine) and almost any pair of smaller peptides on G-10, just by manipulating with the acetic acid concentration and the salt concentration, which are still rather mild conditions.

ACKNOWLEDGEMENT

The author would like to express his appreciation to Dr. D. EAKER for putting original data at his disposal and for useful discussions.

SUMMARY

It is a well known fact that the more tightly cross-linked dextran gels of the Sephadex series interact with certain solutes, causing anomalously high elution volumes. In this paper the molecular basis for some of these interactions under the influence of different solvent media is discussed. A few examples from the literature are given, where advantage has been taken of these effects in certain separation problems.

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# GEL FILTRATION IN ORGANIC SOLVENTS

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Gel filtration in aqueous systems as developed by FLODIN AND PORATH in the years 1958–1960 has many valuable features, the most important of which are:

(1) Fractionation according to molecular size.
(2) Easy handling of the material.
(3) Good stability of the chromatographic bed.
(4) Possibility of chromatography of labile substances without denaturation.
(5) Permissibility of high sample concentrations because of the linearity of partition isotherms.

There is therefore considerable interest in extending the technique to include organic solvents.

It was very soon found that Sephadex® of the G-series, the classical material for gel filtration in aqueous solutions, could be used in some polar organic solvents. Suitable solvents include glycol, formamide and dimethylsulphoxide, although these have not been frequently used. The use of mixtures of water and the lower alcohols, notably ethanol has, however, often been reported in the literature.

In 1960, VAUGHAN reported the use of cross-linked polystyrene as a chromatographic medium with aromatic hydrocarbons as solvents[1]. This material could be used for the determination of molecular weight, for distribution analysis etc. CORTIS-JONES also used the same material[2], while BREWER used vulcanized rubber latex[3]. Since then, polystyrene gels have been developed by MOORE for use mainly with non-polar organic solvents[10]. DETERMANN *et al.* have prepared a copolymer of methyl methacrylate and ethyleneglycol dimethacrylate that was used for fractionation of low molecular weight polystyrenes[11].

A quite diffrerent approach to the problem of gel filtration in organic solvents was taken by NYSTRÖM AND SJÖVALL[4,5]. These authors modified the existing types of Sephadex to make them suitable for work in polar organic solvents. Methylation was performed with dimethylsulphate by a rather laborious method involving several steps. The most highly methylated Sephadex can be used even with non-polar organic solvents such as hydrocarbons. With the modified Sephadex, SJÖVALL and coworkers have been able to separate a considerable number of lipids, steroids[4], protected oligopeptides[6] and vitamins of the K group[7]. In some experiments they have used a chromatographic tedchnique very similar to that used in gas chromatography, with a 1.5 mm diameter teflon tube, approximately 2 m long, as the column.

At the same time that NYSTRÖM AND SJÖVALL developed their methylated Sephadex, Pharmacia Fine Chemicals independently developed another derivative of Sephadex for use with organic solvents. This derivative has properties fairly similar to those of the methylated Sephadex of NYSTRÖM AND SJÖVALL. It is commercially

available under the name Sephadex LH-20. It is produced by the hydroxypropylation of Sephadex G-25 and has a solvent regain value of approximately 2 ml/g in many solvents. The swelling properties and other technical data for Sephadex LH-20 are presented in Table I. The fractionation range is slightly different in different solvents. In most solvents the exclusion limit falls somewhere between 2,000 and 10,000.

TABLE I

SOLVENT REGAIN FOR SEPHADEX LH-20 IN DIFFERENT SOLVENTS

| *Solvent* | *Approximate solvent regain (ml solvent/g dry gel)* | *Approximate bed volume (ml/g dry gel)* |
|---|---|---|
| Dimethylformamide | 2.2 | 4.0–4.5 |
| Water | 2.1 | 4.0–4.5 |
| Methanol | 1.9 | 4.0–4.5 |
| Ethanol | 1.8 | 3.5–4.5 |
| Chloroform, stabilized by 1 % ethanol | 1.8 | 3.5–4.5 |
| Chloroform | 1.6 | 3.0–3.5 |
| *n*-Butanol | 1.6 | 3.0–3.5 |
| Dioxane | 1.4 | 3.0–3.5 |
| Tetrahydrofuran | 1.4 | 3.0–3.5 |
| Acetone | 0.8 | |
| Ethyl acetate | 0.4 | |
| Toluene | 0.2 | |

It is well-known that in aqueous solutions aromatic substances are retarded on Sephadex of the G-series[12]. This effect is particularly noticed in the highly cross-linked gels G-10, G-15 and G-25. This retardation of aromatic compounds is found for Sephadex LH-20 with alcoholic solutions, although the effect is far less pronounced than with aqueous solutions. In chloroform solutions the effect is not noticeable[8].

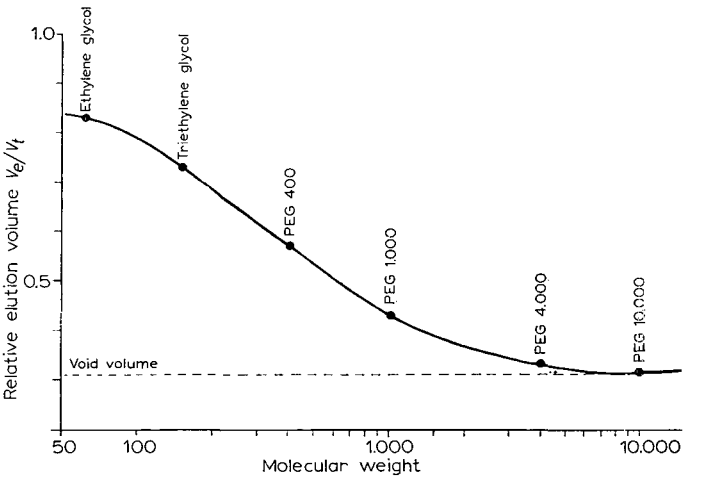


Fig. 1. Correlation between relative elution volume and molecular weight for polyethylene glycols on Sephadex LH-20 in ethanol.

It has been noticed that in chloroform solutions substances containing carboxyl groups and hydroxyl groups are retarded relative to the corresponding substances not containing these groups. This specific retardation may, in some cases, be used to obtain fractionation of substances which are otherwise closely similar. Some examples of fractionations obtained with Sephadex LH-20 will now be discussed:

The relationship between elution volume and molecular weight for polyethylene glycols on Sephadex LH-20 follows the expected pattern (see Fig. 1). At low molecular weight $K_D$ values approach unity and decrease within the fractionation range to $K_D = 0$ at approximately mol. wt. = 5,000 in ethanol solution. In chloroform solution, very high $K_D$ values are obtained at low molecular weight. This may be explained by the presence of terminal hydroxyl groups in the polyethylene glycol chains. The relationship between elution volume and molecular weight for polyethylene glycols in chloroform on LH-20 is illustrated in Fig. 2.

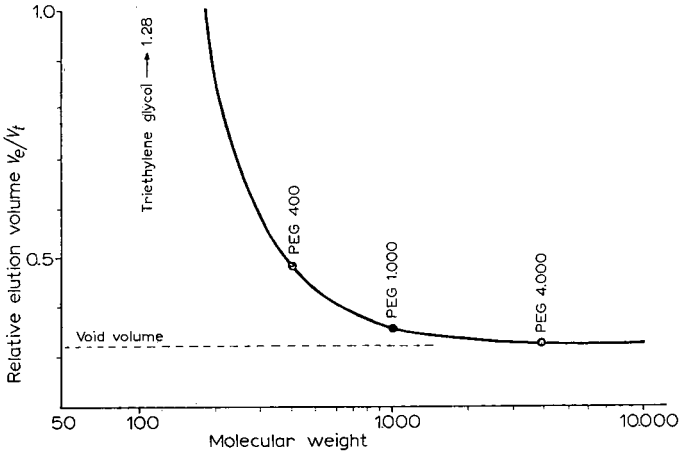


Fig. 2. Correlation between relative elution volume and molecular weight for polyethylene glycols on Sephadex LH-20 in chloroform.

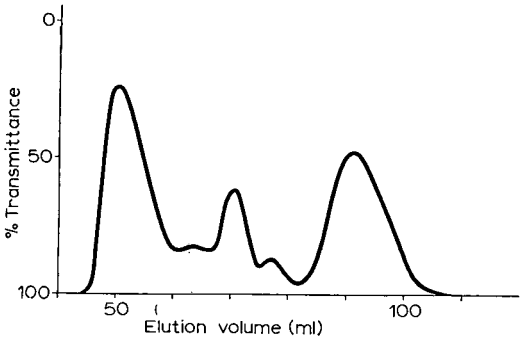


Fig. 3. Elution diagram of a commercial polystyrene (Dow Resin PS-3) on Sephadex LH-20 in chloroform.

Separation of substances of high and low molecular weight can be used for analytical purposes. Fig. 3 shows the separation of a commercially available polystyrene, Dow-Resin PS-3, on Sephadex LH-20 in chloroform. The first peak eluted corresponds to the polymer and the last peak corresponds to the monomer. The second peak probably corresponds to the plasticizer or some similar material.

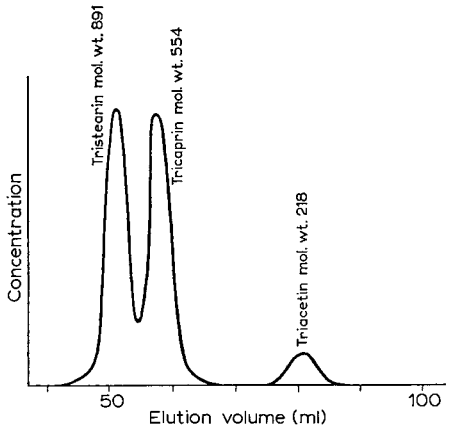


Fig. 4. Elution diagram for glycerol esters on Sephadex LH-20 in chloroform.

The separation of molecules according to molecular weight is illustrated in Fig. 4, giving the separation of tristearin, tricaprin and triacetin on Sephadex LH-20 in chloroform solution.

The separation of dipalmitins with primary and secondary hydroxyl groups illustrated in Fig. 5, is based on the structure rather than on the molecular size of the substances.

Finally the use of mixed solvents should be mentioned. VIKHO has reported the separation of cholesterol and dehydroepiandrosterone sulphate[9] on Sephadex LH-20 in a mixture composed of chloroform, methanol and water. In this case, the content of water in the solvent mixture has a profound influence on the separation obtained.

In mixed solvents, the composition of the solvent inside and outside the gel

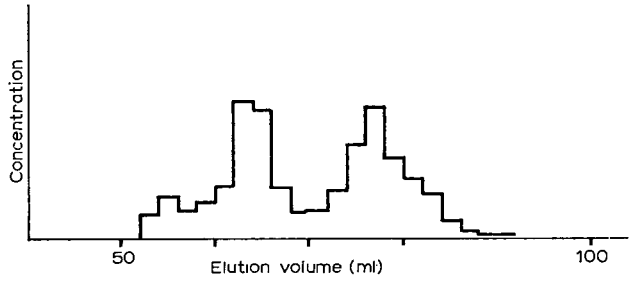


Fig. 5. Elution diagram for dipalmitins with primary and secondary hydroxyls on Sephadex LH-20 in chloroform.

grain is different. Thus, in addition to the sterical factors, partition of a solute between the mobile and the gel phase is also influenced by the difference in the composition of the liquids.

SUMMARY

The development of gel filtration in organic solvents is briefly reviewed. The properties of Sephadex LH-20 in some organic solvents are discussed in more detail, *viz.* solvent regain values, the elution behaviour of polyethylene glycols in ethanol and chloroform, the retardation of hydroxyl containing substances in chloroform.

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# DEXTRAN-GEL FILTRATION OF RAT LIVER α-GLUCOSIDASES

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At least two enzymes are responsible for the hydrolysis of α-glucosyl disaccharides in mammalian liver: the acid and the neutral α-glucosidases[1-3]. The first enzyme is active at acid pH, is present in lysosomes and seems to be able to degrade glycogen; the other is active at neutral pH, is localized for the most part in the microsomes and degrades oligosaccharides like maltose and maltotriose but, at least in some mammals, not glycogen[3].

At present the functions of these enzymes are not completely clear. The neutral enzyme probably degrades oligosaccharides such as those yielded by the action of liver α-amylase. A function for the acid glucosidase may be inferred from a consideration of Pompe's disease. The only known enzymic defect associated with this glycogen storage disease is the lack of an acid glucosidase[4]. Thus this enzyme might function by degrading glycogen from its outer chain ends to free glucose, so that besides the degradation of glycogen *via* phosphorolysis and α-amylolysis, it is now supposed that degradation *via* glucamylolysis in mammalian tissues can also occur[5].

The three pathways of glycogen degradation and the possible role of the two glucosidases are briefly represented in Fig. 1.

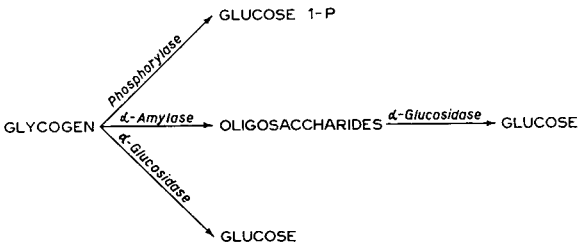


Fig. 1. Pathways of glycogen degradation.

TORRES AND OLAVARRÍA were able to purify both α-glucosidases from dog liver[2]. The acid enzyme was purified about 30 times with a recovery of 2.6%.

As the acid enzyme has a weak dextranase activity[5] the behaviour of the two α-glucosidases on dextran gel was studied. An interaction between the acid glucosidase and dextran gel (for instance of the enzyme-substrate type) was possible, and would be expected to "retard" the elution of the acid glucosidase from the gel column, thus separating the acid from the neutral glucosidase and from other proteins which are not adsorbed on dextran gel. The results show that it is possible to separate the neutral from the acid glucosidase by dextran-gel filtration. By using this tech-

nique the acid enzyme from rat liver was purified about 700 times with a recovery of 20 % of the initial activity present in the crude extract.

## MATERIALS AND METHODS

Glucose was obtained from Merck, Darmstadt, Germany; shell fish glycogen, peroxidase and glucose oxidase were from Sigma Chemical Co., St. Louis, Mo., U.S.A.; Tris was supplied by C.F. Boehringer und Soehne G.m.b.H., Mannheim, Germany; *o*-dianisidine by British Drug Houses Ltd. All other reagents were of analytical grade.

The α-glucosidase activity was measured by the formation of glucose from maltose. The incubation mixture contained 20 μmoles of buffer (acetic acid–sodium acetate at pH 3.6, imidazole–HCl at pH 7.5) and 4 mg of maltose per ml. The formation of glucose from glycogen was measured only at pH 3.6 (the glycogen concentration was 8 mg per ml of the reaction mixture). The reaction was run at 37° for 0.5–1 h and stopped by the addition of NaOH and $ZnSO_4$. The glucose in the protein-free solution was determined enzymatically *via* glucose oxidase and peroxidase according to HUGGET AND NIXON[6] with the exception that 0.5 *M* Tris–HCl, pH 7, was used instead of phosphate buffer. Protein was measured by the method of LOWRY and coworkers[7], the modified reagent B introduced by EGGSTEIN AND KREUTZ being used[8].

### *Gel filtration*

Sephadex G-100 was suspended in a solution of 6 *M* urea, stirred with a magnetic stirrer for about 1 h and exhaustively washed with water. The smallest particles were removed by decantation and a suspension of the gel was deaerated and packed into columns (1.5 cm diameter × 67 cm long). Columns of gel were equilibrated with the desired solution, which was allowed to pass through until the height of the gel bed remained constant. The flow rates of the columns were approximately 15 ml/h.

## RESULTS

Five or six Wistar male rats were starved for 30 h and then killed. The livers were perfused with ice-cold 1 m*M* EDTA–25 m*M* NaCl, pH 6.7. The same solution was used in every step of the experiment. The livers were homogenized for 3 min with an equal volume of liquid. The homogenate was frozen and thawed, and was then centrifuged at 105,000 × *g* for 60 min. The supernatant was concentrated by dialysis at reduced pressure. 7 or 8 ml of the sample, containing about 200 mg protein per ml, were pipetted on to the top of a gel column and filtered. When the sample was applied on the column a reduction of about 5 ml of the gel bed volume occurred.

The eluted fractions were examined for extinction at 280 mμ, for maltase activity at pH 3.6 and 7.5, and for glucose formed from glycogen at pH 3.6. It should be noted that maltase activity in liver is due to the α-glucosidases and that if the acid α-glucosidase is a glucamylase it forms glucose from glycogen.

Fig. 2 shows the elution diagrams from a Sephadex G-100 column. (A) is the extinction at 280 mμ, (B) the maltase activity at pH 7.5, (C) the maltase activity at pH 3.6, and (D) the glucose formed from glycogen at pH 3.6.

Curve B shows that the neutral maltase activity is eluted with most of the protein.

Graph C shows the elution profile of acid maltase activity; at least two peaks are recognizable. The first peak corresponds to the peak of neutral maltase activity and it is possible that it is due to the activity of the neutral α-glucosidase at this pH. The second peak, eluted after the total volume of the gel column, is a peak of activity which has clearly been retarded. There are three interesting facts to emphasize at this point: (1) the negligible extinction at 280 mμ, corresponding to the second peak of acid maltase activity, (2) the negligible neutral maltase activity, and (3) a peak of glucose production from glycogen at acid pH visible from the elution diagram presented in D. The fact that the second peak of acid maltase activity and the single peak of glucose formed from glycogen have the same elution volume from the gel column, and that both activities are retarded, supports the claim of several authors that both activities are due to the same enzyme, the acid α-glucosidase.

The specific enzymic activity of the fraction corresponding to the descending part of the elution profile of the second peak of acid maltase activity, where the neutral activity is absent, was measured. The acid maltase activity was found to have been purified 700 times after the gel filtration with a recovery of 20% of the initial activity in crude extract.

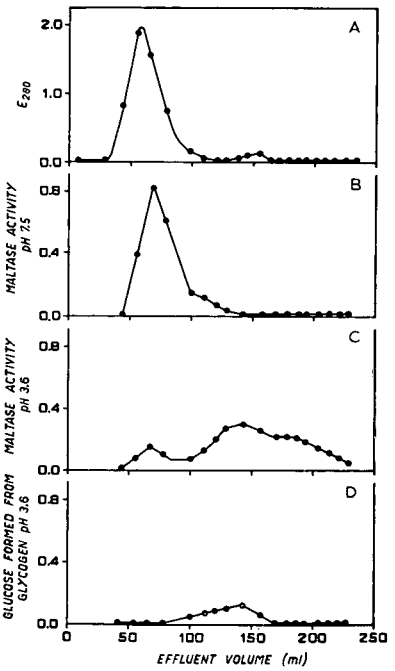


Fig. 2. Elution diagrams from a Sephadex G-100 column (1.5 cm × 67 cm) of (A) extinction at 280 mμ, (B) maltase activity at pH 7.5, (C) maltase activity at pH 3.6, and (D) glucose formed from glycogen at pH 3.6.

SUMMARY

The behaviour of the acid and neutral α-glucosidases from rat liver on dextran gel was studied.

As the acid enzyme has dextranase activity an interaction between the acid α-glucosidase and dextran gel was possible and would be expected to "retard" the elution of the enzyme from the gel column, thus separating the acid α-glucosidase from proteins which are not adsorbed on dextran gel. By using dextran gel filtration, the acid α-glucosidase was purified about 700 times with a recovery of 20 % of the initial activity in crude extract.

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# SEPARATION OF DEOXYRIBONUCLEIC ACID AND RIBONUCLEIC ACID BY GEL FILTRATION

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In a current investigation in collaboration with the Microbiological Laboratories of this Institute, it was necessary to work out a method of separating ribonucleic acid (RNA) and deoxyribonucleic acid (DNA) fractions from an extract of nucleic acids. It was important to avoid any degradation of such substances and to obtain each fraction with less than 1 % of the other component.

At first the two substances were obtained from the mixture by enzymatic hydrolysis of DNA and RNA, respectively, and removal of the hydrolysates by gel filtration on Sephadex G-50 in water. But, owing to the incomplete hydrolysis of RNA, the DNA so obtained was not free of RNA. A direct separation of the two components by gel filtration was then tried, in view of the fact that these substances, even though they have a molecular weight very much greater than those generally fractionated by gel filtration (*e.g.* proteins), are very different in structure from proteins and from each other. Sephadex G-200, a dextran of maximal porosity, was used, and to obtain the optimal experimental conditions it was tried with different buffers of increasing ionic strength and with artificial mixtures of nucleic acids.

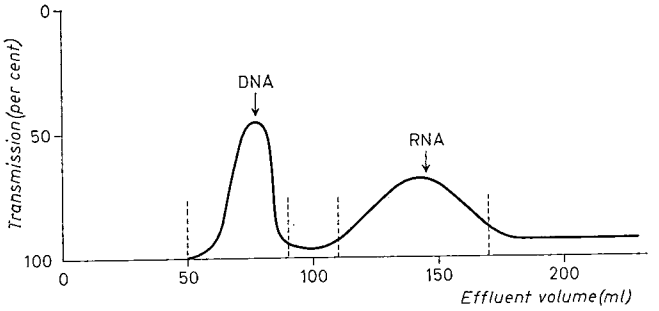


Fig. 1. Separation of DNA and RNA. Experimental conditions as reported in Table I. The vertical dotted lines limit the fractions I and II mentioned in Table II.

As a result of these tests, a complete separation of the DNA and RNA mixture into two fractions was possible as shown in Fig. 1, which illustrates the separation of a nucleic acid mixture extracted from Rhesus monkey liver by the phenol method of GIERER AND SCHRAMM[1]. The conditions are given in Table I.

The curve in Fig. 1 gives the optical transmission at 254 mμ (recorded by an LBK Uvicord ultraviolet absorptiometer) *versus* the eluate volume. Peak I is formed by DNA and the sharp peak proves that the DNA does not diffuse into the gel particles. Peak II, formed by RNA, has a long tail representing a great quantity of

TABLE I

SEPARATION OF DNA AND RNA BY GEL FILTRATION

| | |
|---|---|
| Sample: | nucleic acids from liver, about 1 mg in 5 ml |
| Column: | diameter = 25 mm, height = 350 mm |
| Packing: | Sephadex G-200 in 0.1 *M* Tris–HCl, pH 7.2; 1 *M* NaCl |
| Eluent: | the same buffer as for the packing |
| Pressure: | 700 mm of solution column |
| Flow: | 30 ml/h |
| Measurement: | continuous recording of optical transmission at 254 mμ |
| *Desalting* | |
| Sample: | fractions I and II evaporated to 5 ml |
| Column: | diameter = 25 mm, height = 350 mm |
| Packing: | Sephadex G-50 in water |
| Eluent: | water |
| Pressure: | 500 mm of water column |
| Flow: | 500 ml/h |
| Measurement: | optical transmission as above and electrical conductivity |

RNA of low molecular weight and oligo- and mono-ribonucleotides which were present in the extract, as shown also by chromatography on methylated albumin.

In the lower part of Table I the experimental conditions for desalting fractions I and II on Sephadex G-50 are reported, as a great quantity of salts present in the eluate must be eliminated.

In Table II the analytical results for the separation given in Fig. 1 are reported. The DNA has been determined by the diphenylamine method according to BURTON[2].

TABLE II

RESULTS OF THE CHEMICAL ANALYSIS OF A SAMPLE OF DNA AND RNA BEFORE AND AFTER SEPARATION AND DESALTING

| | *Volume (ml)* | *DNA (μg)* | *RNA (μg)* |
|---|---|---|---|
| Sample | 5 | 270 | 850 |
| Peak I | 29 | 264 | < 0.5 |
| Peak II | 39 | < 0.5 | 325 |
| Peak I after desalting | — | 261 | — |
| Peak II after desalting | — | — | 284 |

Small amounts of RNA in the DNA fraction were not determined by the orcinol method because the great quantity of DNA present interferes and gives elevated values for RNA. Therefore the following method was adopted: The samples (2 ml) are digested at 37° for one hour with an equal volume of 2 *N* NaOH. One millilitre of cooled 4 *N* $HClO_4$ is then added to the cooled solution, which is centrifuged at 4000 r.p.m. for 10 min (according to DEFRANCE *et al.*[4]). The RNA in the supernatant is determined as P according to BEREMBLUM AND CHAIN[3].

As shown in Table II the traces of RNA and DNA in fractions I and II, respectively, are near the sensitivity limits of the methods.

When the nucleic acid solution contains a DNA of low molecular weight, the RNA fraction is treated with DNAase before desalting on Sephadex G-50.

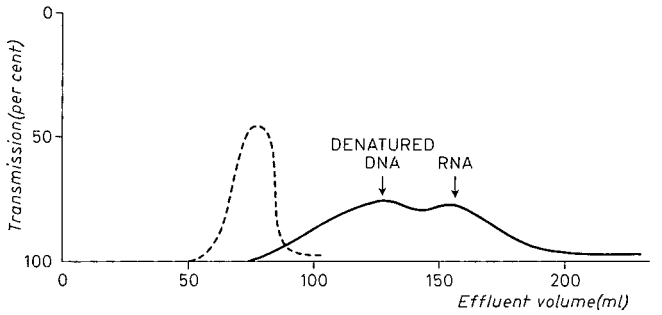


Fig. 2. Chromatogram of a sample of nucleic acids, as in Fig. 1, after alkaline denaturation. The dotted line is the DNA peak from Fig. 1.

It is probable that the separation of DNA and RNA on Sephadex G-200 is due to the difference in structure rather than the difference in molecular weight. According to this hypothesis a sample of DNA chromatographed under the conditions given in Table I after alkaline denaturation gives the elution curves shown in Fig. 2 (for comparison the non-denatured DNA is shown as a dotted line).

ACKNOWLEDGEMENT

The authors thank Mr. D. ZORRETTA for his skilled assistance.

SUMMARY

This paper describes the gel filtration of an extract of nucleic acids on Sephadex G-200. The experiments were performed on 2.5 × 30 cm columns with 0.1 *M* Tris–HCl pH 7.2 and 1 *M* NaCl at a flow rate of 30 ml/h. The optical transmission of the eluate was recorded.

Two peaks were obtained: peak I is due to deoxyribonucleic acid, peak II to ribonucleic acid and oligo- and mono-ribonucleotides which were present in the extract.

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# ADSORPTION PROPERTIES OF IODOTYROSINES AND DERIVATIVES ON SEPHADEX*

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Separation of iodotyrosines and derivatives can be achieved by many techniques, however no rapid preparative procedure is so far available. During our studies on the mechanism of the synthesis of thyroxine from diiodotyrosine and its ketoacid derivative, 4-hydroxy-3,5-diiodo-phenylpyruvic acid (DIHPPA)[1,2], it became evident that a method was needed by which the separation of the three components (diiodotyrosine, DIHPPA and thyroxine) and measurement of their concentration could be achieved.

Dextran gel chromatography had already been employed in other laboratories to separate iodotyrosines and thyroxine[3,4]. We have improved and extended this method, finding conditions in which separation of diiodotyrosine, DIHPPA and thyroxine is achieved in a single step by elution of a Sephadex G-25 column with 0.02 *N* NaOH. The behaviour of other iodotyrosines and tyrosines, as well as that of their acetic acid derivatives, has been studied under the same conditions.

## MATERIALS AND METHODS

The following pure reagents have been used: 3-iodotyrosine, 3,5-diiodotyrosine, 3,3',5-triiodothyronine and thyroxine (Sigma); 3,5-diiodothyronine and 3-iodothyronine (Warner-Chilcott); thyronine, 3',5'-diiodothyronine and 3,3',5'-triiodothyronine (Calbiochem); 3,5-diiodothyroacetic acid and 3,3',5-triiodothyroacetic acid (Aldrich Chem. Co.). DIHPPA (Osaka Synthetic Chem. Labs.) was recrystallized twice before use.

$^{125}$I-diiodotyrosine was obtained from New England Nuclear Co. Sephadex G-25 was purchased from Pharmacia.

### *Column chromatography*

A column (1.5 × 18 cm) of Sephadex G-25 was used, and eluted at room temperature with 0.02 *N* NaOH at a flow rate of 0.55 ml/min. Fraction volumes of 3.25 ml or smaller have been collected using an automatic fraction collector.

## RESULTS AND DISCUSSION

Thyroxine synthesis from $^{125}$I-diiodotyrosine and DIHPPA was achieved with a yield of 43 %[3]. The reaction mixture, containing diiodotyrosine, DIHPPA and the newly synthesized thyroxine, was put on a G-25 column at the end of the incubation

* Supported in part by the PHS research grant AM-06860/03.

time. The column was eluted with 0.02 *N* NaOH. The elution profile, followed by measuring the optical density at 325 mμ and the $^{125}$I-radioactivity of each fraction, is shown in Fig. 1. Three peaks are clearly visible. These peaks, analyzed by paper

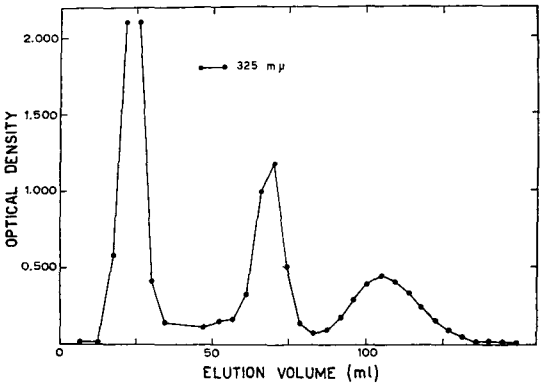


Fig. 1. Fractionation on Sephadex G-25 of a mixture of $^{125}$I-diiodotyrosine and DIHPPA incubated in the conditions for the synthesis of thyrosine. Elution medium: 0.02 *N* NaOH.

chromatography in *n*-butanol–ethanol–0.5 *N* ammonia (5:1:2) and by U.V. spectrophotometry, have been identified as diiodotyrosine (first peak), thyroxine (third peak) and diiodobenzaldehyde (DIBA) (middle peak); this aldehyde derivative of DIHPPA is quantitatively produced from DIHPPA[5,6] under our elution conditions. Table I shows the values of the partition coefficients ($K_d$) for the pure compounds diiodotyrosine, DIBA and thyroxine.

This method has the advantage that purified thyroxine can be obtained very easily in a single step. It can be used for both micro- and macro-scale preparation of pure thyroxine. Furthermore, kinetic studies, which so far could not be easily per-

TABLE I

PARTITION COEFFICIENTS OF TYROSINE AND THYRONINE DERIVATIVES

| *Compound** | $K_d$ |
|---|---|
| Tyrosine | 0.32 |
| 3-Iodotyrosine (MIT) | 0.36 |
| 3,5-Diiodotyrosine (DIT) | 0.52 |
| 3,5-Diiodobenzaldehyde (DIBA) | 3.10 |
| Thyronine ($T_0$) | 0.52 |
| 3-Iodothyronine | 0.93 |
| 3,5-Diiodothyronine | 1.13 |
| 3,5,3′-Triiodothyronine ($T_3$) | 2.35 |
| 3,5,3′,5′-Tetraiodothyronine (thyroxine) | 5.20 |
| 3′,5′-Diiodothyronine | 1.95 |
| 3,3′,5′-Triiodothyronine (reverse $T_3$) | 4.40 |
| 3,5-Diodothyroacetic acid | 1.54 |
| 3,5,3′-Triiodothyroacetic acid | 2.60 |

* 1.5 mg in 0.5 ml 0.02 *N* NaOH.

formed, can now be made because the amount of thyroxine synthesized, as well as the concentration of unreacted diiodotyrosine and DIHPPA, can be determined very easily.

The $K_d$ values of DIBA and thyroxine clearly show that they are adsorbed on the dextran. In order to get a clearer picture of the mechanism of adsorption of these compounds, other related iodoamino acids have been eluted under the same conditions. Table I shows that the partition coefficients of tyrosine and its iodinated derivatives (MIT and DIT) are not very different.

The iodothyronines, however, behave differently. The elution volume is greatly increased with the increasing number of iodine atoms per molecule. Also, the position of the iodine atoms on the two phenyl rings influences the elution volume and the $K_d$ (see Fig. 2 and Table I). With regard to the dependence of elution volume on the

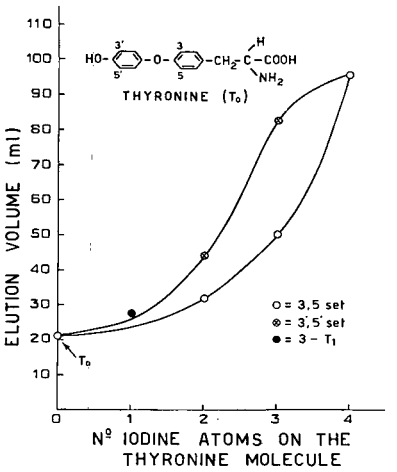


Fig. 2. Dependence of the elution volume of thyronine derivatives on the number of iodine atoms on the molecule.

number of iodine atoms per molecule, two groups of iodothyronines can be distinguished: (1) the 3,5 and (2) the 3',5' group, in which the 3 and 5 positions or the 3' and 5' positions, respectively, are always occupied by iodine (see Fig. 2). The 3',5' group shows a larger increase in the elution volume with increase of the number of iodine atoms on the molecule. This indicates that adsorption is probably effected somehow through the phenolic hydroxyl group.

From the results reported in Fig. 1, *i.e.* the separation between diiodotyrosine and DIBA, a further possibility appears, namely that the nature of the side chain residue modifies the degree of adsorption of the two substances (DIT and DIBA). The situation seems to be similar in the thyronines. In Table I, the $K_d$ values of diiodo and triiodothyroacetic acids can be compared with those of diiodo and triiodothyronine. The $K_d$ values of the acetic acid derivatives in both cases slightly exceed those of their alanine analogs. The presence of the amino group on the side chain of the thyronines seems to decrease slightly the adsorption of the iodothyronines to dextran.

SUMMARY

Iodinated derivatives of tyrosine and thyronine have been eluted through a Sephadex G-25 column with 0.02 *N* NaOH as eluent. Under these conditions these compounds become reversibly adsorbed to the dextran matrix, and this, in most cases, allows their separation.

A list of the partition coefficients ($K_d$) of the substances tested is presented. Furthermore, a tentative relationship between the structure of the compound and its elution volume on Sephadex has shown that the presence of iodine on the hydroxyl-carrying phenol of the thyronine derivatives strongly influences the degree of adsorption, more than the presence of iodine on the alanine-carrying phenol. Side-chain effects have been studied by means of the acetic acid analogues of diiodo- and triiodothyronine; the amino group slightly decreases the degree of adsorption of these substances.

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# GEL FILTRATION APPLIED TO SOME CLINICAL PROBLEMS

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In the present communication the preliminary results obtained by applying gel filtration to the study of some urinary enzymes and serum proteins, in clinical problems, will be briefly summarized.

## URINARY ENZYMES

Gel filtration was used to study the possible occurrence of amylase isoenzymes, and it was shown that two amylolytic enzymes which could be separated by Sephadex G-100 gel filtration (Fig. 1)[1] occur in human urine. This was in apparent contrast with the data of other workers[2]. Peak II, on the basis of its catalytic properties, appeared to be an α-amylase[3]. Peak I, on the other hand, exhibited, in addition, maltase activity, and could be further fractionated into two enzymes by means of Sephadex G-200, as shown in Fig. 2. These two enzymes differ from α-amylase in several catalytic properties[4,5]. By means of gel filtration it was thus possible to find in human urine two polysaccharide-splitting enzymes, which can be regarded as gluco-amylases (E.C. 3.2.1.3) or α-glucosidases (E.C. 3.2.1.20). They probably bear some relationship

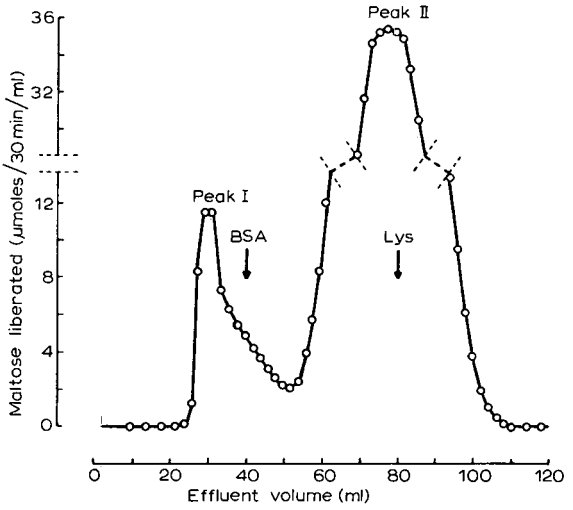


Fig. 1. Elution pattern of amylolytic activity from a Sephadex G-100 column. 1 ml of 1000-fold concentrated urine was applied to the column, and amylolytic activity (substrate: starch) was determined in the eluted fractions. The elution volumes of beef serum albumin (BSA) and lysozyme (Lys) are marked by arrows, for comparison.

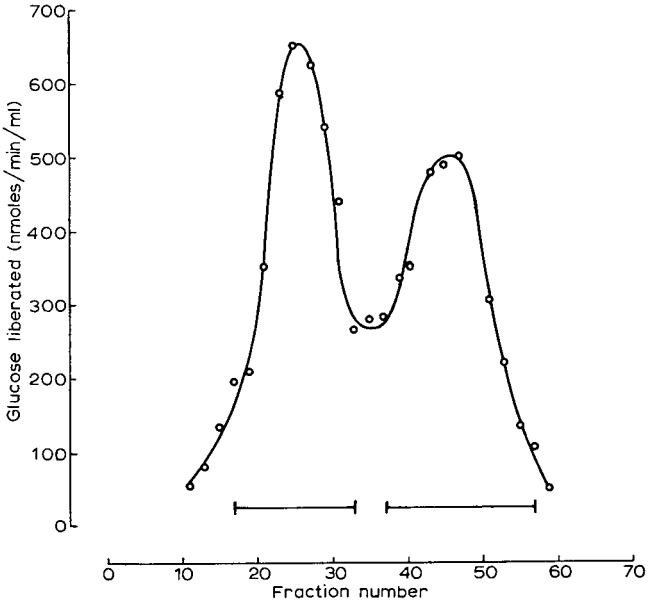


Fig. 2. Elution pattern of maltase activity from a Sephadex G-200 column: pooled, concentrated fractions, corresponding to peak I of Fig. 1, were applied to the column, and enzyme activity was determined in the eluate with maltose as a substrate.

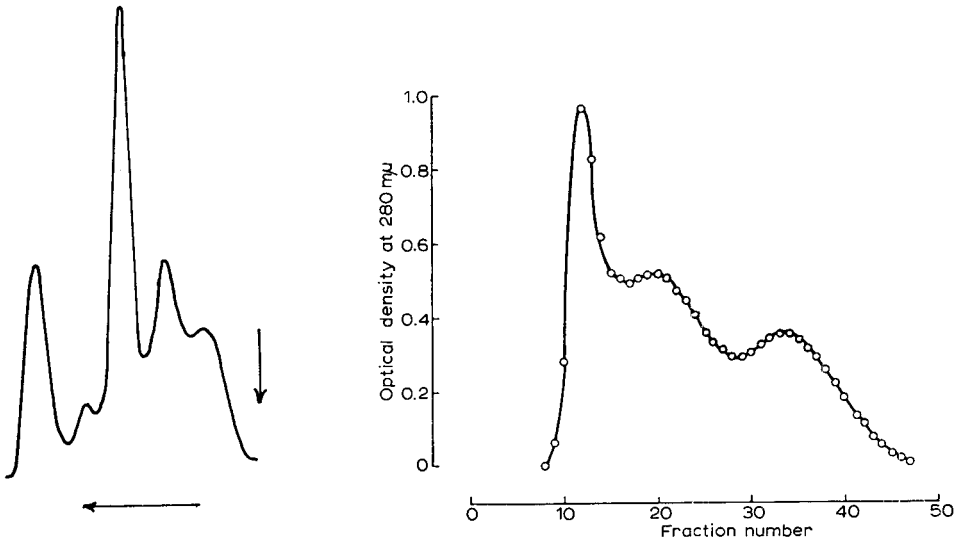


Fig. 3. Electrophoretic pattern (paper electrophoresis) of serum proteins from a patient suffering from malignant granuloma. Note the high increase of $\alpha_2$-globulin.

Fig. 4. Gel filtration pattern of the same serum as in Fig. 3. Note the increase in the 19 S fraction.

to the "maltase" previously described in human urine[6], to the enzymes referred to by Dr. AURICCHIO in this Symposium[7], and to some enzymes described by several workers in some animal organs (*cf.* refs. 8 and 9).

## SERUM PROTEINS

A method has been studied for the quantitative determination of $\gamma_1$- and $\alpha_2$-macroglobulins, by combined Sephadex G-200 gel filtration and cellulose acetate electrophoresis. The method has been applied to the study of the sera from patients suffering from malignant granuloma, where an increase of $\alpha_2$-globulin takes place[10]. In some cases we found this to be due, at least in part, to an increase of the $\alpha_2$-macroglobulin[11]. A clear example is shown in the following figures: Fig. 3 shows the electrophoretic pattern of the serum from a malignant granuloma patient, with a very high increase in $\alpha_2$-globulin. Fig. 4 shows the gel filtration pattern of the same serum (on Sephadex G-200), with the increase in the 19 S fraction. Fig. 5 shows the electrophoretic pattern of the 19 S fraction, compared with whole serum. Clearly most of the 19 S fraction is made up of $\alpha_2$-macroglobulin, which accounts for about 20 % of total serum proteins.

The possibility of assessing the molecular size of antibodies is a further application of gel filtration in the field of serum proteins[12,13]. The opportunity of studying a serum with a very high level of cold agglutinin[14] was presented. By means of gel filtration on Sephadex G-200 an increase of the 19 S fraction was demonstrated, which is in accordance with data obtained by means of ultracentrifugal analysis[15], and the molecular class of the agglutinin itself, which, as known, belongs to the 19 S group (Fig. 6) was characterized.

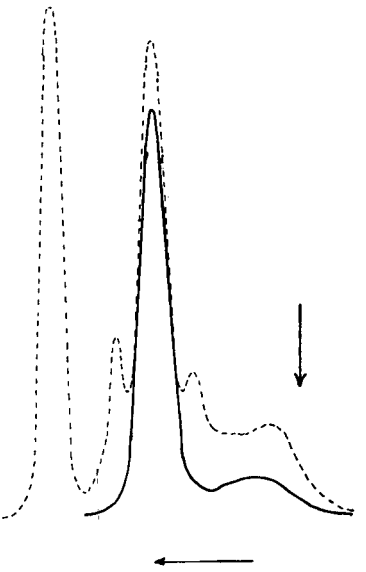

Fig. 5. Electrophoretic pattern (cellulose acetate) of the 19 S fraction obtained by the gel filtration experiment of Fig. 4 (continuous line), in comparison with the whole serum from the same subject (dotted line). The 19 S fraction is made up almost entirely of $\alpha_2$-globulin.

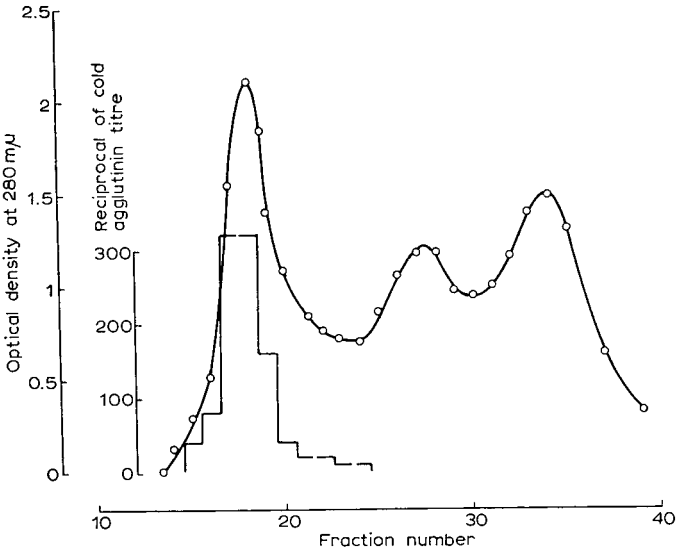


Fig. 6. Elution pattern of protein and cold agglutinin from a Sephadex G-200 column. 2 ml of a serum with a very high cold agglutinin titre (1:51, 200) were fractionated. The cold agglutinin is eluted with the 19 S peak.

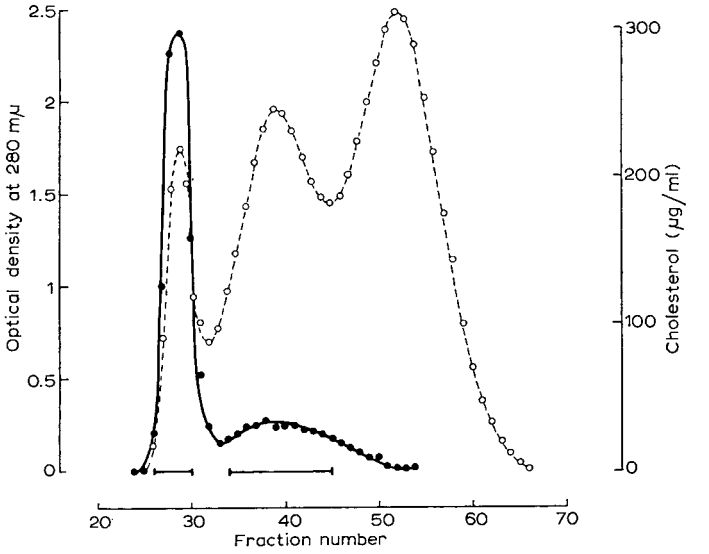


Fig. 7. Elution pattern of cholesterol (continuous line) and protein (as optical density at 280 mμ, dotted line), obtained by fractionating whole serum on a Sephadex G-200 column. Cholesterol is eluted into two peaks.

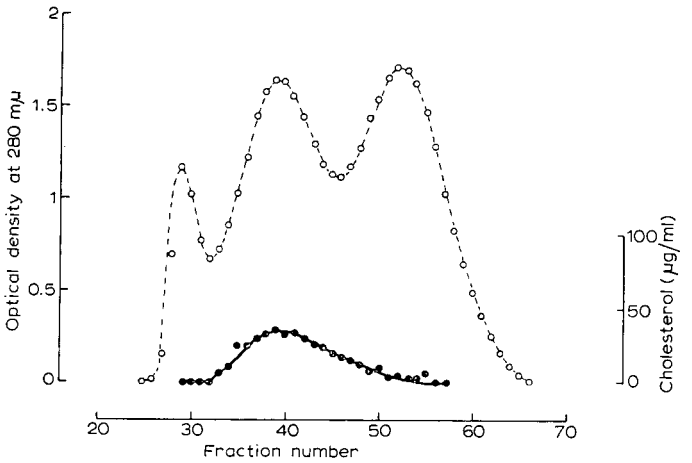


Fig. 8. Same as Fig. 7, obtained by fractionating $\beta$-lipoprotein-depleted serum.

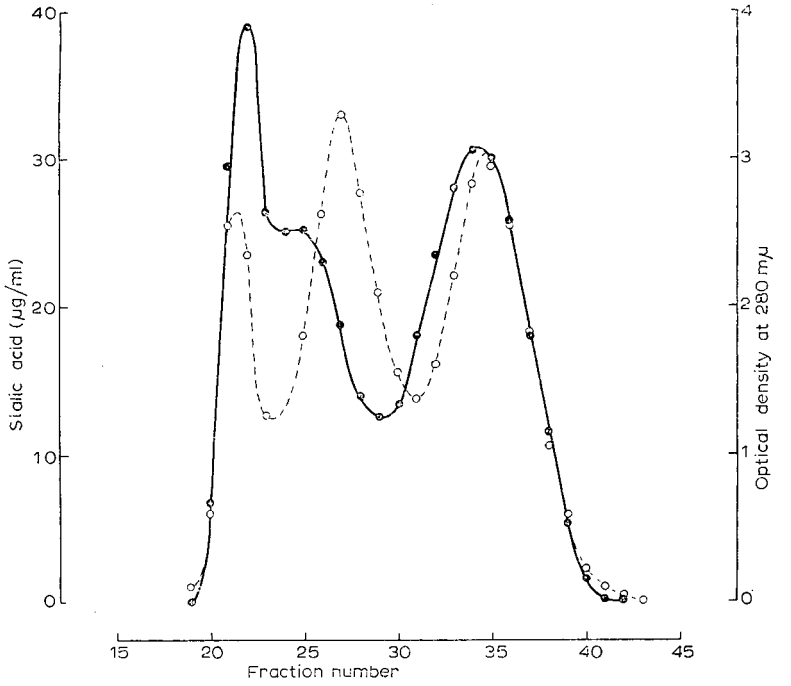


Fig. 9. Elution pattern of sialic acid (continuous line) and protein (as optical density at 280 m$\mu$ dotted line) obtained by fractionating adult serum on Sephadex G-200.

Conjugated proteins of human serum were also studied, in our laboratory, by means of gel filtration.

Lipoprotein distribution in the effluent fractions from a Sephadex G-200 column was monitored by means of cholesterol determination. Two peaks of cholesterol were obtained with whole serum (Fig. 7), and one only with $\beta$-lipoprotein-depleted serum (Fig. 8). The correspondence between the two peaks and $\beta$- and $\alpha$-lipoproteins, respectively, was established by several criteria, including electrophoretic mobility and cholesterol/phospholipid ratios[16].

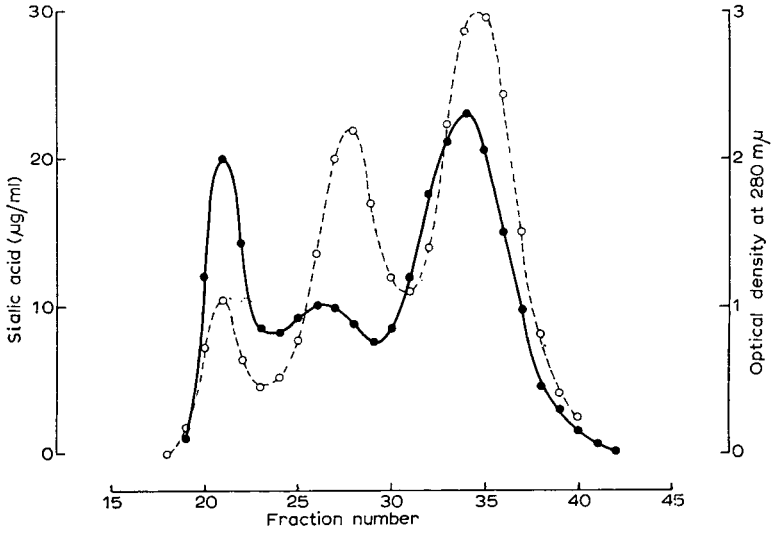


Fig. 10. Same as Fig. 9, obtained by fractionating cord serum on the same column.

Glycoprotein distribution was studied by determining the sialic acid content in the effluent fractions from a Sephadex G-200 column[17]. In search for an explanation for the low sialic acid levels in cord sera[18], both adult and cord sera were fractionated. Results are shown in Fig. 9 for an adult serum, and in Fig. 10 for a cord serum. In both cases sialic acid was recovered in three main peaks, roughly corresponding to the three protein peaks, the second being somewhat displaced. Quantitative distribution of sialic acid among the three peaks is shown in Table I. All the three fractions from

TABLE I

DISTRIBUTION OF SIALIC ACID AMONG THREE FRACTIONS OBTAINED BY SEPHADEX G-200 GEL FILTRATION

The data are the mean values from five experiments and are expressed both as mg/100 ml serum and as percentage of total recovered.

| | *19 S fraction* | | *7 S fraction* | | *4 S fraction* | |
|---|---|---|---|---|---|---|
| | *mg/100 ml* | % | *mg/100 ml* | % | *mg/100 ml* | % |
| Adult sera (5) | 16 | 21 | 20 | 24 | 43 | 55 |
| Cord sera (5) | 12 | 25 | 9 | 18 | 29 | 57 |

cord sera contained a lower amount of sialic acid, the more pronounced lowering being found in the 7 S fraction. The reasons for these findings, as well as for the displacement of the second peak of sialic acid, are now being investigated in our laboratory.

All the gel filtration experiments referred to, herein, were performed with columns of cross-linked dextran gels, marketed by Pharmacia (Uppsala) under the trade name Sephadex.

SUMMARY

When gel filtration was applied to the study of human amylolytic enzymes, it was possible to show in human urine the occurrence of two α-glucosidases, which could be separated from α-amylase.

In the field of serum proteins the following results were achieved: the hyper-$\alpha_2$-globulinemia of malignant granuloma was found to be due, at least in part, to a high level of $\alpha_2$-macroglobulin; an elevation of the 19 S peak was found to occur in cold agglutinin disease, and the antibody itself eluted in the same peak; α- and β-lipoprotein eluted separately; the quantitative distribution of sialic acid among the gel filtration fractions was studied in adult and cord sera.

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# INVESTIGATIONS ON FICIN

## III. PURIFICATION OF FICIN BY GEL FILTRATION AND THE CHARACTERIZATION OF OTHER PROTEIN FRACTIONS OF FICUS LATEX

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After earlier success in the column chromatographic purification of ficin on CM cellulose and then DEAE cellulose[1], attention has now been turned to a technique permitting not only the purification of ficin, but also the characterization of other protein fractions present in Ficus latex, used as a proteolytic agent in certain countries[2]. The choice fell on gel filtration, an efficient technique for the separation of biologically active proteins[3].

### EXPERIMENTAL AND RESULTS

Ten grams of a powder obtained in the crude form from the latex of *Ficus antihelmintica** were dissolved in 200 ml of water, 60 g of ammonium sulphate were added, and the solution was allowed to stand for 1 h at 5°. The resulting precipitate was separated off by centrifuging the solution at 10,000 r.p.m. for 10 min, washed three times with a 23% solution of ammonium sulphate, redissolved in 150 ml of water, and dialysed in a cold-room for 48 h with 10 l of distilled water. The resulting precipitate was removed by successive centrifuging, and, on being rendered lyophilic, the remaining clear solution gave about 5 g of pure sample.

50 mg of the sample were dissolved in 5 ml of a 0.005 *M* tris–phosphate buffer (pH 7.2, 0.9% of NaCl), placed on a 90 × 3 cm column packed with Sephadex G-100, and eluted with the same buffer at an average flow rate of 30 ml/h. The eluent fractions were monitored spectrophotometrically for proteins, which showed an extinction maximum at 280 mμ (broken curve in Fig. 1A). A 400 μl portion of each fraction was then incubated at 37° for 1 h with 1 ml of a 1% casein solution, the proteins were precipitated with 5% trichloroacetic acid, centrifuged, and the supernatant liquid was used to determine the extinction at 280 mμ. The enzymatic activity of the various eluent fractions, found in this manner, is shown by the full curve in Fig. 1A.

Whereas the broken curve (protein concentration) exhibits five peaks (and an inflexion high up in the central peak), the full curve (enzymatic activity) stops after the large central peak coinciding with the third and highest maximum in the dashed curve, and, therefore, this is attributed to ficin.

*Effect of temperature*

To study the effect of temperature on the enzymatic activity of *Ficus* proteins,

* Purchased in Colombia by Prof. G. Navarro, whom we wish to thank.

a number of solutions were prepared, each from 50 mg of the lyophilized sample and 5 ml of 0.005 *M* tris–phosphate buffer (pH 7.2). The solutions were incubated at 37° for various periods, and then eluted down a 90 × 3 cm Sephadex G-100 column. After incubation for two or more hours, the enzymatic activity of the fraction corresponding to ficin is reduced to about 25 % of the initial activity (Fig. 1B).

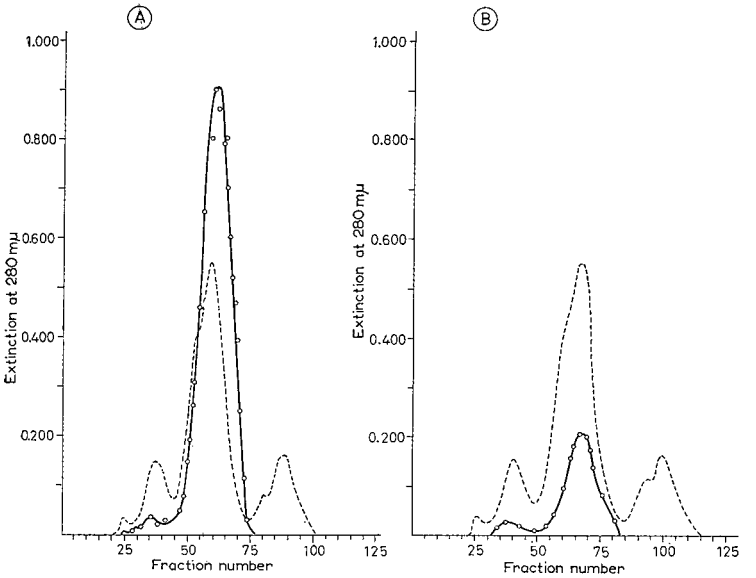


Fig. 1. (A) Extinction (at 280 mμ) of Ficus proteins separated on Sephadex G-100 (broken curve), and the corresponding enzymatic activity determined by Kunitz's casein method (full curve). (B) Enzymatic activity, after preincubation of the original protein mixture for 2 h at 37°. Extinction and enzymatic activity are indicated as in (A).

To find the cause of the inactivation of ficin, a 300 mg portion of the lyophilized mixture was first separated on Sephadex G-100, the resulting fractions were then individually incubated at 37° for various periods, and the enzymatic activity was finally determined at hourly intervals with the aid of 400 μl of casein solution. The results show that ficin almost entirely retains its activity even after 2 h at 37°. The inhibition observed in the previous experiment must therefore be due to a factor present outside the enzyme molecule, though still within the mixture.

Fig. 2 shows furthermore that incubation raises the enzymatic activity of the first two protein fractions F-1 and F-2. The question now is whether these contain ficin of other Ficus enzymes activated by incubation. To find out whether both F-1 and F-2 are complexes formed between a substrate and an enzyme that is activated by incubation and gives hydrolysates, 50 mg samples of F-1 and F-2 were dissolved in 5 ml of buffer and re-eluted through Sephadex G-100. A 400 μl portion of each eluent fraction was used to determine the enzymatic activity with casein, and the rest was kept at 37° for 5 h, treated with trichloroacetic acid, and centrifuged. The supernatant liquid containing the autolysates was used to determine the extinction at 280 mμ. The resulting curves (see Fig. 3) show the same variation in the enzymatic activity and in the autolysates. This indicates that each of the two protein fractions F-1 and F-2 is

composed of an enzyme and a substrate forming a higher-molecular-weight complex.

In order to confirm this result, 400 mg of F-1 and F-2 were fractionated on Sephadex G-100, the elution fractions were kept at 37° for 5 h, combined, dialysed, lyophilized, dissolved in tris–phosphate, and separated on Sephadex G-100. The protein determination was carried out by the Lowry-Folin method, the results being shown in Fig. 4. These peaks, the first two of which are very large, are probably

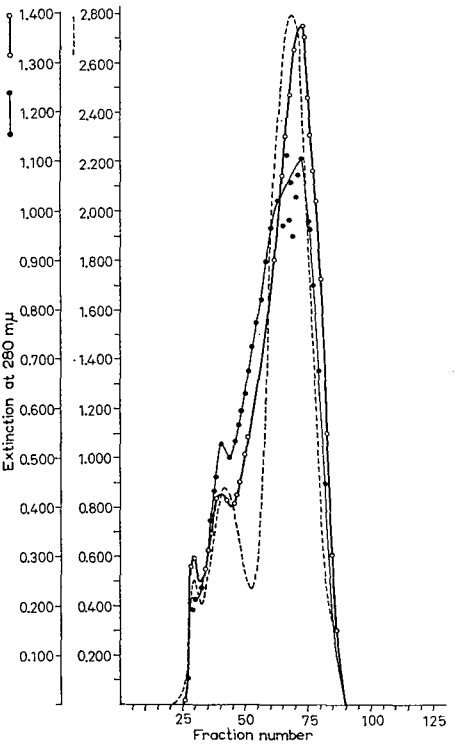


Fig. 2. Enzymatic activity without incubation of the fractions (o–o–o) and after incubation for 2 h at 37° (•–•–•). The protein concentration is shown by the broken curve.

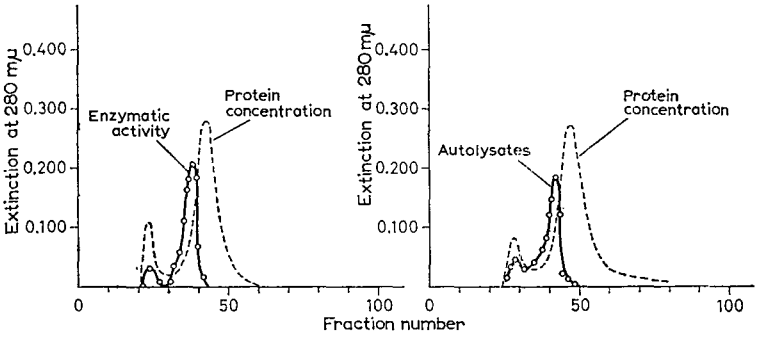


Fig 3. Correspondence between the enzymatic activity and the autolysates of the first two protein fractions shown in Fig. 1A.

ascribable to enzymatically active ficin and two inactive peptides. The protein fractions F-1 and F-2 are thus relatively stable, and cleavage leads to the same components as those found in nature. The curve for the enzymatic activity determined with casein and shown in Fig. 4 gives an even clearer representation of the process: a large activity maximum is found in the eluent volume corresponding to ficin, and since the enzyme is known to have been absent from the starting material used in this experiment, it must have been formed by the action of heat on the complex.

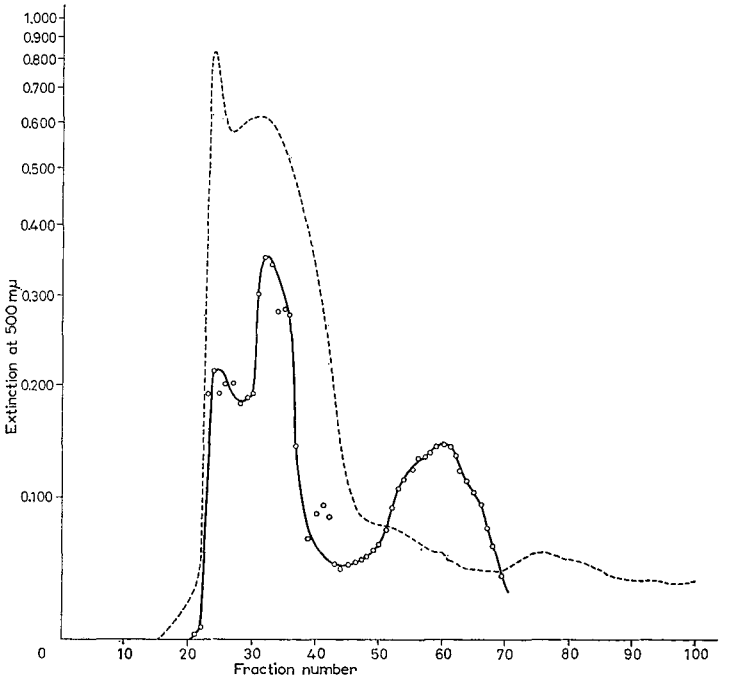


Fig. 4. Concentration of the two proteins F-1 and F-2 determined by the Lowry-Folin method (broken curve), and their enzymatic activity (full curve) determined with casein after autolysis. The thermal treatment gives rise to protein fragments, one of which coincides with the elution volume of enzymatically active ficin.

## DISCUSSION

Sephadex G-100 is particularly useful for the separation of Ficus proteins extracted with ammonium sulphate, and represents considerable labour saving with respect to earlier techniques[1].

The presence of an inflexion in the ficin peak (Fig. 1) and the asymmetrical disposition of the enzymatic activity curve about the protein concentration curve suggest the existence of two enzyme forms—probably isoenzymes—differing in activity and also somewhat in molecular weight.

ACKNOWLEDGEMENTS

The author wishes to thank Mrs. M. RANIERI and Mr. M. DI JORIO for their assistance.

SUMMARY

Purified ficin obtained by repeated precipitation with ammonium sulphate was passed through a column packed with Sephadex G-100.

Ficin and other protein fractions were obtained. The first two fractions, by heat effect (37°), give rise to proteolytic active fraction.

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# GEL FILTRATION OF MODIFIED AND DENATURED SERUM PROTEINS ON SEPHADEX G-200 AND ON PEARL CONDENSED AGAR

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An experimental blood volume expander has been prepared in our Institute by heat denaturation and formolation of bovine serum in the presence of partially degraded gelatin and by the oxidation of this mixture with hydrogen peroxide. This preparation was investigated from various points of view and was found to consist of a very complex mixture of modified protein molecules and their aggregates. Attempts made to characterize this heterogeneous system by different methods of fractionation (salting out, electrophoresis (Fig. 1), chromatography, sedimentation analysis) were not successful.

Relatively good fractionation, however, was achieved by gel filtration on Sephadex G-200 and on pearl condensed agar (see Fig. 2). Some of the experimental results will be referred to in the present communication.

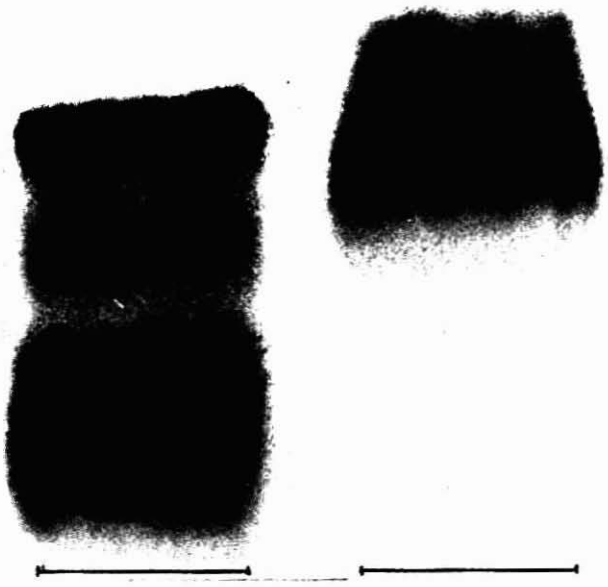

Fig. 1. A typical electrophoretic pattern of MBS. Buffer, pH 8.6 (veronal–citrate–oxalate buffer), native bovine serum on the left, MBS on the right.

* Director: Prof. J. HOŘEJŠÍ, M.D., D.Sc.

The columns used for gel filtration were usually 80 cm high and 1.6 cm in diameter. Two millilitre portions of 1–5 % protein solutions were applied on the top of the column and were eluted by Tris–HCl buffers, pH 7 and 8, at different salt concentrations from 0.01 $M$ up to 1 $M$ NaCl. The rate of flow was 2–5 ml/cm²/h at laboratory temperature. The concentrations of the eluted protein fractions were determined

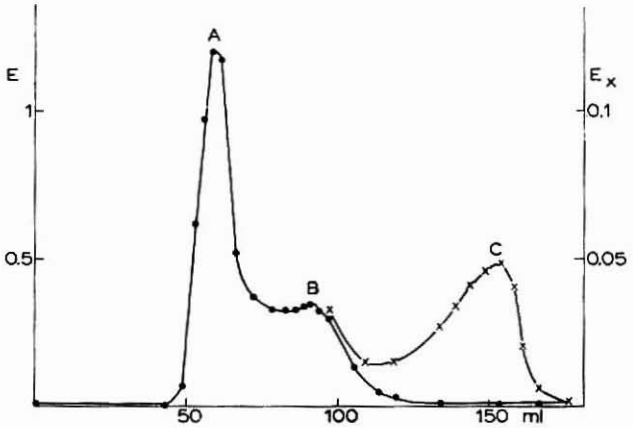


Fig. 2. Gel filtration of MBS on Sephadex G-200. Column: 76 × 1.4 cm, buffer: 1 $M$ NaCl + 0.1 $M$ Tris, pH 8.0. $E$ = Absorbance at 280 nm; $E_x$ = absorbance from the turbidimetric estimation of oxygelatin.

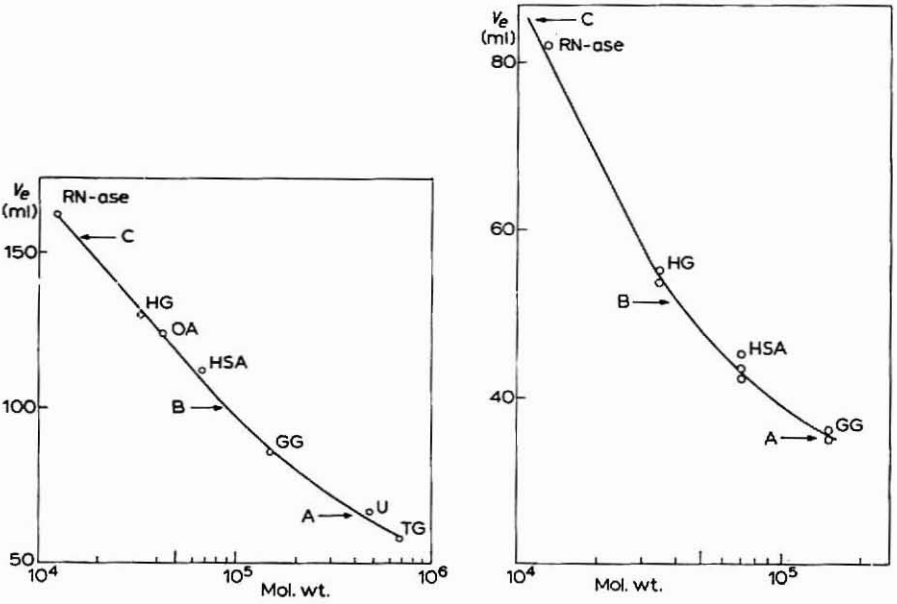


Fig. 3. Calibration curve of molecular weights of proteins on Sephadex G-200. RN-ase = Ribonuclease; HG = hemoglobin; OA = ovalbumin; HSA = human serum albumin; GG = human gamma globulin; U = urease; TG = thyreoglobulin. For A, B, and C, see Fig. 2.

Fig. 4. Calibration curve of molecular weights of proteins in 8 $M$ urea. For nomenclature see Fig. 3. Sephadex G-200; column: 41 × 1.6 cm, Tris buffer, pH 8.0 (*cf.* Fig. 2).

either by U.V.-spectrophotometry or turbidimetrically. Sephadex G-200 was of particle size 40–120 $\mu$m. Beads of 4 % Difco agar gel were prepared according to HJERTÉN by stirring and stepwise cooling an emulsion of a hot aqueous agar solution in a mixture of toluene (316 ml) and carbon tetrachloride (86 ml) in the presence of Tween 60 (0.2 ml). After washing and sieving, beads of 25–100 mesh were used to fill the column.

Fig. 2 shows the elution curves of modified bovine serum (MBS) on Sephadex G-200 indicating the presence of at least three groups of molecules of different molecular sizes. Peaks A and B belong to modified serum proteins, peak C to modified gelatin. When an attempt was made to estimate roughly the average molecular weights of peaks A, B and C from a calibration curve constructed on the basis of elution data of several native proteins (see Fig. 3), substantially higher values were attained than those found by sedimentation analysis (450,000 instead of 160,000 for A, 100,000 instead of 40,000 for B and 17,000 instead of 11,000 for C). This was due most probably to the unfolded irregular shape of the modified proteins, which could not penetrate the gel as easily as the globular calibrating proteins.

Interesting results in this respect were found when the gel filtration of all samples (*i.e.* of calibrating substances as well as of MBS) was carried out in buffers containing 8 *M* urea. Under these hydrogen bond breaking conditions the shapes of the various proteins under study were assumed to have greater similarity to each other. It was further assumed that no profound changes of the molecular weight took place. As is shown in Fig. 4, a relatively good agreement was found between the molecular weights of peaks A, B and C estimated from sedimentation data and from this calibration curve in 8 *M* urea.

However, the elution volume of the main peak of MBS was already near to the exclusion limit of the Sephadex G-200 column, so that the presence of relatively large particles could be expected especially in the front part of the peak. Since a higher content of such molecular aggregates in a blood volume expander could cause complications during infusion in the organism of the acceptor, it was important to investigate this question. Gel filtration of MBS on a looser gel than G-200, *e.g.* 4 % pearl condensed agar was found to be suitable for this purpose.

As can be seen in Fig. 5, the elution curve achieved on agar gel was quite dif-

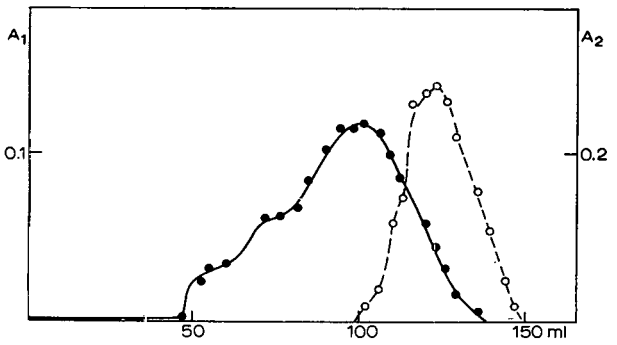


Fig. 5. Gel filtration of MBS on pearl-condensed 4 % agar. Column: 84 × 1.4 cm. Dotted line: oxygelatin. Buffer, *cf.* Fig. 2.

ferent from that on Sephadex G-200 (*cf.* Fig. 2), showing four peaks, the first being relatively small. The average molecular weight of this smallest fraction was of the order of $10^6$. At lower salt concentrations (in buffers diluted 50 and 100 times by water) the content of the high molecular weight fraction rose substantially (Fig. 6). This was probably due to the formation of aggregates stabilized mostly by electrostatic interactions since the aggregation was reversible after the addition of salt. This seemed to be confirmed also by viscosity measurements (see Fig. 7).

For a quantitative evaluation of the curves, with special attention to the high molecular weight fraction, the area under the first peak was expressed as a percentage of the total area limited by the elution curve and the ordinate. The following results

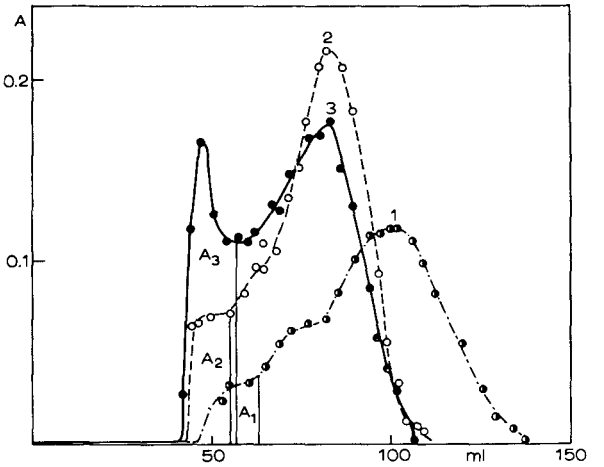


Fig. 6. Gel filtration of MBS on agar pearls at different salt concentrations. (1) Buffer 1 *M* NaCl + 0.1 *M* Tris, pH 8.0; (2) the standard buffer (1) diluted 1:50; (3) the standard buffer (1) diluted 1:100.

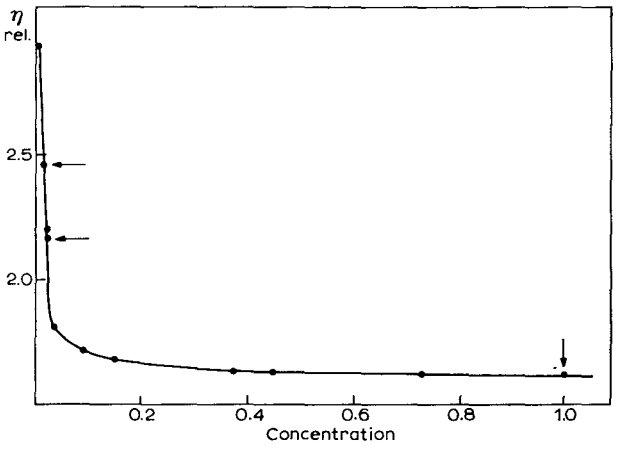


Fig. 7. The dependence of viscosity of MBS on salt concentration. Dots indicated by the arrows correspond to curves 1, 2 and 3 in Fig. 6.

were obtained: 8 % for curve 1, 11 % for curve 2 and 29 % for curve 3. In other MBS preparations (*e.g.* "Resorba", Germany) the content of the aggregates remained relatively high (35 %) even at physiological salt concentrations or at 1 *M* NaCl (see Fig. 8). It seems reasonable to expect that the high molecular weight fraction here consists of molecular aggregates bound by stable methylene bridges formed during the reaction of proteins with a greater concentration of formaldehyde.

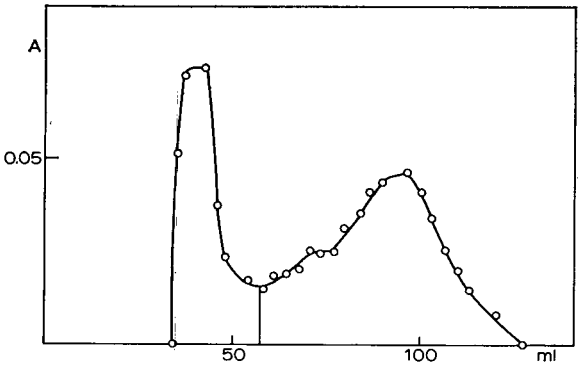


Fig. 8. Gel filtration of the plasma expander "Resorba" on agar pearls. Column: 84 × 1.4 cm. Buffer: 1 *M* NaCl–0.1 *M* Tris, pH 8.0.

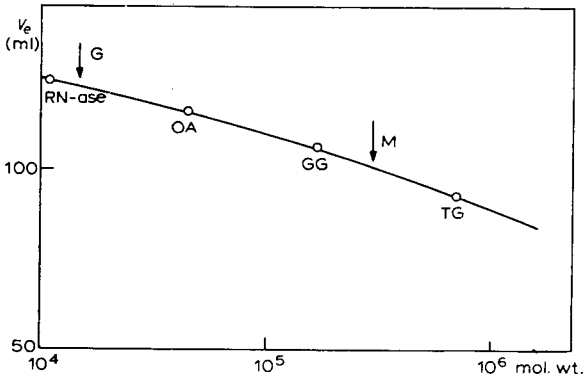


Fig. 9. Calibration curve of molecular weights of proteins on pearl condensed agar. For nomenclature see Fig. 3. $V_e$ of oxygelatin (G) and of the main peak of MBS (M) are indicated by arrows.

A calibration curve of molecular weights was also constructed from data obtained on agar gel columns. However its use has the same limitations as was mentioned in connection with Sephadex G-200 when estimating molecular weights of modified and denatured proteins (see Fig. 9).

It may be concluded that gel filtration on Sephadex G-200 and on 4 % pearl condensed agar proved very useful for the characterization of modified serum proteins. This technique is recommended as a method for checking the standard quality of different types and batches of blood volume expanders of this kind.

SUMMARY

Denatured and chemically modified bovine serum (MBS) proteins of a blood volume expander were characterized by gel filtration on Sephadex G-200 and pearl condensed 4 % agar gel. The presence of three respectively four main macromolecular fractions differing in particle size was detected in MBS. On Sephadex G-200 a satisfactory estimation of the molecular weights of some fractions of MBS was achieved using elution data from gel filtration in 8 *M* urea. The dependence of the aggregation of MBS proteins on salt concentration was studied by gel filtration and viscometry. A simple way for a quantitative evaluation of the elution curves on agar pearls was proposed as a means for checking the standard quality of MBS.

# THE SEPARATION OF TRYPTIC PEPTIDES BY GEL FILTRATION

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Our research work on the structural characterization of human abnormal hemoglobins involves the separation and purification by chromatographic and electrophoretic techniques of the hemoglobins studied, and also of the peptides resulting from their tryptic digests.

It was thought that it would be of some interest to report in this symposium on a problem that occurred in the course of the structural characterization of an abnormal human hemoglobin that was solved by means of gel filtration[1].

Some years ago, SILVESTRONI AND BIANCO[2,3] reported the occurrence of an abnormal fast hemoglobin in four Italian families, one of them of Sardinian origin, the others living in the outskirts of Cosenza. The hemoglobin had been classified by these authors as hemoglobin N (Hb N) on the basis of its electrophoretic mobility; in addition, as a result of hybridization and recombination experiments the hemoglobin had been proved to be an alpha chain variant. Samples of this hemoglobin have been subsequently committed to our research group for further characterization.

The abnormal Hb N was separated from hemoglobin A (Hb A), contained in the hemolysate, by starch block electrophoresis; both the purified hemoglobins A and N were subsequently submitted to digestion with trypsin[4]. The fingerprints[5] of Hb N were compared with those of Hb A (Figs. 1 and 2). One spot observed in Hb N was not present in Hb A; this abnormal spot is indicated in Fig. 1 with an arrow and was

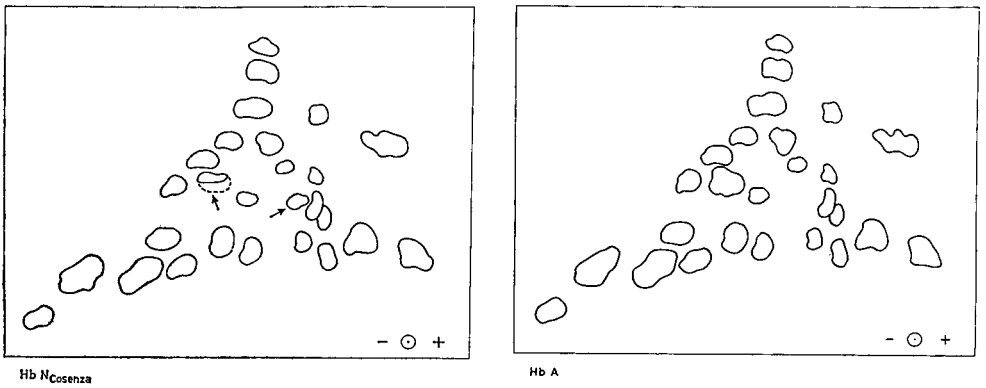


Fig. 1. Finger print tracing of Hb N. The arrow on the right indicates the abnormal peptide $\alpha^{N}$TpIII. The position of the homologous normal peptide $\alpha^{A}$TpIII is sketched with a dashed line and indicated by the arrow on the left.

Fig. 2. Finger print tracing of Hb A.

present in all the fingerprints from three different samples of hemoglobin N. One of the peptides normally present in Hb A, the position of which is indicated by a dashed line and the second arrow in Fig. 1, was found to be missing in Hb N; on the basis of its position this peptide has been identified as $\alpha^{A}$TpIII according to the system of nomenclature recommended by GERALD AND INGRAM[6]. It was therefore possible to deduce that the abnormal spot present in the Hb N fingerprint is due to a modified peptide $\alpha^{A}$TpIII and can be designated as $\alpha^{N}$TpIII. This identification is also supported by the fact that this peptide, as well as $\alpha^{A}$TpIII, shows a positive tryptophan reaction[7].

Peptide $\alpha^{A}$TpIII actually contains five amino acid residues of the alpha chain, namely alanyl-alanyl-tryptophanyl-glycyl-lysyl, which occupy the position 12 to 16 among the 141 residues composing this chain.

A further step in the characterization of the abnormal hemoglobin was the purification on a preparative scale of the modified peptide $\alpha^{N}$TpIII with a view to analysing its amino acid content and indentifying the substituent amino acids by comparison with the known amino acid composition of the corresponding peptide, $\alpha^{A}$TpIII, contained in Hb A.

The purification of the peptides is generally obtained by a combination of paper electrophoresis and paper and column chromatography; however, in this particular case, it could be expected that the purification of the abnormal peptide would be troublesome since the peptide $\alpha^{N}$TpIII, is positioned in a region of the fingerprint particularly rich in peptides, as already shown in Fig. 1.

Fortunately, the peptide $\alpha^{N}$TpIII studied contains tryptophan as had already been stated. It is well known that the amino acids containing an aromatic ring interact with dextran gel; it would therefore be expected that gel filtration on Sephadex would give better results, since the retardation due to the relative smallness of the peptide, composed of only five residues, would be enhanced by the retardation due to the interaction of tryptophan with the dextran gel; in other words a real chromatographic process could, in this instance, augment the gel filtration.

On the basis of the foregoing considerations we developed the following procedure: 50 mg of the tryptic digest of Hb N were chromatographed on a 0.9 ×

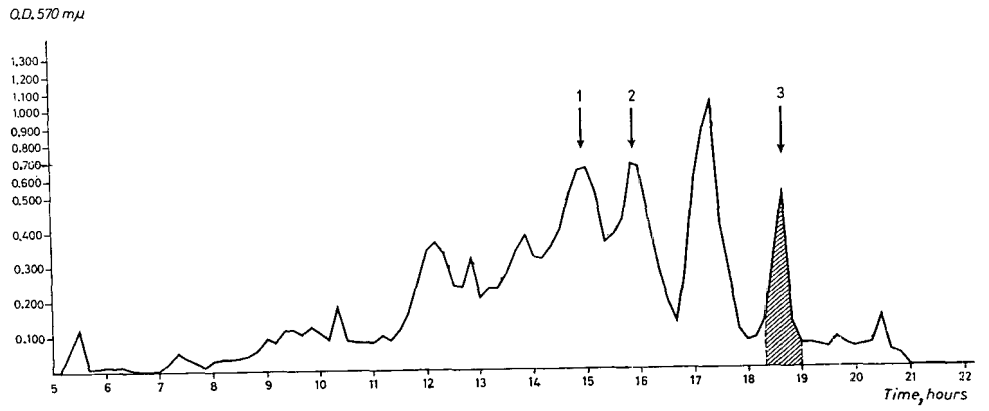


Fig. 3. Elution pattern of Hb N tryptic peptides from a Sephadex G-25 0.9 × 120 cm column (0.2 *N* acetic acid; flow rate 60 ml/h). Arrow No. 1 = $\beta^{A}$TpIV; arrow No. 2 = $\beta^{A}$TpII; arrow No. 3 = $\alpha^{N}$TpIII.

120 cm column of Sephadex G-25 equilibrated with 0.2 *N* acetic acid. The separation obtained with a flow rate of 60 ml/h is shown in Fig. 3. The peptide content has been determined on an aliquot of each fraction of the effluent by reading, at 570 mμ, the color developed by YEMM AND COCKING[8]. The normal Hb A contained in the blood of the same subject was also submitted to tryptic digestion and to gel filtration on Sephadex G-25 under the same experimental conditions. The elution curve of Hb A tryptic peptides is identical with that of Hb N.

Human hemoglobin contains three tryptophanyl residues, one of them present in the alpha chain, the others in the beta chain. In the elution curve the tryptophan-containing peptides are indicated by the arrows Nos. 1, 2 and 3 and appear among the last peaks as was to be expected owing to the interaction between tryptophan and dextran gel. The tryptophan-containing peptides have been identified by submitting aliquots of the effluent to the same chromatographic and electrophoretic procedure followed in the development of the fingerprint of the whole tryptic digest, and, of course, to the specific staining for tryptophan. The peak marked with arrow No. 1 contains $\beta^{A}$TpIV peptide constituting ten amino acid residues. In the next peak, marked with arrow No. 2, peptide $\beta^{A}$TpII, containing nine residues, is eluted; the dashed peak, marked with arrow No. 3, contains the abnormal $\alpha^{N}$TpIII peptide, or alternatively the normal peptide $\alpha^{A}$TpIII when the tryptic digest of Hb A is submitted to gel filtration. This was again to be expected since both peptides contain five residues including the tryptophanyl one and therefore should emerge from the column with the same $K_d$.

As it is evident, the tryptophan-containing peptides emerge from the Sephadex column with a $K_d$ which increases according to the decrease in their molecular size. This affords further evidence that in this experiment the molecular sieving and a real chromatographic process augment each other.

In this instance the technique of gel filtration has thus proved to be extremely useful in simplifying the preparation and purification of the peptide studied. This concludes our report on the work directly concerning gel filtration, however it could be of some interest to mention the results of the experiments we spoke of.

Both the abnormal peptide $\alpha^{N}$TpIII and the normal one, $\alpha^{A}$TpIII, were subjected to acid hydrolysis with 6 *N* hydrochloric acid at 110° for 24 h. The quantitative amino acid analysis[9] of the acid hydrolysate gave the results reported in Table I.

As is well known, tryptophan undergoes almost complete destruction in the course of acid hydrolysis.

TABLE I

AMINO ACID ANALYSIS OF $\alpha^{A}$TpIII AND $\alpha^{N}$TpIII PEPTIDES (μMOLES/μMOLE PEPTIDE)

| | *$\alpha^{A}$TpIII* | *$\alpha^{N}$TpIII* |
|---|---|---|
| Ala | 1.9 μmoles | 1.8 μ moles |
| Gly | 1.1 μmoles | 0.1 μmoles |
| Asp | 0.1 μmoles | 1.0 μmoles |
| Lys | 1.1 μmoles | 1.0 μmoles |
| Try | + | + |

These results prove that in the abnormal peptide an aspartyl residue is present instead of the glycyl residue normally occurring in $\alpha^{A}$TpIII peptide. It is most likely that the aspartyl residue occupies position 15 in the amino acid sequence of the Hb N alpha chain, which is normally occupied by a glycyl residue in the corresponding chain of Hb A.

As it is well known that asparagine is quantitatively converted into aspartic acid at the beginning of acid hydrolysis, it was necessary to establish whether the substituent amino acid in the abnormal peptide was originally an aspartyl or an asparagyl residue. The peptide was therefore digested first with carboxypeptidase B and then with carboxypeptidase A.

Carboxypeptidase B liberated one residue of lysine per mole of the peptide, but carboxypeptidase A did not act any further. It is known[10] that carboxypeptidase A does not liberate acidic amino acids under the experimental conditions which have been used in the present work. An aspartyl residue should therefore occupy position 15 immediately preceding that of the carboxyl-terminal lysyl residue of the abnormal peptide. Had it, on the other hand, been an asparagyl residue, which is not an acidic amino acid, it should have been liberated by the action of carboxypeptidase A.

The abnormality present in the Hb N studied can therefore be identified as the substitution of aspartic acid for glycine 15 which normally occurs in the amino acid sequence of the Hb A alpha chain. An identical structural abnormality has been already reported[11] under the name of hemoglobin $J_{Oxford}$ in the hemoglobin present in the blood of some subjects in an English family.

SUMMARY

Gel filtration has proved most useful in the separation on a preparative scale of the abnormal peptide $\alpha^{N}$TpIII present in the human hemoglobin variant $\alpha_2{}^{15Asp}\beta_2$.

The abnormal peptide contains a tryptophanyl residue whose aromatic ring interacts with dextran gel; in this instance, therefore, a real chromatographic process augments the separation due to gel filtration.

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# ESTIMATION OF BLOOD LIPOPROTEINS BY RADIAL IMMUNODIFFUSION AFTER AGAROSE GEL FILTRATION

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Gel filtration on columns of 2 % agarose gel beads is capable of separating nearly spherical particles in the molecular weight range $10^5$ to $10^7$ (ref. 1). This allows the fractionation of blood plasma lipoproteins: The framework of the gel is sufficiently loose to retard $\alpha_1$-lipoproteins, $\beta$-lipoproteins and lipid particles up to the $S_f$ 400 class; larger particles elute with the front. In addition to monitoring the eluates for turbidity (at 700 m$\mu$), and protein content (at 280 m$\mu$) it is possible to analyze their total lipid content, and its varying components. Since the lipoprotein classes differ in their composition, this will give characteristic patterns. More direct information would however be obtained by quantitation of the individual lipoproteins themselves in the eluates.

This paper describes analysis of serum lipoproteins, and other proteins, after fractionation by gel filtration by radial immunodiffusion[2]. In this method a specific antibody is incorporated into a layer of gelled agarose. Wells are punched out, and samples of the elution fractions placed in them. The antigen-containing sample then diffuses into the antibody-containing agarose, where specific antigen–antibody complexes precipitate in an extending circular area until all antigen is used up. The final area of precipitation is proportional to the antigen concentration in the sample.

## EXPERIMENTAL

### *Preparation and operation of columns*

Details have been previously described[1]. Columns with a total bed volume of 200 to 230 ml (height 40 to 45 cm, inner diameter 2.5 cm) were prepared from gelled agarose beads (mesh size 30 to 60). Phosphate buffer (0.2 *M*, pH 7.4) was used as eluent. The high molarity was chosen to exclude adsorption, and aggregation of lipoproteins. Usually 10 ml freshly drawn serum was used as sample.

### *Preparation of radial immunodiffusion plates*

An exactly 0.1 cm thick polyethylene strip (about 0.5 cm width) was put between two glass plates (20 × 10 cm) along the two narrow ends. The plates were then made watertight by sealing with polyethylene adhesive tape (Shandon Labortechnik, Frankfurt) the two narrow edges, and one long side of the plate, thus forming

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a flat chamber. Before pouring the agarose–antibody mixture into this, the plates were warmed to 50° in an incubator.

0.25, 0.50 or 0.75 g agarose (Industrie Biologique Française) were dissolved in 20 ml Michaelis buffer (sodium diethylbarbituric acid 0.1 *M*, pH 8.6) in a water bath at 90° to 100°. The solution was cooled to about 48°, antiserum added in the desired concentration, and the volume of the mixture brought to 25 ml with buffer (final agarose concentration: 1 %, 2 %, or 3 %). Antiserum and agarose solution were mixed with a glass rod, avoiding the formation of air bubbles. The mixture was then poured immediately between the pre-warmed glass plates. Once the agarose has gelled the plates are ready for use. They can be kept at 4° for weeks.

Plasma protein antisera from the rabbit (Behringwerke, Marburg) were used in the following concentrations: anti-$\alpha_1$-lipoprotein serum, 2 %; anti-$\beta$-lipoprotein serum, 1 %; anti-$\alpha_2$-macroglobulin serum, 0.2 % and 0.5 %; anti-antitrypsin serum, 2 %; anti-acid-$\alpha_1$-glycoprotein serum, 2 %; anti-$\gamma$G-globulin serum, 4 %. The concentrations had to be adjusted to the size of the original serum sample, and to the potency of the antiserum.

*Radial immunodiffusion*

The adhesive tape was removed from the plates, and one glass plate detached from the agarose layer. (It is possible to siliconize one glass plate prior to use to facilitate this.) Sample wells (1.6 mm diameter) were punched out with a suction needle (as used for immunoelectrophoresis, Beckmann Instruments). Usually a distance of 12 to 15 mm from well to well was chosen. 2 $\mu$l of antigen-containing sample solution were placed in each well with a Hamilton pipette. For immunodiffusion the plates were kept in a high humidity chamber at room temperature (usually 24 h). The non-precipitated proteins were then eluted with saline for 24 h, the precipitates fixed, and stained with saturated amidoblack solution (amidoblack 10 B 25 g, methanol 1250 ml, distilled water 1000 ml, acetic acid 250 ml), and the plates decolorized with methanol–glacial acetic acid (9:1; v/v). The diameters of the precipitates were measured in two directions at right angles with a lens fitted with a scale reading to 1/10 mm, the square of the radius being used as a measure of the precipitation area. For documentation the agarose layer can be dried under a moist filter paper, and removed from the glass plate as a film.

Calibration curves were made from dilutions of a standard serum for each individual plate. The results were expressed in per cent concentration of this standard.

*Other analytical methods*

Optical density measurements at 280 m$\mu$ and 700 m$\mu$ were made spectrophotometrically on a Zeiss PMQ spectrophotometer. Lipids were extracted according to the method of FOLCH *et al.*[3], and total lipids determined by the dichromate method[4,5]. Alternatively the lipids were estimated directly in the eluates with the sulfophosphovanillin method[6,7] after lyophilization (100 $\mu$l samples). This method, while avoiding tedious extraction of lipids, gave the same results as the dichromate method. Cholesterol was estimated directly in the eluates by the method of SEARCY, BERGQUIST AND JUNG[8], with slight modifications. For thin-layer chromatography of the lipid extracts mixtures of petroleum ether–ethyl ether (90:10, v/v) were used. Spots were rendered visible by charring with 50 % $H_2SO_4$ and heating at 200° for 1 h.

RESULTS

Fig. 1 is the photograph of an immunodiffusion plate, where $\alpha_1$-lipoproteins have been precipitated by a specific antiserum. Samples of successive elution fractions of human serum separated on a column of 2 % granulated agarose were placed in the different wells. Reading from the top and each row from right to left, the antigen concentration increases to a maximum, then decreases. Dilutions of a standard serum used for calibration are in the bottom row. Samples stored for a prolonged period at 4° sometimes produced a second inner ring, when $\alpha_1$-lipoprotein was precipitated. It only appeared if the $\alpha_1$-lipoprotein concentration was high and was always proportional to the main outer ring. No other antiserum produced double rings.

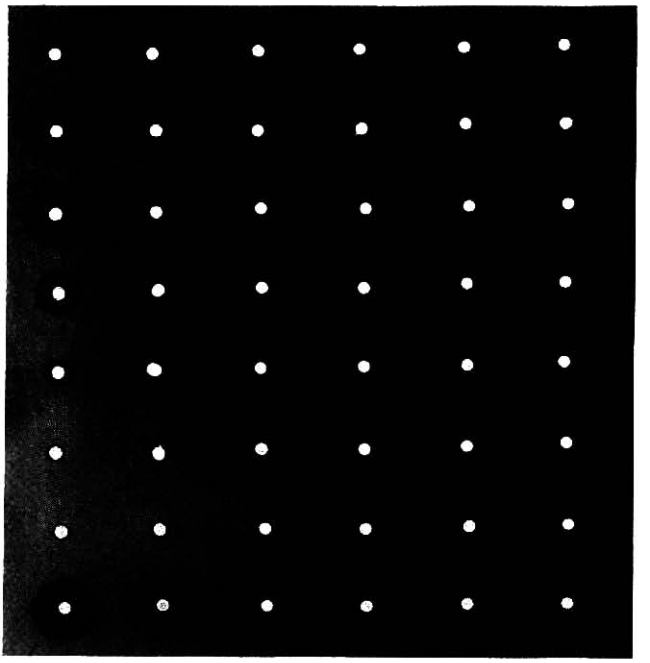

Fig. 1. Radial immunodiffusion plate: 2 % (v/v) of anti-$\alpha_1$-lipoprotein was gelled in a 1 mm thick layer of 2 % agarose. Well diameter 1.6 mm. 2 $\mu$l of individual fractions of serum separated by gel filtration were placed in the wells. The bottom row are dilutions of a standard serum. Amido-black stain of the antigen–antibody precipitates.

Fig. 2 shows the pattern obtained from normal blood serum, drawn 3 h after a load of 250 ml heavy cream (total lipids 705 mg/100 ml), separated on 2 % granulated agarose. Measurement of optical density at 280 m$\mu$ showed two well separated peaks, where the first at the front ($K_d$ 0) contained all the turbid liquid, as measured at 700 m$\mu$. $\alpha_1$-Lipoproteins eluted in a single symmetrical peak with a maximum between $K_d$ 0.77 and $K_d$ 0.85. $\beta$-Lipoproteins eluted earlier with a maximum between $K_d$ 0.50 and $K_d$ 0.62, in a skewed peak. Its shape was identical, whether 1 or 3 % agarose was used in the radial immunodiffusion. Total lipid content of the eluates

attained a maximum between the $\beta$- and $\alpha_1$-peaks. Some lipid was present in the early fractions where the large chylomicrons appeared; these did not react with either the $\alpha_1$- or $\beta$-antibody. Antitrypsin eluted in a symmetrical peak at $K_d$ 0.81 to $K_d$ 0.85. $\alpha_2$-Macroglobulin eluted earlier with a maximum between $K_d$ 0.62 and $K_d$ 0.65 in a skewed peak, due to molecular size variations.

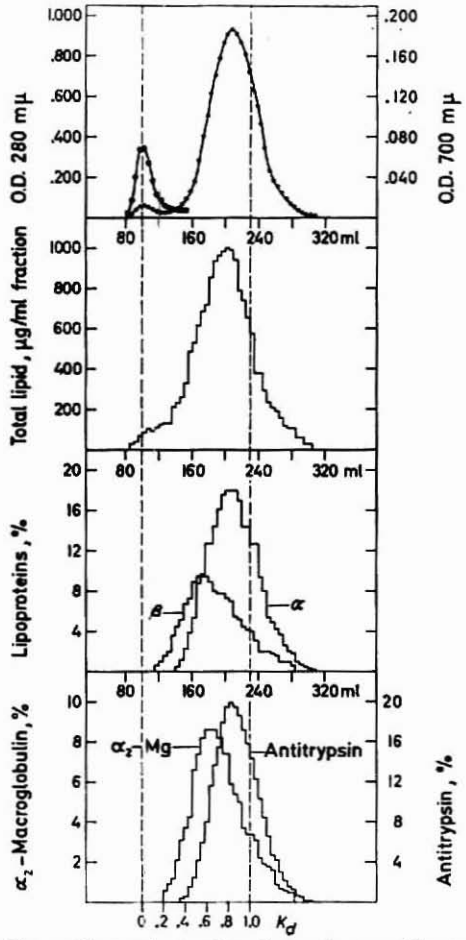


Fig. 2. Chromatography of 10 ml normal human blood serum, withdrawn 3 h after peroral lipid load (total lipids 705 mg %), on a column of 2% agarose beads. Optical densities at 280 m$\mu$ (eluates diluted 1:10), and 700 m$\mu$. Total lipid content of eluates. $\alpha_1$-Lipoprotein, $\beta$-lipoprotein, $\alpha_2$-macroglobulin and antitrypsin concentrations of eluates, expressed in per cent of a standard serum.

The major lipoprotein classes and their estimated molecular weights[9] are listed in Table I, at the left. On the right are the $K_d$ values expected from the calibration of 2% granulated agarose beds[1], and the $K_d$ values found by immunodiffusion. Except for a slight retardation of the $\alpha_1$-lipoproteins the values agree well. No appreciable interaction occurs between the bed material and the lipoproteins, which are known to be easily adsorbed on charged surfaces.

TABLE I

MOLECULAR WEIGHTS OF DIFFERENT LIPOPROTEIN CLASSES (DATA TAKEN FROM FREEMAN *et al.*[9]) EXPECTED $K_d$ VALUES ON A COLUMN OF 2 % AGAROSE GEL BEADS (DATA TAKEN FROM WERNER[1]) AND $K_d$ VALUES FOUND BY RADIAL IMMUNODIFFUSION

| *Lipoprotein class* | *Molecular weight* | *$K_d$ value* | |
|---|---|---|---|
| | | *Expected* | *Found* |
| Alpha | 150,000 — 400,000 | 0.80 — 0.74 | 0.85 — 0.77 |
| $S_f$ 0 — 20 | $1 \times 10^6$ — $3 \times 10^6$ | 0.65 — 0.52 | 0.62 — 0.50 |
| $S_f$ 20 — 400 | $5 \times 10^6$ — $12 \times 10^6$ | 0.42 — 0.16 | |
| $S_f$ 400 — $10^5$ | $12 \times 10^6$ — $3 \times 10^{11}$ | Front — 0.16 | |

A summary of $K_d$ values of various proteins on 2 % and 4 % granulated agarose as determined by radial immunodiffusion is given in Table II. 2 % agarose is more suitable for the separation of chylomicrons ($K_d$ 0 to $K_d$ 0.42) from lipoproteins, but practically no resolution is obtained among proteins smaller than $\gamma$G-globulin. On 4 % agarose, the $\beta$-lipoproteins shift towards the front and are better separated from the $\alpha_1$-lipoproteins, but most chylomicrons will elute in bulk with the front. On the other hand separation is effective down to molecular weights of 45,000.

The diagrams in Fig. 3 were obtained with two blood samples from the same patient with hyperlipemia due to alcoholism and liver cirrhosis (total lipids 1140 mg %). The sample on the left was taken 3 h after a load of 300 ml heavy cream. Chylomicrons in large quantity eluted in a sharp peak at the front (optical density 700 m$\mu$). Measurement at 280 m$\mu$ showed two well separated peaks. The total lipid curve also showed two peaks: The smaller, first one, eluted at the front; the larger, second one, preceded its normal $K_d$ value slightly. $K_d$ values for $\beta$- and $\alpha_1$-lipoproteins coincided with those in normals. The sample on the right was taken 8 h after the same meal. Chylomicrons were only partially cleared, turbid liquid still eluted at the front. Although the total lipid content of the serum remained unchanged, there was less lipid at the front and comparatively more in a shoulder between $K_d$ 0.2 and $K_d$ 0.4; the main peak retained its position. Simultaneously the $\beta$-lipoprotein concentration increased, while that of $\alpha_1$-lipoprotein remained unchanged.

Serum obtained in the fasting state from a woman with essential hyperlipemia (total lipids 3700 mg %) gave a different pattern (Fig. 4). The turbid liquid (optical density 700 m$\mu$) eluted in a strongly skewed peak at the front. Measurement of optical

TABLE II

$K_d$ VALUES OF VARIOUS PROTEINS DETERMINED BY RADIAL IMMUNODIFFUSION AFTER FRACTIONATION ON COLUMNS OF 2 % AND 4 % AGAROSE GEL BEADS

| *Protein* | *Molecular weight* | *$K_d$ on 2 % agarose* | *$K_d$ on 4 % agarose* |
|---|---|---|---|
| $\beta$-Lipoproteins | $1 \times 10^6$ — $3 \times 10^6$ | 0.62 — 0.50 | 0.35 — 0.26 |
| $\alpha_2$-Macroglobulin | 820,000 — 950,000 | 0.65 — 0.62 | 0.40 — 0.35 |
| $\alpha_1$-Lipoproteins | 150,000 — 400,000 | 0.85 — 0.77 | 0.65 — 0.61 |
| $\gamma$G-Globulin | 150,000 | 0.85 — 0.81 | 0.65 — 0.57 |
| Antitrypsin | 45,000 | 0.85 — 0.81 | 0.70 — 0.65 |
| Acid $\alpha_1$-glycoprotein | 44,000 | 0.88 — 0.81 | 0.70 — 0.65 |

density at 280 mμ showed a shoulder in the ascending limb, starting at the front and continuing uninterruptedly to the main peak. Total lipid eluted in a single peak, starting at the front. Its maximum at $K_d$ 0.4 appeared earlier than in normals and in the previous case. In spite of the large quantity of chylomicrons the proteins reacting with β- and $\alpha_1$-antigen retained their peak positions. β-Lipoproteins were present in larger amounts than normal. Some $\alpha_1$-lipoprotein reaction was found at early $K_d$ values, almost up to the front. Thin-layer chromatography showed slightly differing maxima for the individual lipid classes; cholesterol esters were maximal at 140 ml, triglycerides and free cholesterol at 160 ml.

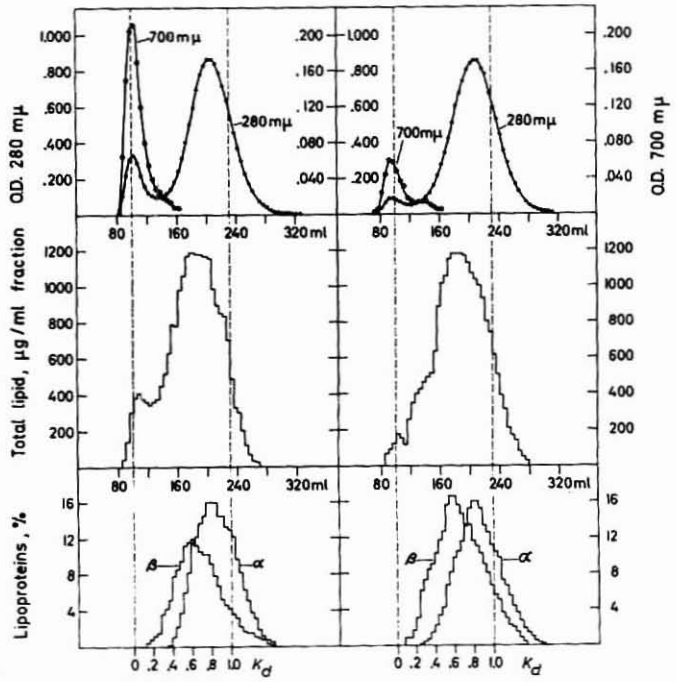


Fig. 3. Chromatography of 10 ml human blood serum of a man with hyperlipemia due to alcoholism and liver cirrhosis (total lipids 1140 mg %) on a column of 2% agarose beads. Left: Serum withdrawn 3 h after peroral lipid load. Right: Serum withdrawn 8 h after the same meal. Optical densities at 280 mμ (eluates diluted 1:10), and 700 mμ. Total lipid content of eluates. $\alpha_1$-Lipoprotein and β-lipoprotein concentrations are expressed in per cent of a standard serum.

A different pattern (Fig. 5) again was obtained from serum drawn in the fasting state from a patient with carbohydrate induced hyperlipemia and xanthomatosis (total serum lipids 3900 mg %). The marked turbidity of the serum eluted mostly at the front. Measurement of optical density at 280 mμ showed two distinct peaks. The maximum of total lipid content was shifted close to the front as lipid analysis and thin-layer chromatography showed. All major lipid classes eluted with a maximum around 110 ml ($K_d$ 0.2).

DISCUSSION

The lipids of plasma do not circulate free, but occur in specific combinations with protein and carbohydrate[10]. Major classes of these complexes can be separated by electrophoresis[11], ultracentrifugation[9], or insoluble complex formation[12]. For the fractionation of larger particles (very low density lipoproteins, chylomicrons) special

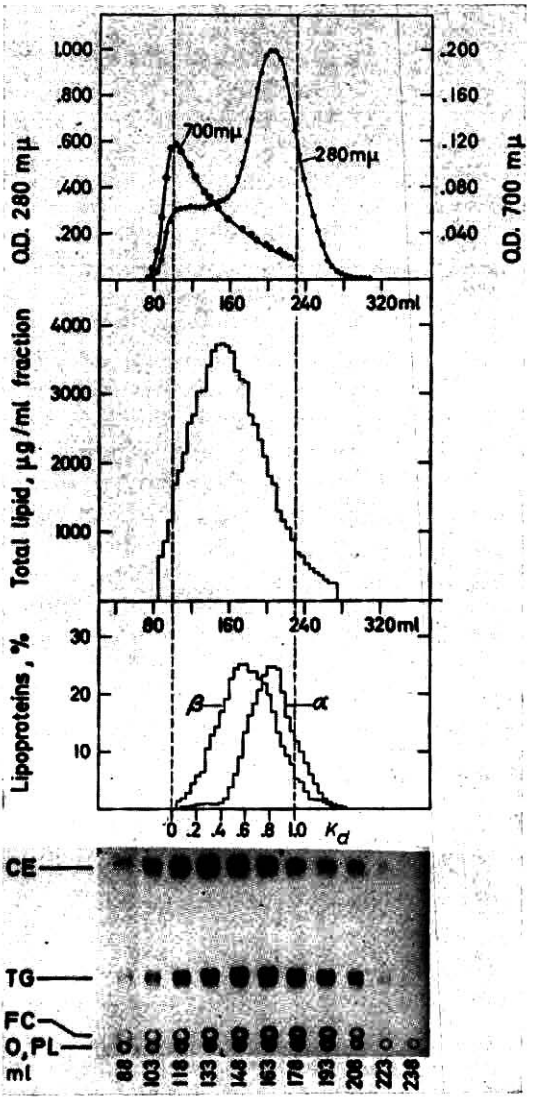


Fig. 4. Chromatography of 10 ml fasting serum of a woman with hyperlipemia (total lipids 3700 mg %) on a column of 2 % agarose beads. Optical densities at 280 mμ (eluates diluted 1:10), and 700 mμ. Total lipid content of eluates. $\alpha_1$-Lipoprotein and $\beta$-lipoprotein concentrations are expressed in per cent of a standard serum. Thin-layer chromatography of lipid extracts of eluates (CE = cholesterol esters; TG = triglycerides; FC = free cholesterol; PL = phospholipids; O = origin).

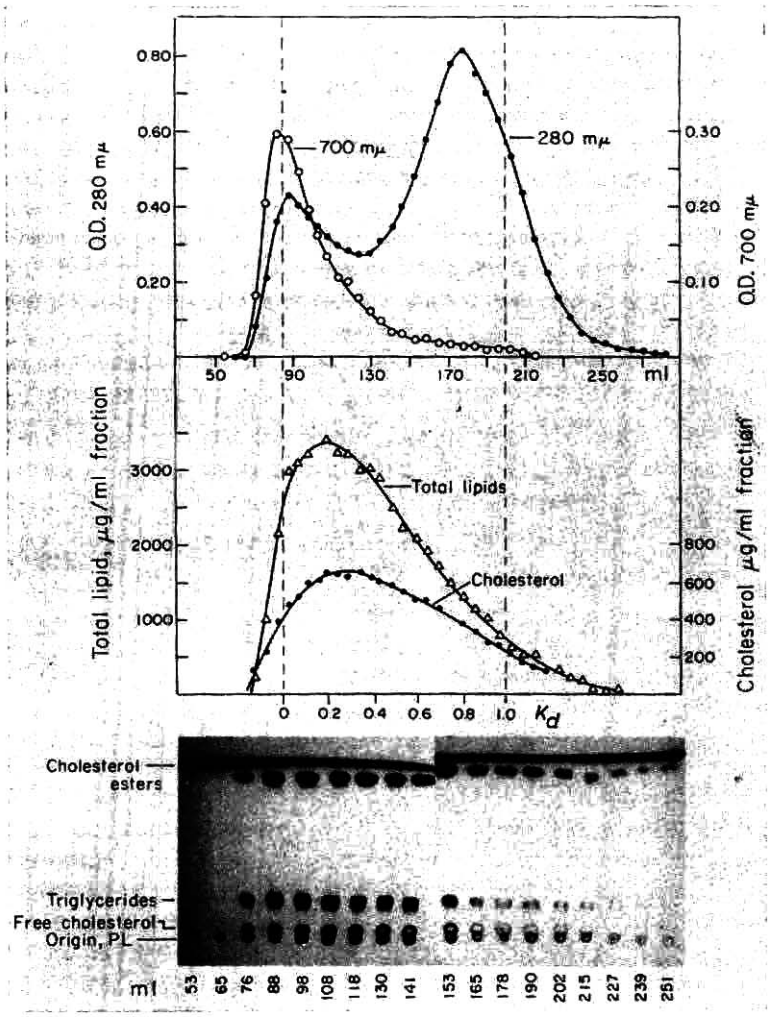


Fig. 5. Chromatography of 8 ml fasting serum of a patient with carbohydrate induced hyperlipemia (total lipids 3900 mg %) on a column of 2 % agarose beads. Top: optical densities at 280 mμ (eluates diluted 1:10) and 700 mμ. Middle: total lipid and total cholesterol content of eluates. Bottom: thin-layer chromatography of lipid extracts of eluates.

techniques have been devised[13–15]. Unfortunately many of these procedures alter the native state in which lipoproteins are present in blood[16,17]. Gel filtration on granulated agarose offers an alternative method for lipoprotein fractionation[1]. The sample is subjected only to the forces of diffusion, and constantly remains in buffered solution. Varying patterns of lipoprotein distribution in hyperlipemia can be distinguished by this method (Figs. 3–5).

There is no general agreement on whether proteins are integral components of the chylomicron surface or whether they should be considered contaminants[16]. Centrifugation in saline or buffer can remove almost all the nitrogen from the larger

fat particles[18], prolonged exposure to serum on the other hand alters the amount and composition of their surface proteins[19,20]. After gel filtration none of the following proteins could be demonstrated by radial immunodiffusion at the front, where large particles elute: $\beta$-lipoprotein, $\alpha_1$-lipoprotein, $\alpha_2$-macroglobulin, acid $\alpha_1$-glycoprotein, antitrypsin, $\gamma$G-globulin. These findings are in some contrast with the assumption that, in the blood stream, small amounts of $\alpha_1$- and $\beta$-lipoproteins form a complex with the lipids absorbed in the intestine (primary particles)[21]. If gel filtration does not destroy these complexes, their content of $\alpha_1$- or $\beta$-lipoproteins was either too small for detection, or their antigenic sites were masked. The possibility that the chylomicrons do not penetrate the gel in immunodiffusion, and that they hold the surface proteins tightly enough to prevent their free diffusion also has to be considered.

It has recently been suggested that $\alpha_1$-lipoproteins combine with $\beta$-lipoprotein to form the very low density lipoproteins ($S_f > 20$, density $< 1.006$, pre-$\beta$-lipoproteins) that take part in the transport of endogenous triglyceride[22]. The serum analyzed in Fig. 4 contained large amounts of very low density lipoproteins. In this case $\alpha_1$-lipoproteins were immunologically detectable in small amounts up to the front, where they do not normally appear.

There is agreement that the low density lipoproteins ($S_f$ 0 to 20, density 1.006 to 1.063, $\beta$-lipoproteins) are antigenically distinct from high density lipoproteins (density $>$ 1.063, $\alpha_1$-lipoproteins)[23,24]. In spite of wide physicochemical heterogeneity, complete antigenic homogeneity of low density lipoproteins[24,28] has been demonstrated. A low density lipoprotein component with a hydrated density of 1.018 ($S_f >$ 12) has been isolated from human serum[29,30]. This fraction, called $\alpha_2$-lipoprotein after its electrophoretic mobility on starch or polyvinyl chloride, has been differentiated from $\beta$-lipoprotein. Immunologically, however, the two cannot be distinguished[31,32]. Since $\alpha_2$-lipoprotein contains 8% polypeptide, and $\beta$-lipoprotein 16.5%[32] the immunological reaction of $\beta$-lipoprotein is relatively stronger. A skewed curve of $\beta$-lipoproteins with tailing to high $K_d$ values was often found by radial immunodiffusion after gel filtration on 2% agarose beads. Plates of 2% agarose were usually chosen to avoid steric hindrance of diffusion of $\beta$-lipoproteins during immunoprecipitation, but identical patterns were found with 1% and 3% concentrations. Since agar interacts with low density lipoproteins[33], agarose should be used exclusively.

In contrast to plasma low density lipoproteins the high density lipoproteins (density 1.063 to 1.21, $\alpha_1$-lipoproteins) are immunologically heterogeneous[10,17,34–36]. Two forms $\alpha LP_A$, and $\alpha LP_B$, have been distinguished, the first being transformed into the second during ultracentrifugation and storage[36]. Antigenic polymorphism of the $\alpha_1$-protein component may be due to the presence in solution of monomeric and polymeric forms[10]. After gel filtration on 2% agarose beads $\alpha_1$-lipoproteins consistently appeared as a symmetrical peak. Double rings observed in immunodiffusion may be due to conversion of $\alpha LP_A$ into $\alpha LP_B$. They were not produced consistently by the same antiserum, and appeared mostly in aged samples. Such conversions may also be the cause of the difficulty encountered in producing a stable standard serum for calibration of immunological methods[37].

SUMMARY

Estimation of plasma lipoproteins, and other proteins by radial immunodiffu-

sion after gel filtration, is described. Gels of 2 and 4 % granulated agarose were used. On 2 % granulated agarose, large chylomicrons elute with the front, very low density lipoproteins ($S_f$ 20–400) at $K_d$ values up to 0.45, low density lipoproteins ($\beta$-lipoproteins; $S_f$ 0–20) at $K_d$ 0.50 to 0.62, and high density lipoproteins ($\alpha_1$-lipoproteins) at $K_d$ 0.77 to 0.85. The latter values confirm those predicted from calibration, proving that lipoproteins are not appreciably adsorbed to the bed material. Different forms of hyperlipemia can be distinguished by this method.

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END OF THE SYMPOSIUM CONTRIBUTIONS

# MOLECULAR WEIGHT DISTRIBUTION ANALYSIS BY GEL CHROMATOGRAPHY ON SEPHADEX

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## INTRODUCTION

The original form of gel chromatography, gel filtration[1,2] on hydrophilic gels, has been used for a vast number of separations of macromolecular compounds, particularly of biological origin.

Using standardized conditions, gel filtration has also been successfully applied to the determination of molecular weights of a wide variety of proteins[3–5]. Hydrophilic gels have also been used for the preparative fractionation of water-soluble polymer homologues[6–10].

In this paper the application of gel filtration to molecular weight distribution analysis is described. In the field of organophilic polymers a similar method, called gel permeation chromatography, is well established today[11–15]. Our work was started primarily to facilitate certain studies on membrane permeability, glomerulus filtration, lymph penetration etc. in healthy humans and also pattern changes caused by certain diseases. Such studies can be carried out with a colloidal plasma volume expander as test substance, provided that it is possible to determine the molecular weight distribution of the penetrated or excreted material. A method of distribution analysis was desired for dealing with very dilute samples of body fluids and small amounts of substance. Preparative fractionation was thus out of the question.

WALLENIUS had earlier developed an advanced technique for turbidimetric titration of different molecular sizes of dextran in order to study their renal clearance after infusion in dog[16] and in humans[17]. Unfortunately, this method is too expensive for general use.

Thus, in view of the wide use of dextran in therapy and in the studies mentioned above, and encouraged by our experiences from the preparative fractionation of dextrans on Sephadex[6,7] the authors began two years ago to examine the possibility of calibrating Sephadex for molecular weight distribution analysis of dextran, preferentially within the $\bar{M}_w$ range 150,000 to 10,000.

Since this method has proved to be rapid and reproducible and well suited for advanced automation and because of its potential application to water-soluble polymers in general, the calibration procedure and some applications are reported below.

## EXPERIMENTAL

### *Column and bed material*

The column used had the dimensions 1.4 × 75 cm (PS 58/30 Quickfit tube) and

was provided with a cooling jacket and an applicator cup. Before packing, the volume of the column was calibrated over its entire length using water. The bed height could then be read from a mm-scale fitted on the mantle. On the basis of our experience from the fractionation of dextrans[7] a mixture of two Sephadex types, G-200 (Wr = 20.1 g/g) and G-100 (Wr = 9.8 g/g) was used. A dry weight ratio of 1 to 2 between G-200 and G-100 was chosen, so that both occupied approximately equal volumes in the swollen state. The mixture was allowed to swell in excess water several days before packing. This was carried out by pouring the homogeneous suspension through an extension tube into the water filled column. The water circulating in the jacket was thermostated at 20° ± 0.1°. To ensure complete equilibration, eluant was percolated through the bed some days before determining the void volume ($V_0$). The absence of any carbohydrate material in the eluate was checked before the column was considered ready for use.

It soon became evident that distilled water was not a satisfactory eluant. Occasionally the bed height changed suddenly even after a long period of operation. 0.3 % NaCl solution, with chlorobutanol as preservative, was therefore used throughout this work. The flow rate was kept at 6–7 ml/h (3.8–4.5 ml/cm²/h) by means of a constant hydrostatic pressure.

## VOID VOLUME ($V_0$) AND PARTITION COEFFICIENT ($K_{av}$)

Blue dextran of high molecular weight was used for the void volume determination. 0.5 ml of 0.5 % solution was transferred using a syringe provided with a Millipore filter adaptor, into the applicator cup just as the eluant soaked into the bed. The eluate was collected from the moment the last of the coloured solution vanished into the bed. For determination of $V_0$, the coloured eluate was collected in fractions of 0.3 ± 0.01 g. The extinction of the fractions was read at 650 mμ. The elution volume at the inflection point of the leading edge of the elution pattern was taken to represent the void volume $V_0$. The determination of $V_0$ was carried out once a week. The accuracy of the determination of $V_0$ was ± 0.1 ml (± 0.4 %).

In order to make the calibration independent of the individual experimental conditions and thus more generally applicable, the concept of the volume fraction available for a solute in the gel phase was introduced according to LAURENT AND KILLANDER[18]:

$$K_{av} = \frac{V_e - V_0}{V_t - V_0}$$

where $V_e$ is the elution volume of the species in question, $V_t$ is the total volume and $V_0$ the void volume of the gel bed used. All these parameters can be determined accurately and the resulting $K_{av}$ expresses the penetrability of a certain molecular size into a gel of defined porosity.

## CORRELATION OF $\overline{M}_w$ AND $K_{av}$ FOR DEXTRAN ON SEPHADEX

Several authors[5,18,19] have found that the elution behaviour of macromolecules primarily depends on the Stokes radius (or diffusion coefficient) of the species. From

this relationship other parameters, like molecular weight, can be derived assuming the necessary data are known. As the gel filtration (GF) method in this case was intended for routine analysis of molecular weight distributions of one particular polymer, a direct calibration of $K_{av}$ *vs.* molecular weight was desirable.

Fractions of dextran, obtained by extraction fractionation[20] or preparative gel filtration, were used for the calibration. They were not sharp enough to be considered as monomolecular samples. Consequently the heterogeneity of the fractions had to be taken into account. The tentative procedure was as follows.

TABLE I

PHYSICAL CHEMICAL DATA FOR THE DEXTRAN FRACTIONS USED IN THE CALIBRATION OF THE GF DISTRIBUTION ANALYSIS

| *Fraction No.* | $\overline{M}_w$ | $\overline{M}_n$ | $K_{av}$ | $D_{20,w} \times 10^{7}$[a] *(cm²/sec)* | *Stokes radius (r) (Å)* |
|---|---|---|---|---|---|
| 1 | 147,000 | 91,000 | 0.04 | 2.6 | 82 |
| 2 | 130,000 | 83,000 | 0.043 | 2.77 | 78 |
| 3 | 96,000 | 66,000 | 0.064 | 3.2 | 67.5 |
| 4 | 76,000 | 53,000 | 0.085 | 3.58 | 60.5 |
| 5 | 58,000 | 46,000 | 0.110 | 4.10 | 53.0 |
| 6 | 48,300 | 38,500 | 0.148 | 4.46 | 48.5 |
| 7 | 41,700 | 33,500 | 0.173 | 4.77 | |
| 8 | 36,000 | 26,500 | 0.215 | 5.10 | 42.5 |
| 9 | 32,400 | 26,500 | 0.227 | 5.38 | |
| 10 | 27,800 | 21,800 | 0.266 | 5.8 | 37.7 |
| 11 | 22,400 | 18,500 | 0.316 | 6.4 | |
| 12 | 19,300 | 16,000 | 0.380 | 6.9 | 31.8 |
| 13 | 13,200 | 9,500 | 0.476 | | (26.5) |
| 14 | 10,000 | 7,100 | 0.556 | 9.4 | 23.3 |
| 15 | 7,500 | 5,100 | 0.620 | | |
| 16 | 6,100 | 4,160 | 0.671 | | |
| 17 | 5,400 | 4,070 | 0.719 | | |

[a] Extrapolated from values measured for a series of dextran fractions[21]. Stokes radius follows from the equation:

$$r = \frac{RT}{6\pi\eta DN}$$

where $R$ = gas constant, $T$ = absolute temperature, $\eta$ = viscosity of the solvent, $D$ = diffusion coefficient and $N$ = Avogadro's number.

Seventeen well defined dextran fractions* within the $\overline{M}_w$ range 150,000 to 5,000 were chromatographed on the Sephadex gel mixture selected for this work. The data for these fractions are given in Table I. The weight average molecular weights, $\overline{M}_w$, were determined by the light scattering method with a Sofica photogoniodiffusometer using essentially the same technique as described previously[21]. The number average molecular weights, $\overline{M}_n$, were determined by the end group analysis using the Somogyi copper phosphate method[22]. Using these data, the theoretical molecular weight distributions of the fractions were then computed by applying the Lansing-Kraemer logarithmic number distribution function according to WILLIAMS AND SAUNDERS[23].

* The parent material was partially hydrolyzed and purified samples of dextran, synthesized by *Leuconostoc mesenteroides*, strain B 512.

The measured $\overline{M}_w$ value of each fraction was then noted on the differential plot of the corresponding Lansing-Kraemer distribution and the two fractional areas under the curve were measured planimetrically (Fig. 1). (Alternatively, the Lansing-Kraemer distribution may be presented as an integral curve and the position of the experimental $\overline{M}_w$ value read off on the Y-axis.)

The procedure adopted for these fractions and for all subsequent samples was as follows. Two milligrams of dextran dissolved in 2 ml water were applied as described for the void volume determination and eluted with 0.3 % NaCl solution. After a certain volume ($\lesssim V_0$), fractions of about 1.5 ml were taken and weighed to $\pm$ 0.01 g each. The dextran concentrations were determined with anthrone[24]. (Later these analyses were automated using the Technicon Auto Analyzer. The above conditions were so chosen that the dextran in the fractions could be determined without further dilution.)

Finally the elution curve, *i.e.* the dextran concentrations ($\gamma$/ml) *vs.* $K_{av}$, was drawn as well as the corresponding cumulative diagram (Fig. 1). The Y-axis position of the experimental $\overline{M}_w$ value according to the determination above was inserted in the cumulative elution diagram and serves as one point on the calibration curve for $\overline{M}_w$ *vs.* $K_{av}$ for dextran on the gel in question.

The cumulative elution profiles of the dextran fractions used in calibration are

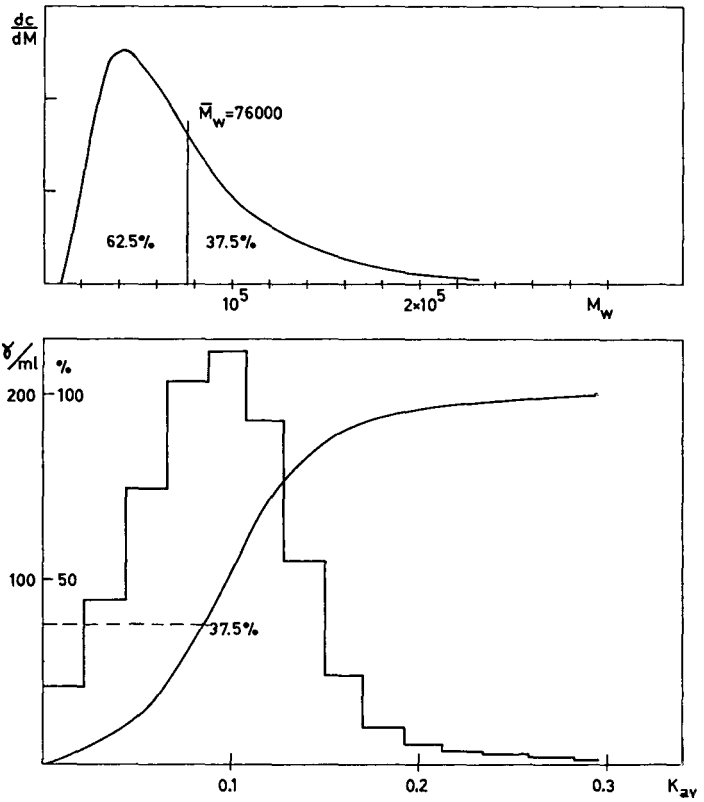


Fig. 1. Lansing-Kraemer distribution and elution diagram of dextran fraction No. 4 (Table I).

shown in Fig. 2. No significative displacement of the curve was observed when the sample charged was 20 mg/2 ml, 2 mg/2 ml or 0.5 mg/2 ml, respectively.

The correlation between log $\overline{M}_w$ and $K_{av}$ for dextran within the range of $\overline{M}_w \sim$ 150,000 to 5,000 is plotted in Fig. 3. The correlation is linear only within a very limited range, thus diverging from the observations of several authors when chromatographing globular proteins on Sephadex[25–27]. This discrepancy very likely depends on a non-Gaussian pore distribution of the mixed gel used. For the same reason the results obtained cannot be treated according to the theories proposed by PORATH[27] and LAURENT AND KILLANDER[18]. The correlation between corresponding Stokes radii and $K_{av}$ was deduced using the diffusion coefficients in Table I.

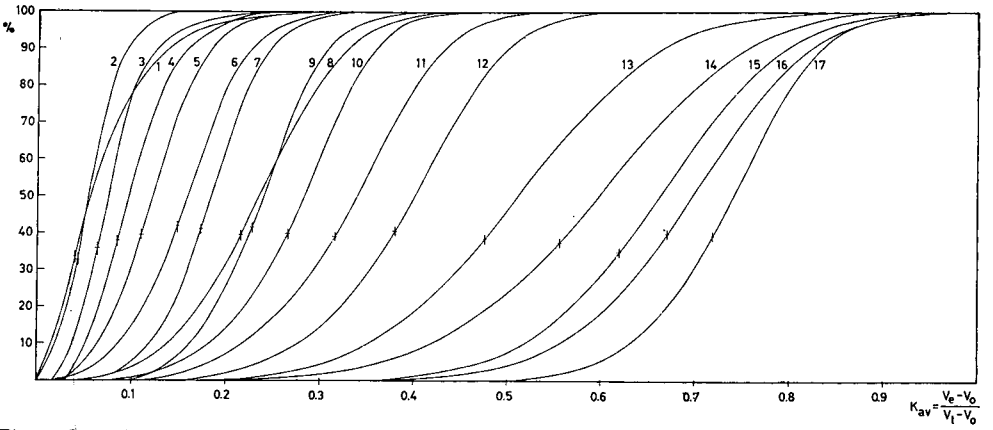


Fig. 2. Cumulative elution curves of the dextran fractions in Table I.

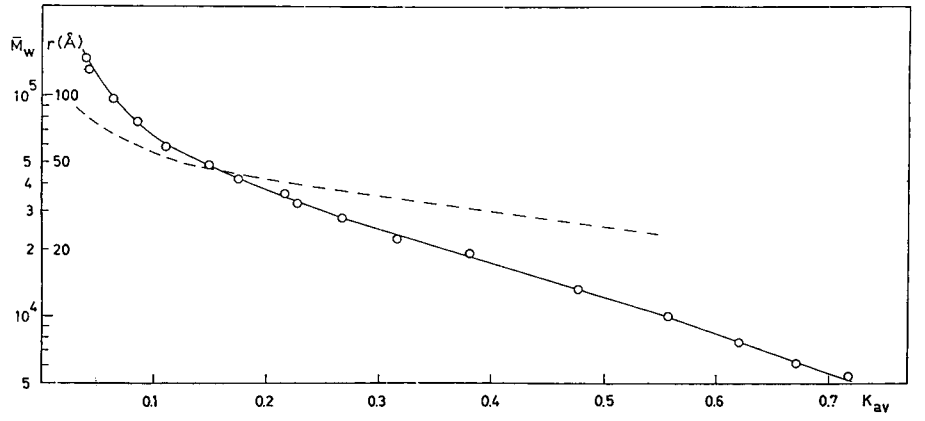


Fig. 3. The correlation curve log $\overline{M}_w$ *vs.* $K_{av}$ for dextran on Sephadex G-200 + G-100. The dotted curve represents Stokes radius *vs.* $K_{av}$.

## DETERMINATION OF MOLECULAR WEIGHT DISTRIBUTIONS

Having calibrated the gel for a specific polymer, the determination of molecular weight distributions of unknown samples of the same polymer is simple and rapid. The experimental conditions are the same as described for the calibration runs. The elutions are usually carried out during the night and the fractions analyzed the following day. The calibration curve is then used to convert the $K_{av}$ values of the cumulative elution curve into molecular weights. Although this can readily be performed manually, a computer program has been worked out, affording both a tabulated and graphical presentation of the cumulative and differential molecular weight distributions and also the molecular weight averages of interest*.

## RESOLUTION

Several authors have studied the gel chromatographic process by applying the general plate height equation. The contribution of longitudinal diffusion to the broadening of the zone was found to be negligible even in rather long columns and with slow flow rates[28,29]. The fact that monomolecular species and very narrow fractions of polymers are eluted as Gaussian curves is primarily due to the non-perfect resolution power of the chromatographic system[30]. In order to check the influence of the broadening phenomenon in our system when dealing with evaluation of polydisperse distributions the following experiments were carried out.

A series of six fractions obtained by preparative gel filtration of a dextran on Sephadex G-200[10] were run separately, charging 2 mg of each fraction, on calibrated columns. The computed $\bar{M}_w$ values were within the range of 63,000 to 15,000. The elution diagrams, with the areas scaled in proportion to the size of each fraction, are presented in Fig. 4a. The distribution of the parent sample was then reconstructed by summing the individual distributions in Fig. 4a. In another experiment, appropriate amounts of each constituent fraction were dissolved to give a total sample of 2 mg/2 ml which was subsequently charged on the gel. Both the calculated and the experimental elution curve are drawn in Fig. 4b. The agreement is good indicating that the broadening in the elution profiles of the subfractions is compensated in the elution of the heterogeneous sample.

The resolving power of the chromatographic system used here was further tested by rechromatography of one of the eluted fractions from a sample of considerable heterogeneity. The experimentally determined values for the parent polymer were $\bar{M}_w = 35{,}000$ and $\bar{M}_n = 22{,}000$ ($\bar{M}_w/\bar{M}_n = 1.6$). The sample applied on the column contained 20 mg/2 ml and it emerged within an eluant volume of 65 ml. A fraction near the peak was selected for rechromatography (1.25 mg/2.04 ml) representing a cut-out between 33,000 and 37,000 on the calculated distribution. The rechromatographed distribution had a nearly symmetrical shape (Fig. 5) and gave $\bar{M}_w = 36{,}500$, $\bar{M}_n = 34{,}500$ ($\bar{M}_w/\bar{M}_n = 1.057$).

The overlap of the adjacent molecular sizes, shown in this experiment, apparently is due to the limited resolution of the column. For a general analysis of the elution diagrams the calibration should be based on both the molecular sizes and the resolution[31].

* The program for dextran was coded by Dr. K. VOGEL in Fortran IV for CD 3600 Computer.

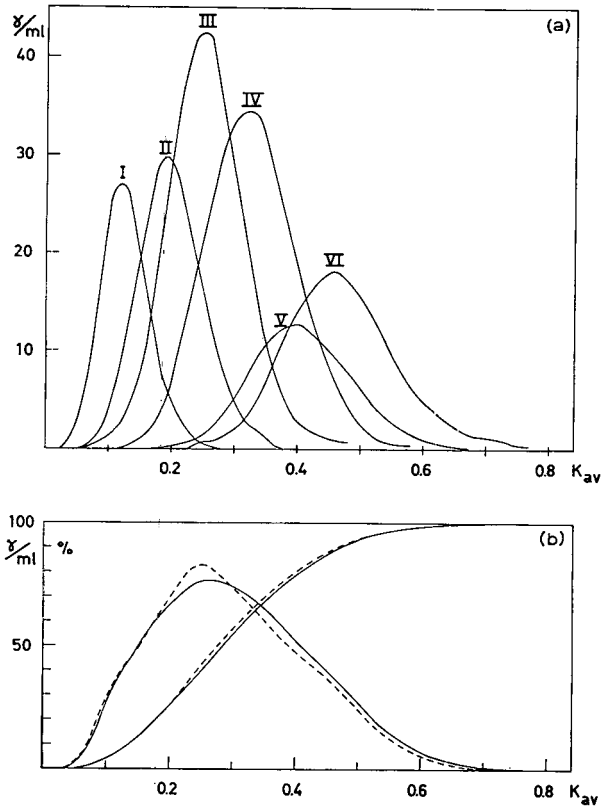


Fig. 4. (a) Elution curves for the constituent fractions of a dextran ($\overline{M}_w \sim 32{,}000$, $\overline{M}_n \sim 24{,}000$) (b) Continuous line: Elution curve after remixing the fractions in Fig. 4a ($\overline{M}_w = 29{,}700$, $\overline{M}_n = 23{,}400$). Dotted line: The calculated summed curve of the elution profiles of the fractions in Fig. 4a ($\overline{M}_w = 30{,}200$, $\overline{M}_n = 24{,}000$).

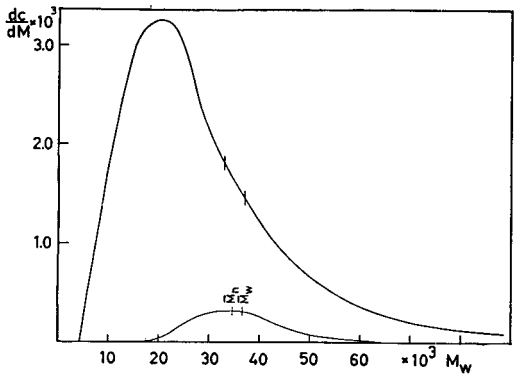


Fig. 5. Molecular weight distribution obtained by rechromatography of a cut-out from an elution pattern of dextran.

During the preparation of this manuscript, a study of the gel permeation chromatographic (GPC) process, partly by experiments similar to those described above, was published by ADAMS, FARHAT AND JOHNSON[14]. Their comparison between calculated molecular weights derived from various fractionation methods and the experimentally determined values showed that fractional precipitation analysis resulted in too narrow molecular weight distributions and consequently in too low $\bar{M}_w/\bar{M}_n$ ratios for the sample, no matter whether the original distributions were narrow or broad. GPC analysis again had a tendency to result in broader distributions than was expected from the experimental molecular weights. An improved resolution was obtained by decreasing the flow rate, however.

Of the very large number of runs in which the molecular weights calculated from the gel filtration analysis above have been compared with measured values, the heterogeneity of the GF distributions is regularly somewhat less than indicated by the experimental values of molecular weights. The difference is due primarily to the higher number average molecular weights (see Table II). This discrepancy, illustrated also by a slight shift of the calibration curve when $\bar{M}_n$ values are correlated with the corresponding $K_{av}$ values, very likely depends on a non-perfect presentation of the true distributions by the Lansing-Kraemer equation.

TABLE II

MOLECULAR WEIGHTS OF DEXTRANS MEASURED AND CALCULATED FROM GF ANALYSIS RESPECTIVELY

| *Measured values* | | | *Calculated values** | | |
|---|---|---|---|---|---|
| $\bar{M}_w$ | $\bar{M}_n$ | $\bar{M}_w/\bar{M}_n$ | $\bar{M}_w$ | $\bar{M}_n$ | $\bar{M}_w/\bar{M}_n$ |
| 110,000 | 80,000 | 1.38 | 108,000 | 82,000 | 1.32 |
| 72,000 | 40,500 | 1.78 | 70,400 | 44,900 | 1.57 |
| 38,400 | 25,900 | 1.48 | 37,700 | 27,800 | 1.36 |
| 20,800 | 14,500 | 1.43 | 19,800 | 15,600 | 1.27 |
| 11,200 | 6,700 | 1.67 | 11,100 | 7,400 | 1.50 |

* Calculated from the GF distribution diagrams according to the equations

$$\bar{M}_w = \Sigma w_i M_i \text{ and } \bar{M}_n = \frac{1}{\Sigma \frac{w_i}{M_i}}$$

## APPLICATIONS

The molecular weight distribution analysis described above has been for the past two years in regular use in our laboratories. It has been used in a great number of investigations pertaining to clinical dextran. Most of this work will be reported in the medical literature[32]. A short review of a few experiments will be given below, however, to illustrate the applicability of this new method.

Fig. 6 shows the shift in the molecular weight distribution of dextran in urine, collected at specified time intervals after the intravenous infusion of 500 ml dextran (Rheomacrodex®, 10%), in a normovolemic healthy person. The dextrans isolated from the urine samples were subjected to molecular weight determination by light scattering and end group analysis. Separately 2 mg of each dextran were applied on a

column and the molecular weight distribution determined (see Fig. 6 and Table III). These figures are complementary to the distribution analyses for dextran in serum performed by ARTURSON AND WALLENIUS[17] using the turbidimetric titration technique. In accordance with these previous studies, the human renal threshold for dextran molecules was found to be about 55,000 ($\overline{M}_w$).

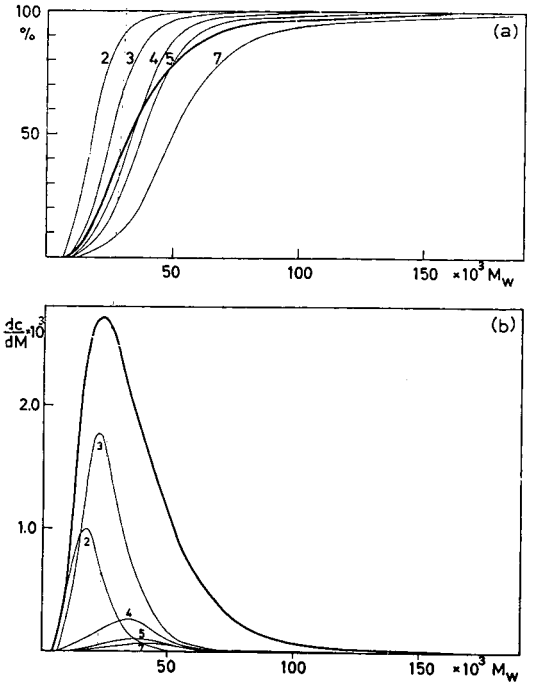


Fig. 6. (a) Integral curves for the molecular weight distributions of dextran in human urine collected at specified times after infusion of 500 ml Rheomacrodex (see Table III). The thick curve shows the distribution of the Rheomacrodex infused. (b) The corresponding frequency curves. The thick curve represents the amount and distribution of the dextran infused. The areas of the other dextran curves are proportional to the amounts excreted in the urine.

| *Hours after infusion* | *Curve* | *Per cent of the amount infused* |
|---|---|---|
| 0–1 | 2 | 18 |
| 1–4 | 3 | 38 |
| 4–8 | 4 | 8 |
| 8–12 | 5 | 3.4 |
| — | 6 | 3.7 |
| 24–48 | 7 | 3.0 |

The distribution analysis of dextran in serum is complicated by the high blank readings caused by proteins. Elimination of proteins by precipitation with zinc sulphate has proved suitable. The serum glucose appears as a separate peak at $K_{av} = 1$ and does not interfere with the evaluation of the molecular weight distribution of dextran in the sample. Occasionally the urine samples, mostly from small animals

TABLE III

MOLECULAR WEIGHT DATA ON EXCRETED DEXTRAN IN URINE COLLECTED AT INTERVALS AFTER INFUSION

| Hours after infusion | *GF analysis* | | | | | |
|---|---|---|---|---|---|---|
| | *Calculated values* | | | *Measured values* | | |
| | $\bar{M}_w$ | $\bar{M}_n$ | $\bar{M}_w/\bar{M}_n$ | $\bar{M}_w$ | $\bar{M}_n$ | $\bar{M}_w/\bar{M}_n$ |
| 0–1 | 19,300 | 16,300 | 1.18 | 20,000 | 16,400 | 1.22 |
| 1–4 | 26,700 | 23,300 | 1.15 | 27,000 | 20,500 | 1.32 |
| 4–8 | 35,100 | 31,300 | 1.12 | — | 26,200 | — |
| 8–12 | 39,000 | 33,400 | 1.17 | 42,000 | 31,300 | 1.34 |
| 12–24 | — | — | — | 49,000 | 31,800 | 1.54 |
| 24–28 | 55,900 | 45,600 | 1.22 | 56,000 | — | — |
| Rheomacrodex® infused | 37,200 | 27,000 | 1.38 | 38,000 | 24,000 | 1.58 |

such as rabbits and mice, cause difficulties and isolation of dextran prior to gel chromatography is recommended. A detailed study of the treatment of biological fluids for GF analysis is proceeding in our laboratories and will be published elsewhere.

An interesting application of the GF analysis was checking the permeability of a Visking membrane used in a dialysis study of different plasma volume expanders. The molecular weight distribution of the dextran in the dialysate is shown in Fig. 7. According to this, only 7.5 % of the permeating material had a molecular weight over 20,000.

Molecular weight distribution of inulin. Preparative fractionations of inulin have been performed previously on Sephadex[7]. These fractions were used for the calibration of the gel filtration method for the distribution analysis of inulin. Average molecular weights for the fractions were determined by light scattering and vapor pressure osmometry. Fig. 8a shows the elution diagram for the inulin preparation studied (Gurr Ltd., London). Two of the fractions are included for comparison. Fig. 8b

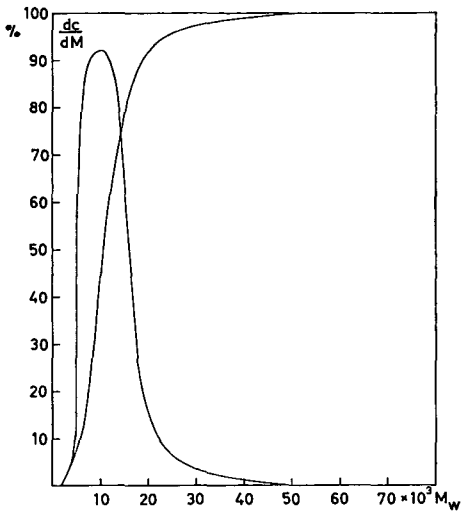


Fig. 7. Molecular weight distribution of dextran dialyzed through the Visking tube 28/32.

presents the resulting molecular weight distribution ($\bar{M}_w$ calc. = 7,900 and $\bar{M}_n$ = 7,000). The corresponding measured values were $\bar{M}_w$ = 7,000 and $\bar{M}_n$ = 6,300. The calibrated correlation, log $\bar{M}_w$ *vs.* $K_{av}$ implies that the inulin molecule has a considerably more compact structure than dextran. This may be expected when considering the 1 → 2 fructosidic linkages of the inulin[33] chain.

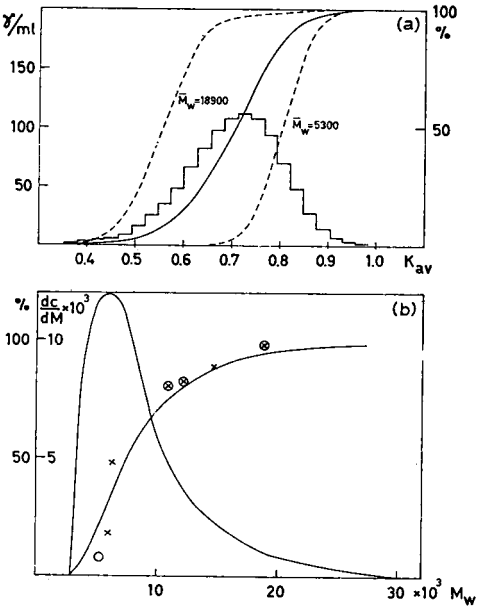


Fig. 8. (a) Elution diagram for inulin. The dotted lines show the cumulative curves for fractions $\bar{M}_w$ = 18,900 and $\bar{M}_w$ = 5,300, respectively. (b) Molecular weight distribution of inulin. Lines are constructed to show the integral and frequency curves, calculated from the elution diagram above. The dots and crosses represent fractions from a preparative gel filtration of the same batch inulin[7].

Fig. 9 presents the elution diagram of a sample of the polysaccharide pullulan*. The native glucan was synthesized by *Pullularia pullulans* with sucrose as the carbon source[34]. The elution pattern is symmetrical and gives, when converted to molecular weights using the dextran calibration curve, a distribution with $\bar{M}_w$ = 14,000 and $\bar{M}_n$ = 10,300. The measured values obtained earlier in our laboratory were $\bar{M}_w$ = 18,000 ($dn/dc$ = 0.152 as for dextran was used in the calculation) and $\bar{M}_n$ = 10,000. These results would suggest a similar molecular shape in solution for the polyglucoses dextran and pullulan.

The gel filtration method has recently been applied in our laboratory to obtain molecular weight distributions of the degraded gelatine and polyvinylpyrrolidone preparations marketed as plasma volume expanders. Details of these experiments will be reported elsewhere.

* This sample was kindly supplied by Dr. H. BOUVENG, Svenska Träforskningsinstitutet, Stockholm.

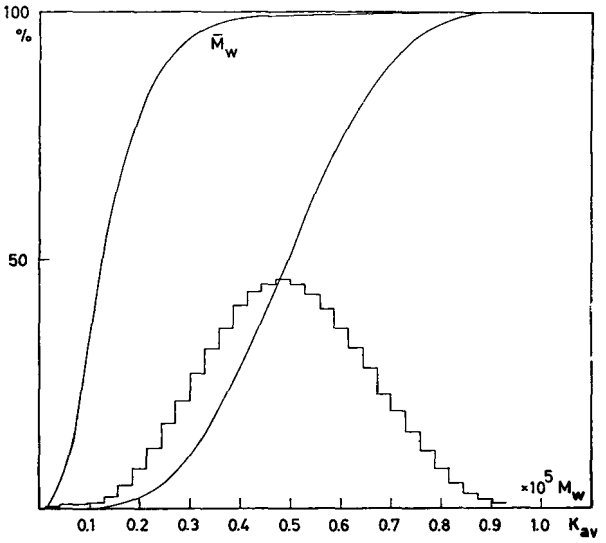


Fig. 9. Elution diagram and corresponding molecular weight distribution of pullulan.

## DISCUSSION

The gel chromatography technique described above provides a new tool for the routine analysis of molecular weight distributions of water-soluble polymers within a fairly broad molecular weight range. The method is essentially equivalent to the gel permeation chromatographic procedure recently developed for polymers in organic solvents.

For routine analyses of a given polymer an initial calibration of the elution properties of a series of fractions from the same polymer is required. Once the polymer/gel system in question has been calibrated, only the void volume of the bed has subsequently to be determined, preferably however, at frequent intervals. An improved check of the total volume by adding KCl into the samples has recently proved feasible. The determination of $V_e$ ($= V_t$) for KCl is carried out by flame photometry. A continuous method, for example precision refractometry or recording spectrophotometry, is recommended for the determination of concentration in the fractions, whereby the collecting of individual fractions is eliminated. The carbohydrates may be assayed by an Auto Analyzer which operates perfectly with the anthrone method and handles about forty samples per hour. The use of a peristaltic pump for attaining a constant flow rate reduces the need of weighing each fraction apart from a few sample tests. The necessary input data, *i.e.* the gel bed parameters, the polymer concentrations and amounts per fraction volume are then recorded together with the corresponding elution volumes and the derived $K_{av}$ values respectively. A computer program using the equation for the calibration curve converts the input information to integral and differential distributions. Molecular weight averages and the printing of the diagrams are easily included in the program.

With polymers for which the calibration $\bar{M}_w$ *vs.* $K_{av}$ is not available a distribution in terms of molecular dimensions may be derived from the gel chromatography

data. Provided the coefficients of sedimentation or diffusion are known, the Stokes radii can then be used to calculate molecular weights and frictional ratios[5,10].

The molecular weight distribution analysis using the present types of Sephadex as the gel material may be applied to molecular sizes below 100 Å (Stokes radius), corresponding to dextran $\bar{M}_w < 200{,}000$. On agarose gels, now available in the bead form (Sepharose®, Pharmacia Fine Chemicals), a considerably greater range of molecular sizes can be resolved. The procedure for the molecular weight distribution analysis on Sepharose is at present being studied and will be presented separately.

SUMMARY

A column chromatographic method for the molecular weight distribution analysis of water-soluble polymers has been developed with Sephadex as gel material. Very small amounts of polymer are needed and the procedure is well suited to an automated routine analysis. Some applications are illustrated.

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# ESTIMATION OF MOLECULAR WEIGHTS OF PROTEINS BY BIO-GEL P GEL FILTRATION

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Molecular sieve chromatography, or gel filtration, is a very well known technique for fractionation, isolation, analysis and purification of proteins, enzymes, hormones, nucleic acids, etc. It was suggested by LATHE AND RUTHVEN[1] that the dimensions of proteins and polysaccharide molecules might be estimated by this technique; although restricted to certain homologous series of macromolecules, correlation between molecular weight and size in gel filtration has been demonstrated by GRANATH AND FLODIN[2]. ANDREWS[3] showed by experiments with agar gel columns that this technique can be used as a comparative method to estimate molecular weights of proteins. This has been suggested by further reports about correlation between molecular weights of globular proteins and their behaviour on Sephadex or agar gel columns[4–11] or polyacrylamide columns[12].

Bio-Gel P is a copolymer of acrylamide and methylenebisacrylamide; the present report is concerned with finding a relationship between the molecular weights and the gel filtration behaviour of some globular proteins. Experiments are described in connection with three of the Bio-Gel P gel filtration materials (P-100, P-200 and P-300).

## MATERIAL AND METHODS

### *Proteins*

The proteins used in this study are listed in Table I. Coproporphyrinogenase was purified as described by BATLLE, BENSON AND RIMINGTON[25] and purified δ-aminolaevulinic acid dehydrase was prepared in this laboratory from cow liver (unpublished work).

### *Preparation and use of gel columns*

Columns were packed in vertical glass tubes (30 cm long and either 1.6 or 1.8 cm I.D.), equipped with capillary outlets. A small glass wool plug and glass beads were added to fill the bottom of the tube to obtain a small mixing chamber. It is advisable to coat the column before use to avoid distortion of the bands which is due to the tendency of the buffer to flow at a faster rate near the glass wall. Desicote (Beckman) used diluted, or a solution of 1 % dichlorodimethylsilane in benzene can be

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used. The solution is heated to about 60°, poured into the column, allowed to stand for 15–20 min, it is then decanted and the column dried in an oven. The procedure is repeated to give a double coat and the column can then be used for several months. Bio-Gel P-100 (Control No. 2880, particle size 50–150 mesh, U.S. standard), Bio-Gel P-200 (Control No. 2810 and No. 2759, particle size 50–150 mesh, U.S. standard) and Bio-Gel P-300 (Control No. 2859 and No. 2760, particle size 50–150 mesh, U.S. standard), were generous gifts from Bio Rad Laboratories, Richmond, Calif., U.S.A.

The dry gel was added slowly, with constant stirring, to the buffer to be used for elution and allowed to swell for 24 h. Each column was packed with the swollen gel, previously deaerated under reduced pressure, as described for Sephadex columns by ANDREWS[9,11]. When packing was completed, the upper surface of the gel was covered with a 1 cm layer of Bio-Gel P-30 and a well fitting filter paper disc (Whatman No. 1). Columns were operated at a flow rate of 0.17 ml/min to 0.5 ml/min.

Except where otherwise stated columns were equilibrated either with 0.05 *M* Tris–HCl buffer, pH 7.4, containing 0.1 *M* KCl or with 0.067 *M* phosphate buffer, pH 6.8, containing 0.1 *M* KCl.

All experiments were done at room temperature. Proteins were dissolved in the equilibration solution (2–4 ml) and applied to the top of the column by layering under the solution already present (the density of the protein solution was increased by the addition of sucrose, when necessary).

Column effluents were collected with an automatic fraction collector (The Locarte Co., London, S.W. 7).

*Examination of the column effluents*

Proteins were estimated either spectrophotometrically at appropriate wavelengths or by suitable enzyme assays (Table I), in a Beckman D.U. Spectrophotometer. Urease was measured by titration, with 0.01 *N* HCl, of the ammonia it liberated from a solution containing urea[26]. Sucrose was estimated by a colorimetric method[27].

## RESULTS

*Behaviour of proteins of known molecular weight in columns of different size*

Two sets of small columns were used. The results shown in Fig. 1 were obtained with 1.6 × 30 cm columns (60 ml bed volume) and those of Fig. 2 with 1.8 × 30 cm columns (76 ml bed volume). Columns of these sizes do not give the best resolution possible; nevertheless the results obtained with the 1.8 × 30 cm columns are good enough for our purpose.

The proteins shown in Table I were used alone and in several combinations, together with sucrose to determine their elution volumes from the Bio-Gel columns. In most experiments cytochrome *c* was also added to the mixture of proteins.

Sometimes differences of 2 or 3 ml were obtained in elution volumes for some proteins in separate experiments with the same column. This may be due to temperature changes or changes in density of the column packing, though swelling of the gel packed in a column was not observed over a period of some weeks. For individual proteins $V_e$ also varied as much as 4 ml to 6 ml from one column to another, which were identical in size but packed with gel from different batches; this is probably due to differences in the degree of cross-linking from batch to batch.

TABLE I

PROTEINS EMPLOYED IN THE STUDY

| *Protein* | *Source* | *Mol.wt.* $\times 10^{-3}$ | *Reference* | *Amounts used (mg)* | *Method of estimation* |
|---|---|---|---|---|---|
| Thyroglobulin (bovine), purified | Nutritional Biochemical Corp., Cleveland, Ohio, U.S.A. | 670 | EDELHOCH[13] | 1–2 | *E* at 230 m$\mu$ |
| Urease, "urease soluble" | British Drug Houses Ltd., Poole, Dorset, England | 470–510 | SHULMAN[14], GORIN *et al.*[15] | 2–6 (crude) | See the text |
| Edestin | D.Th. Guchardt G.M.B.H., Chemische Fabrik Görlitz | 310 | FRUTON AND SIMMONDS[16] | 2 | *E* at 230 or 280 m$\mu$ |
| Catalase (from ox liver) | Boehringer & Soehne G.M.B.H., Mannheim, Germany | 230–250 | SAMEJIMA AND YANG[17] | 0.04–0.1 | Disappearance of $H_2O_2$ followed at 230 m$\mu$ (TOHUHIKO AND PETERS[24]) |
| Serum albumin dimer (present in bovine serum albumin) | | 134 | (2 × mol. weight of serum albumin) | ~0.4–0.5 | *E* at 220 m$\mu$ |
| Serum albumin bovine; fraction V from bovine plasma | Armour Pharmaceutical Co. Ltd., Chicago, Ill., U.S.A. | 67 | PHELPS AND PUTNAM[18] | 1–8 | *E* at 230 or 280 m$\mu$ |
| Haemoglobin | Sigma Chemical Co., St. Louis, Mo., U.S.A. | 64.5 | HILL *et al.*[19] | 2–10 | *E* at 410 m$\mu$ |
| Ovalbumin | Schering-Kahlbaum A.G., Berlin | 45 | WARNER[20] | 1–2 | *E* at 230 m$\mu$ |
| Pepsin | British Drug Houses Ltd., Poole, Dorset, England | 35 | FRUTON AND SIMMONDS[16] | 2 | *E* at 230 or 280 m$\mu$ |
| Chymotrypsinogen, purified | Worthington Biochem. Corp., Freehold, N.J., U.S.A. | 25 | HARTLEY[21] | 1 | *E* at 220 or 230 m$\mu$ |
| Ribonuclease, purified | Worthington Biochem. Corp., Freehold, N.J., U.S.A. | 13.7 | HIRS *et al.*[22] | 0.5–2 | *E* at 220 m$\mu$ |
| Cytochrome *c* from horse heart, type II | Sigma Chemical Co., St. Louis, Mo., U.S.A. | 12.4 | MARGOLIASH[23] | 0.4–2 | *E* at 412 m$\mu$ |
| Insulin, purified | Eli Lily & Co., Liverpool, England | 6 | FRUTON AND SIMMONDS[16] | 1 | *E* at 230 or 280 m$\mu$ |
| Sucrose | British Drug Houses Ltd., Poole, Dorset, England | 0.348 | | 4–8 | See the text |

The elution volume of many of the proteins listed in Table I was observed for at least two concentrations, which usually differed by three or four fold; except with haemoglobin, no significant effect on the elution volumes was shown over the range of concentrations studied. The smallest quantity that can be used is 0.2 to 0.3 mg, or less with purified enzymes which are generally used in microgram amounts and can be estimated by specific and sensitive assay methods.

Elution volumes for haemoglobin at pH 6.8 and 7.4, showed variation with protein concentration, being lower than expected from their molecular weight when small amounts of protein were used and suggesting some degree of dissociation of the molecules into subunits under these conditions. ANDREWS[9,11] has already found the same behaviour for haemoglobin, β-lactoglobin and glutamate dehydrogenase with his Sephadex columns, which makes proteins such as haemoglobin, unsuitable as reference substances in gel filtration.

Most experiments were carried out with columns equilibrated with potassium phosphate buffer pH 6.8 or Tris–potassium chloride buffer pH 7.4, but for some experiments with urease, columns equilibrated with 0.15 *M* potassium chloride were used; urease examined under both conditions showed similar behaviour.

*Relationship between elution volume and molecular weight*

Figs. 1 and 2 show plots of elution volumes ($V_e$) from Bio-Gel P-100, P-200 and P-300 columns against log molecular weight, for proteins listed in Table I.

The results plotted in Fig. 1, were obtained with small columns and except for Bio-Gel P-100, the relationship between $V_e$ and log (mol.wt.) is not completely linear;

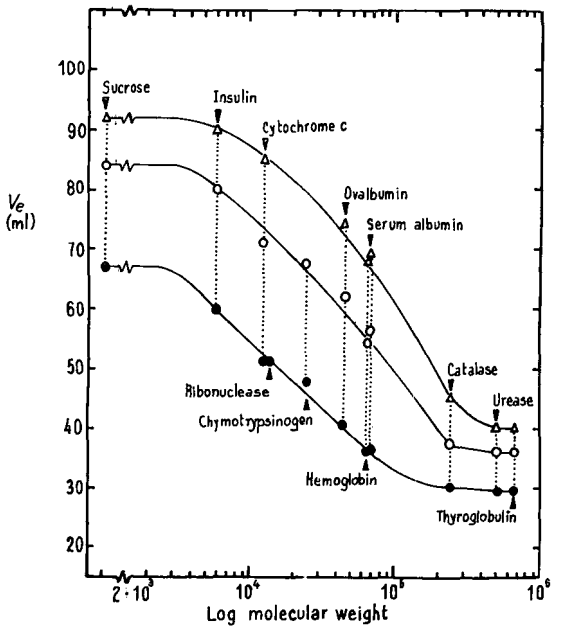


Fig. 1. Plots of elution volume ($V_e$) against log(mol.wt.), for proteins on Bio-Gel P-100 (●——●), P-200 (○——○) and P-300 (△——△). Columns (1.6 cm × 30 cm), equilibrated with 0.067 *M* potassium phosphate buffer, pH 6.8, containing 0.1 *M* potassium chloride.

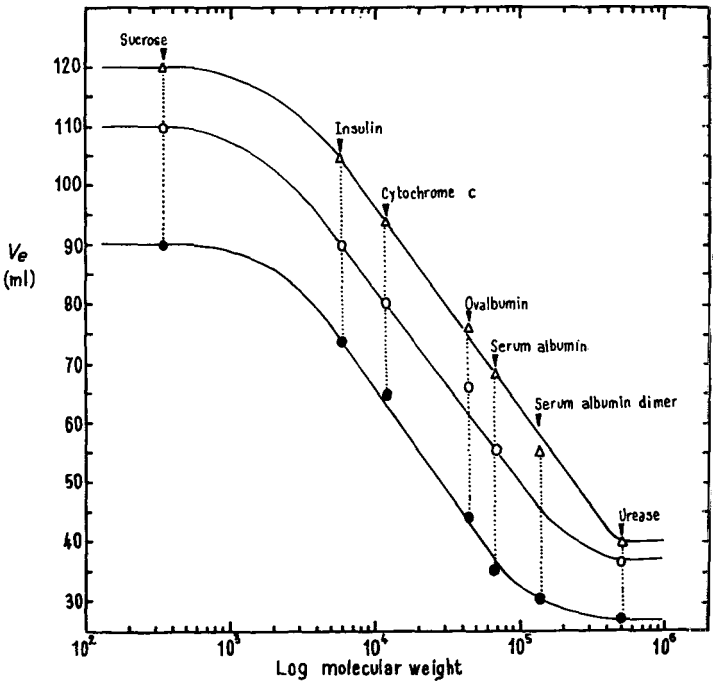


Fig. 2. Plots of elution volume ($V_e$) against log (mol.wt.), for proteins on Bio-Gel P-100 (●——●), P-200 (○——○) and P-300 (△——△). Columns (1.8 cm × 30 cm) equilibrated with 0.067 *M* potassium phosphate buffer, pH 6.8, containing 0.1 *M* potassium chloride.

however, data are reproducible. The relationship between $V_e$ and log (mol.wt.) for bigger columns is shown in Fig. 2. For Bio-Gel P-100 the experimental points lie close to a straight line in the molecular weight range 4,000–80,000, for Bio-Gel P-200 the range is 4,000–150,000, and for Bio-Gel P-300 the range is 5,000–400,000. However, the useful working range of P-100 and P-200 extends above these values; but it must be noted here that the upper limit for these gels depends upon the extent to which the gel has swollen. As with Sephadex gels[9,11] the exclusion limit, in terms of molecular weight, is greater than that specified by the manufacturer.

Experiments with insulin, cytochrome *c*, ovalbumin, serum albumin and pepsin, using Bio-Gel P-100 and P-300 columns (1.8 × 30 cm), were carried out at pH 4.0, 5.0, 6.0, 6.8, 7.4 and 8.0. Plots of $V_e$ against log (mol.wt.) at each pH were similar to those at pH 7.4 (Fig. 2), plots at pH 6.8 and 7.4 fitted identical curves; but at lower pH's, the slope of the linear parts of the graphs slightly increased as the pH decreased.

Experiments with several proteins indicate that quantitative recovery is obtained (in all cases greater than 90 %) when using these Bio-Gel columns.

*Molecular weight estimations*

Elution volumes of coproporphyrinogenase and δ-aminolaevulinic acid dehydrase were determined on calibrated Bio-Gel P-200 columns, and their molecular weights were estimated from calibration curves. Each enzyme was put through the columns alone, and they were also run in admixture with cytochrome *c*. For

coproporphyrinogenase a value of 85,000 was found, while 80,000 was obtained using Sephadex columns[25]. The molecular weight of δ-aminolaevulinic acid dehydrase was estimated to be 140,000.

## DISCUSSION

The relationship between the molecular weight of proteins used in these experiments and their elution volumes from Bio-Gel columns (Fig. 2) is similar to that obtained for Sephadex columns[9,11].

The linear parts of the curves shown in Fig. 2, indicate the molecular weights between which each gel gives optimum separation of proteins, but the useful parts of the curves extend beyond the linear stretches. However, the 30 cm columns used in this study did not give the best resolution obtainable with these gels; better results can be attained by using longer columns.

The amount of any protein necessary for a molecular weight estimation depends on the method used to measure its concentration in the column effluents; but generalizing, 0.2 mg of material is sufficient for such an estimation.

As has already been found by ANDREWS[9], dissociation of haemoglobin occurs while it passes through the column, therefore haemoglobin is not convenient as a protein standard.

Because of the anomalous behaviour of glycoproteins in gel filtration[11] only globular proteins were used for calibration of Bio-Gel columns, as most of the enzymes belong to this group of proteins the method seems to be useful.

Estimates of the molecular weights of simple proteins with Bio-Gel P-100, P-200 and P-300 (Fig. 2) are accurate to within 12 %, though estimates for other types of protein may be less accurate. On the whole, molecular weight estimations by Bio-Gel are, however, fairly reliable and the absence of ionic groups on the inert matrix of polyacrylamide diminishes the undesirable adsorption effects to a minimum.

The use of Bio-Gel offers advantages over other gel filtration materials. It swells more rapidly in various solvents; as stated above, it has an inert matrix which minimises adsorption; the Bio-Gel P series covers a wide range of molecular weights from 200–> 400,000 and its cost is less than similar materials.

In spite of the possible causes of error, also found with other gel filtration media, the method proves to be very useful and practical.

## ACKNOWLEDGEMENTS

I wish to thank Professor C. RIMINGTON and Mrs. A. LATTER for reading, discussing and correcting this manuscript.

## SUMMARY

Correlation between elution volumes, $V_e$, and molecular weight was investigated by Bio-Gel filtration of proteins of known molecular weight on Bio-Gel P-100, P-200 and P-300 columns at pH 6.8 and 7.4. The relationship between $V_e$ and molecular weight was similar over the pH range 4.0 to 8.0.

The molecular weights of two enzymes, coproporphyrinogenase and δ-amino-

laevulinic acid dehydrase, were estimated on calibrated Bio-Gel P-200 columns; their respective values were in agreement with those obtained by using calibrated Sephadex columns.

The uncertainty in values estimated by this procedure was put at 12 %.

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# MICROCHROMATOGRAPHY OF PROTEINS ON WEDGE-COMPRESSED NITROCELLULOSE MEMBRANES

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In another paper[1] we described in detail a simple procedure for compressing nitrocellulose membrane filters into a wedge thus forming a continuous pore size gradient along the membranes. Examples were presented of the chromatographic behaviour and separation of phenolsulphophthalein and a high-molecular denatured protein on membranes compressed at different angles. It was evident from those first experiments that the proposed principle of filter chromatography on compressed membranes really operated. The present communication refers to further results achieved by this technique during the chromatography of very small amounts of proteins.

## EXPERIMENTAL

VUFS membranes (VCHZ Synthesia, Uhříněves, Czechoslovakia; the membranes are now distributed by "Chemapol", Prague, under the name "Synpore 8"), 2 × 4 cm, impregnated with 2 % Tween 60 as described before[2,3], were used. A 0.1 *M* TRIS–HCl + 1 *M* NaCl buffer, pH 7.9, was used in all gel filtration and chromatographic experiments. Ovalbumin, human γ-globulin, dog and bovine serum, two protein fractions isolated by gel filtration on agar pearls[4] from the plasma expander Resorba[5], a Czechoslovak modified bovine serum MBS[6] and phenolsulphophthalein were chromatographed. Amido black 10 B, Ponceau S and nigrosine were used for the detection of the proteins[1,7].

Gel filtration of Resorba was done on a 84 × 1.4 cm column of 4 % pearl condensed agar as described before[5]. The isolated fractions were concentrated twice by dialysis against 25 % crude dextran in the TRIS buffer. The compression of the membranes was done between polished steel blocks, the values *a* and *b* (*cf.* ref. 1) on the pressing device were constant (8 mm and 15 mm respectively), the controlled pressures *P* were in the range 15–100 atm, the supports *c* were 0.08 mm and 0.16 mm respectively. Horizontal chromatography was performed as usual[8], 1–3 % samples were applied in amounts of the order $10^{-5}$–$10^{-4}$ ml. For photographic documentation the chromatograms were made transparent with paraffin oil.

## RESULTS AND DISCUSSION

Fractionation of the blood expander Resorba (Fig. 1) led to the isolation of two

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main peaks[9], A (mol. wt. of the order $10^6$) and B (mol. wt. of the order $10^5$), which were very suitable for further chromatographic experiments.

It was assumed[1] that in the course of chromatographing macromolecules on wedge-compressed membranes from the larger pores towards the smaller ones the larger particles would be intercepted at a certain distance $d$ from the line of maximum compression $h_2$. Fig. 2 shows that this phenomenon actually takes place. In one experiment, the samples were applied onto starting points at different distances from $h_2$. After 7–8 h when the indicator dye had passed to the other end of the strip the protein spots gathered at a line (I) or showed a tendency to approach a line (see also II, III) perpendicular to the direction of pore size gradient, regardless of the position of the starting points. Beyond this line the pores were evidently too small and therefore unpenetrable to the macromolecules under study. This can be seen very well with the sample nearest to $h_2$ which could not even diffuse from the start. The degree of penetration towards $h_2$ depended on the pressure and the angle of compression (*cf.* Fig. 2, II, III) as has already been shown before[1], and on the molecular size (*cf.* Fig. 2, I, II) as was expected. A sufficient "overflowing" of the buffer, as indicated by the indicator dye, is assumed here. Otherwise, in shorter chromatographic runs (about 4 h), where the larger particles were only retarded but not fully immobilized, the time factor also played an important role. To get comparable results in those shorter runs, it was necessary to stop the chromatography at the moment when the indicator dye reached the same distance behind the line $h_2$ on all chromatograms. Experiments were made under such conditions to obtain information on the role of the pressures used to deform the membranes. The results led to the following conclusions (Fig. 3):

Pressures higher than 25 atm led to constant results as far as the distances $d$ from $h_2$ are concerned. However, the running time (over 8 h) was too long to be practical when higher pressures up to 100 atm, had been used, so that 25 atm was found to be most convenient in our experiments when the parameter $c = 0.08$ mm. For $c = 0.16$ mm, $P = 20$ atm was sufficient. When lower pressures were applied (the

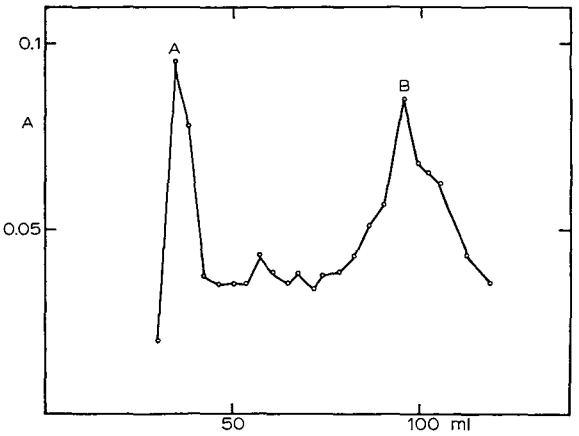


Fig. 1. Gel filtration of the plasma expander Resorba[5] on pearl condensed agar. Buffer: 0.1 *M* TRIS–HCl + 1 *M* NaCl, pH 7.9; 4% agar pearls; column: 84 × 1.4 cm. Estimation of proteins was made turbidimetrically. Fractions A and B were used for further experiments.

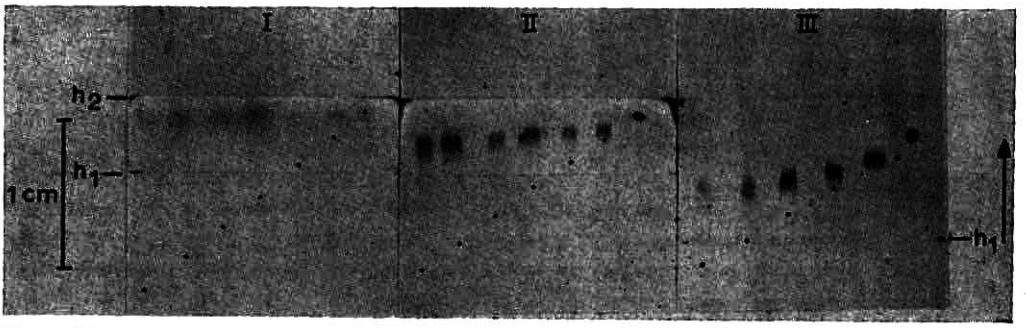


Fig. 2. Chromatography of modified proteins on wedge-compressed VUFS membranes impregnated with Tween 60. $h_1$ = Line of minimal compression; $h_2$ = line of maximal compression. Strip I: fraction B of Resorba. Strip II: fraction A of Resorba; both strips were compressed under the same conditions at the pressing device[1]: $P$ = 20 atm, $a$ = 8 mm, $b$ = 15 mm, $c$ = 0.16 mm. Strip III: fraction A of Resorba; the strip was compressed at $P$ = 25 atm, $a$ = 8 mm, $b$ = 15 mm, $c$ = 0.08 mm. Starts are indicated by dots. Buffer (see Fig. 1). Chromatographed for 4.5 h.

other conditions remaining constant) the membranes were compressed insufficiently so that fraction B often reached or even passed through the line $h_2$ (Fig. 3).

However, proteins having molecular weights less than about 100,000 (*e.g.* ovalbumin, serum albumin), passed the line $h_2$ even when pressures up to 100 atm had been used. This indicated that there was a certain limit in the sizes of particles which could penetrate the "highly compressed" membranes. For VUFS membranes and globular proteins this limit seemed to be at a mol. wt. of about 100,000.

To confirm the applicability of this technique to the fractionation of macromolecules according to their size, a model experiment was made using ovalbumin, $\gamma$-globulin, fraction "B" and indian ink. Whereas indian ink did not penetrate even the uncompressed membrane VUFS, and remained entirely on the start, the other substances differed very distinctly in their chromatographic behaviour (Fig. 4, I). Thus ovalbumin penetrated "freely" through the line $h_2$, fraction "B" and $\gamma$-globulin

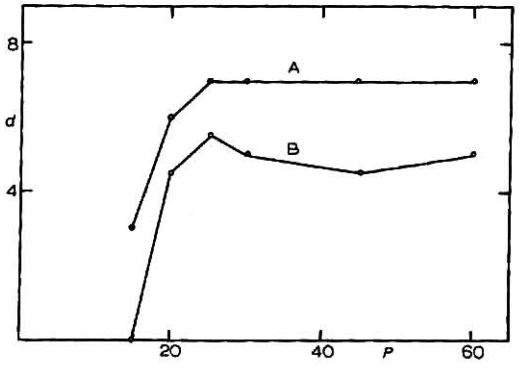


Fig. 3. Migration of protein samples on membranes compressed at different pressures. $d$ = Distance (in mm) of the centre of the spot from $h_2$; $P$ = pressure (in atmospheres) applied to deform the VUFS membrane; other conditions (*cf.* ref. 1): $a$ = 8 mm, $b$ = 15 mm, $c$ = 0.08 mm. Fractions A and B of Resorba (*cf.* Fig. 1) were applied. Phenolsulphophthalein migrated 2 mm over line $h_2$. Chromatography lasted 4 h.

were intercepted at different distances in the compressed area. A mixture of ovalbumin and fraction "B" was resolved effectively into both original components.

In a parallel run the same protein samples were chromatographed on an uncompressed membrane, where all had the same mobility (Fig. 4, II). These results seem to give a positive answer as to whether this method is effective or not.

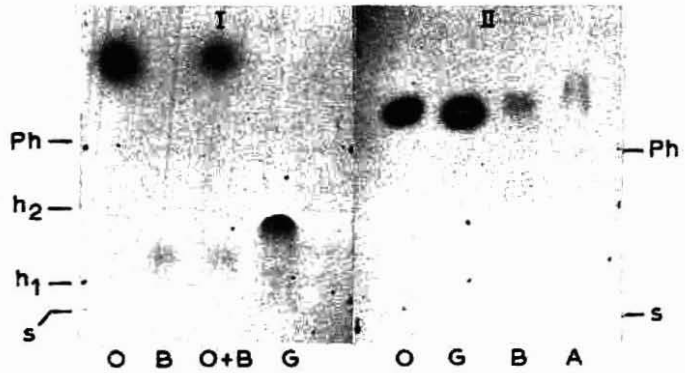


Fig. 4. Chromatography of various protein samples on VUFS membranes impregnated with Tween 60. s = Start; Ph = position of the phenolsulphophthalein (centre of the spot). Strip I: O = ovalbumin; B = fraction B of Resorba; O + B = mixture of both samples; G = $\gamma$-globulin. The strip was compressed at $P = 25$ atm, $a = 8$ mm, $b = 15$ mm, $c = 0.16$ mm. Chromatography lasted 4.5 h. Strip II: O = ovalbumin; G = $\gamma$-globulin; B = fraction B of Resorba; A = fraction A of Resorba. The VUFS membrane was not compressed. Chromatography lasted 1 h.

In other experiments, more complex mixtures of macromolecules were chromatographed to investigate the possibilities of the microchromatographic technique on wedge-compressed membranes. As is shown in Fig. 5, certain characteristic separations were achieved, suitable for some special although rather informative qualitative microestimations. Thus a characteristic difference can be seen between the chromatogram of native serum with a well separated albumin fraction and the chromatogram of the modified serum of a plasma expander MBS (Fig. 5). This change is somewhat similar to the changes of the electrophoretic patterns[10] but here some information is

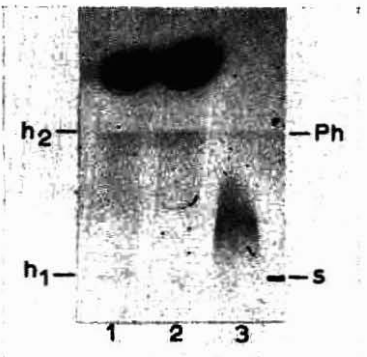


Fig. 5. Chromatography of native and modified sera. 1 = Dog serum; 2 = bovine serum; 3 = MBS. Membrane VUFS, impregnated with Tween 60, compressed at $P = 25$ atm, $a = 8$ mm, $b = 15$ mm, $c = 0.08$ mm. Buffer TRIS–HCl, pH 7.9. Chromatography for 6 h.

also given indicating the increase of particle size. However, no significant fractionation of Resorba or MBS was achieved comparable to gel filtration (*cf.* Fig. 1 and ref. 4) although separate fractions A and B had different mobilities (*cf.* Fig. 2). It is possible that a partial "stopping" of the pores by particles having very similar particle sizes causes interference here.

It can be concluded that the technique in its present form has some limitations which still have to be studied and eventually overcome, in order to achieve more accurate small scale separations of complex systems. For this purpose other types of membranes with a narrower distribution of pore sizes, as well as membranes of another chemical nature, are under investigation now. The results will be published later.

ACKNOWLEDGEMENT

The author is grateful to Mrs. ZD. NOVOTNÁ for technical assistance.

SUMMARY

Small scale chromatography of various protein samples was done on wedge-compressed nitrocellulose membrane filters impregnated with Tween 60. Evidence is presented that particles of different sizes can be separated according to the principle of filtration chromatography during migration along the continuous pore size gradient from the greater pores towards the smaller ones. Some important factors influencing the separation were examined.

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# SEPARATION OF COMPLEX MIXTURES OF POLYHYDROXY PHENOLS ON COLUMNS OF SEPHADEX

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## INTRODUCTION

As part of an investigation of protein-flavonoid complexes in beers, it became necessary to have a column chromatographic method by which proteins, protein-flavonoid complexes and free polyphenols present in malt and hop extracts could be fractionated and estimated under conditions which were mild enough not to cause changes in the system. The polyphenols involved ranged from phenolic acids like vanillic, protocatechuic, gallic, caffeic and chlorogenic to coumarins, catechins, anthocyanogens, flavonols and their glycosides, together with polymers of increasing molecular size.

Various column packing materials have been used successfully for fractionating phenols including cellulose[1], ion exchange resin (*e.g.* Amberlite IRC-50)[2,3], polyamide[4–7] and silica gel[8]. All these methods use more or less complex organic solvent mixtures for elution which are not suitable where proteins and protein complexes are involved.

GELOTTE[9] has shown that the cross-linked dextran gel, Sephadex, has a tendency to adsorb aromatic compounds especially phenols. BOCK[10] indicated that flavonoids and tannins were so strongly adsorbed that they could only be eluted with alkaline solution conditions, under which rapid auto-oxidation of many flavones occurs. However, by using G25 Sephadex and eluting with ammonia NILSONN[11] was able to separate a mixture of isoflavones without being able to discern any relationship between elution volume and structure. SOMERS[12] eliminated the adsorptive effects of the Sephadex by preparing the gel and eluting in aqueous alcohol media.

In this communication the behaviour of a range of phenols on Sephadex columns using aqueous eluants is described and conditions suitable for fractionation defined making use of the adsorptive properties of the gel. At the same time, the molecular sieving properties of the gel permit separation of free phenols from high molecular weight proteins and phenol polymers[13].

## MATERIALS AND METHODS

### *Column preparation and operation*

G25 Sephadex in the medium particle size grade (AB Pharmacia, Uppsala, Sweden) has been used throughout the investigation. Packing, handling and general

operation have been described in detail by FLODIN[14] and these techniques have been generally followed. Columns were 35 cm long and 2.5 cm in diameter with a total bed volume of 120 ml. Each phenol sample was dissolved in the eluting medium to give a concentration of 200 $\mu$g/ml and 1 ml of this was loaded onto the column. Even the least soluble flavonoids dissolved sufficiently to be detected. The flow rate of 25 ml/h was governed by a peristaltic pump and was constant throughout the series of experiments. The eluate from the column was either pumped to a fraction collector or to an AutoAnalyser assembly previously described[15]. In the former case the presence of the phenols in the eluate was detected by the absorption at 280 m$\mu$ whilst in the latter the phenols were coupled with diazotized *p*-aminobenzoic acid and the resultant yellow colour monitored at 420 m$\mu$. Continuous monitoring had the advantages of convenience and accuracy in determining peak elution volumes. In addition reference to a calibration curve permitted peak area to be related to amount of the phenol present in the sample. $10^{-4}M$ concentrations of phenols could be determined in this way with a coefficient of variation of 2.7 %.

Characteristics of the column were determined in the usual manner (FLODIN[14]) and $K_D$ values calculated from the equation $V_e = V_o + K_D V_i$. Under the conditions used $V_o$ and $V_i$ were found to be 40 and 55, respectively. Under standardised conditions the peak elution volume was characteristic of a particular phenol and quite reproducible whether the phenol was alone or in a mixture. Peaks were symmetrical and sharp if eluted early but very strong adsorption and consequently long elution times resulted in the spreading of the bands to some extent. This was not in fact a problem, since the strongly adsorbed compounds were usually very well separated.

*Separation of flavonoids as molybdate complexes*

In the case of flavonoids containing several hydroxyl functions it was necessary to weaken the adsorptive forces between them and the column material so as to achieve elution under mild aqueous conditions in a reasonable time. The formation of complexes between molybdate ions and phenols containing *ortho*-hydroxyl groups is well known. These complexes form readily in mixing aqueous solutions and are yellow in colour. They were usually formed *in situ* on the column by first loading the phenol and then adding 1 ml of 10 % sodium molybdate solution before commencing elution with water. Different groups of flavonoids show characteristic differential spectra some of which are shown in Fig. 1, which, combined with elution volume, serves to identify unknowns.

Molybdate complexes of flavonoids were adequately separated on smaller columns (0.9 cm × 30 cm, bed volume 40 ml) with a saving in time.

## RESULTS AND DISCUSSION

*Simple phenols*

Table I shows $K_D$ values calculated for a range of simple phenols using water, 0.05 $M$ sodium chloride, 0.1 $M$ ammonium hydroxide, 0.1 $M$ acetic acid and, in some cases, 0.01 $M$ sodium molybdate, as eluting media.

With water, elution occurred according to the number of hydroxyl groups the phenol contained. Adsorption occurred in all cases, hence fractionation was achieved not on the basis of molecular size and shape but as a result of differing degrees of

bonding between the phenol and the residual carboxyl groups present in the gel matrix. There was little or no difference between *ortho* and *meta* substituted compounds. Hydroquinone was anomalous in its elution as it is in its structure and this may be accounted for by its tendency to assume a quinone form in solution. Incorporation of neutral electrolyte into the eluting medium caused the $K_D$ values for the phenols to

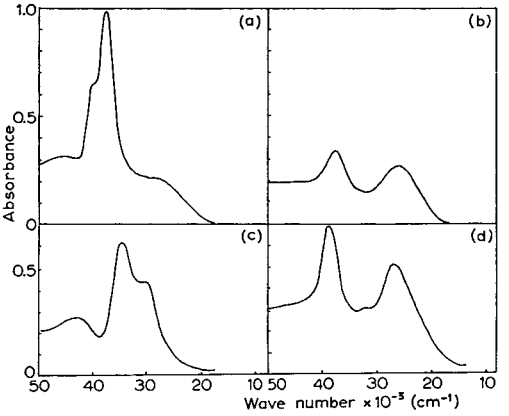


Fig. 1. Spectra of molybdate complexes of some representative flavonoids. Sample: flavonoid dissolved in 0.001 *M* sodium molybdate. Reference: 0.001 *M* sodium molybdate. (a) Catechin; (b) quercetin; (c) dihydroquercetin; (d) rutin.

fall into two series. The *ortho* substituted catechol and pyrogallol formed one group and phenol, resorcinol and phloroglucinol the other with $K_D$ values 0.7 units greater than the corresponding *ortho* compounds. The behaviour of hydroquinone was no longer anomalous and its $K_D$ fell between those for resorcinol and catechol. In acid medium the hydrogen bonding to the column matrix was increased, the extent depending on the number of hydroxyl groups present. $\Delta K_D$ was 0.15, 0.45 and 0.9 for one, two and three hydroxyl groups respectively. The order of elution was otherwise the same as for water. In alkaline media, adsorption was almost eliminated and any small differences in $K_D$ may be accounted for by the shape and size of the ionic species present at these pH's.

Presence of *ortho*-hydroxyl groups permits the formation of complexes in the

TABLE I

COMPARISON OF $K_D$ VALUES FOR SIMPLE PHENOLS IN AQUEOUS SOLUTIONS

| | *Eluting medium* | | | | |
|---|---|---|---|---|---|
| | *Water* | *0.5 M NaCl* | *0.1 M* $NH_4OH$ | *0.1 M* $CH_3COOH$ | *0.01 M* $Na_2MoO$ |
| Phenol | 1.8 | 2.0 | 1.1 | 1.95 | — |
| Hydroquinone | 1.05 | 2.4 | 1.7 | 2.4 | — |
| Resorcinol | 2.1 | 2.65 | 1.6 | 2.5 | — |
| Catechol | 2.05 | 1.9 | 1.05 | 2.5 | 2.2 |
| Phloroglucinol | 2.3 | 3.15 | 1.1 | 3.25 | — |
| Pyrogallol | 2.4 | 2.4 | 1.3 | 3.25 | 2.0 |

presence of molybdate ions. Under these circumstances complex formation reduced the bonding of pyrogallol, but apparently increased slightly that of catechol. This may be due to the different shape and size of the complex.

Table II shows the behaviour of a range of phenol derivatives in the same eluting solvents. Here the effects of solvent were much less marked. As in the case of phenol itself, both electrolyte and acid tended to increase the $K_D$ but generally not to a very marked extent.

TABLE II

$K_D$ VALUES OF PHENOL DERIVATIVES IN AQUEOUS MEDIA

| *Phenol derivative* | *Eluting medium* | | | |
|---|---|---|---|---|
| | *Water* | *0.05 M NaCl* | *0.1 M $NH_4OH$* | *0.1 M $CH_3COOH$* |
| Phenol | 1.8 | 2.0 | 1.1 | 1.95 |
| *o*-Hydroxybenzoic acid | 1.0 | 2.1 | 1.6 | 3.4 |
| Arbutin | 1.45 | 1.55 | 0.85 | — |
| *o*-Nitrophenol | 1.45 | 2.45 | 1.8 | 2.1 |
| Saligenin | 1.85 | 2.1 | 1.55 | 1.9 |
| Guaiacol | 2.2 | 1.8 | 1.45 | 2.1 |
| *o*-Hydroxybenzaldehyde | 2.3 | 2.3 | 1.55 | 2.3 |
| *o*-Cresol | 2.6 | 2.55 | 1.85 | 3.0 |
| Orcinol | 3.0 | 2.6 | 1.5 | — |
| *o*-Chlorophenol | 3.15 | 3.4 | 1.55 | 3.0 |
| *p*-Hydroxydiphenyl | 4.8 | 5.4 | 2.9 | — |

It will be noted from inspection of the quoted $K_D$ values that separation of mixtures of these phenols should be possible and water or acid are probably the most suitable media. Fig. 2 shows two tracings of the elution pattern obtained automati-

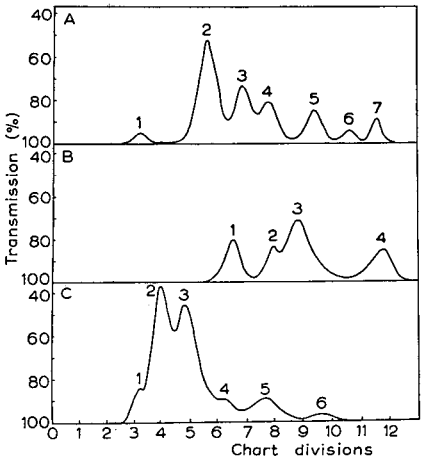


Fig. 2. Separation of phenol mixtures on columns of G25 Sephadex. (A) 35 cm × 2.5 cm column eluted with water. 1 = Hydroquinone; 2 = phenol; 3 = catechol + resorcinol; 4 = guaiacol; 5 = pyrogallol + phloroglucinol; 6 = cresol; 7 = orcinol. (B) Same column eluted with 0.1 *M* acetic acid. 1 = Phenol; 2 = hydroquinone; 3 = resorcinol + catechol; 4 = pyrogallol + phloroglucinol. (C) 0.9 × 25 cm column eluted with 0.1 *M* acetic acid containing 0.05 *M* sodium chloride. 1 = Saligenin; 2 = nitrophenol + guaiacol; 3 = salicylaldehyde; 4 = catechol; 5 = cresol + chlorophenol; 6 = salicylic acid.

cally with the AutoAnalyser. The upper one (Fig. 2A) was obtained with water as eluant, the middle one (Fig. 2B) with dilute acetic acid. Fig. 2C shows the separation of various phenol derivatives using acetic acid as eluant. In this case acid was used because of the anomalous behaviour of hydroxy acids in water.

The behaviour of even simple phenols on Sephadex columns is complex and it is difficult to predict $K_D$ from the structure. The factors determining elution volume include both number and position of hydroxyl groups, substituent groups which effect strength of bonding and medium. In addition steric factors may govern penetration of gel grains. Neither $K_D$ or change in $K_D$ on passing from neutral to alkaline medium seemed to be related directly to the p$K$s of the phenol. Only nitro and carboxylic acid groups decreased bonding. However, it will be seen from Table III that the groups shown causing increased bonding do so in the order expected from their electron donating properties.

TABLE III

EFFECTS OF NUCLEAR SUBSTITUTION IN THE STRENGTH OF HYDROGEN BONDING AND HENCE ON THE ELUTION VOLUME FROM SEPHADEX COLUMNS

Water is used as eluant.

| *Increased bonding* | $\Delta K_D$ | *Parent phenol* | *Decreased bonding* | $\Delta K_D$ |
|---|---|---|---|---|
| *o*-Cresol (2.6) | + 0.8 | | Salicylic acid (1.0) | −0.8 |
| Guaiacol (2.2) | + 0.4 | Phenol (1.8) | | |
| Catechol (2.05) | + 0.25 | | *o*-Nitrophenol | −0.35 |
| Orcinol | + 0.9 | | 3,5-Dihydroxybenzoic acid (1.5) | |
| Phloroglucinol (2.3) | + 0.2 | Resorcinol (2.1) | | −0.6 |

*Phenolic acids*

Monocarboxylic acids were not separated on Sephadex when eluted with water. As reported by GELOTTE[9], the carboxylic acid group has a "negative sorption effect" and in most cases elution occurred much earlier than would be imagined from the parent phenol. Intra-molecular hydrogen bonding is not always responsible since there is no difference between *o*- and *p*-hydroxybenzoic acid in water and the 2,4-dihydroxy acid was eluted early while the 2,3-substituted one was not. In electrolyte solution this "negative sorption" effect disappeared in all the acids and there were stronger absorptive forces. The rate of elution seemed to depend on the number, and orientation of free hydroxyl groups, (*i.e.* those not involved in internal hydrogen bonding) and the extent to which these groups are accessible to the carboxyl group of the gel. Most of the acids were eluted very early with ammonia since they also carry a negative charge and will only be repelled by similar acid groups in the gel. Formation of a complex with molybdate resulted in earlier elution as would be expected except in the case of the 2,6-dihydroxy acid which, although both groups can complex, was very strongly adsorbed. Methylation, as in vanillic and syringic acid, resulted in behaviour very much like a monohydroxy acid. They were not differentiated on Sephadex columns.

From Table IV it is thus apparent that electrolyte is required if separation of mixtures of phenolic acids is to be achieved. Fig. 3 shows the separation of a mixture of acids already reported in barley. Resolution in the first stage is not complete but fractions can be collected as shown and completely resolved by re-running the samples

TABLE IV

$K_D$ VALUES OF PHENOLIC ACIDS IN AQUEOUS ELUTING MEDIA

| *Phenolic acid* | *Eluting medium* | | | |
|---|---|---|---|---|
| | *Water* | *NaCl* | $NH_4OH$ | $Na_2MoO$ |
| *o*-Hydroxybenzoic acid | 1.0 | 2.1 | 1.6 | 1.2 |
| *m*-Hydroxybenzoic acid | 1.0 | 1.8 | 0.85 | — |
| *p*-Hydroxybenzoic acid | 1.0 | 1.45 | 0.8 | — |
| 2,3-Dihydroxybenzoic acid | 2.6 | 1.6 | 0.4 | 2.0 |
| 2,4-Dihydroxybenzoic acid | 0.85 | 2.7 | 1.1 | 1.4 |
| 2,5-Dihydroxybenzoic acid | 1.0 | 3.0 | 1.7 | 1.4 |
| 2,6-Dihydroxybenzoic acid | 0.7 | 1.45 | 0.4 | 2.95 |
| 3,4-Dihydroxybenzoic acid | 1.7 | 2.1 | 0.7 | — |
| 3,5-Dihydroxybenzoic acid | 2.2 | 1.9 | 0.7 | — |
| 3,4,5-Trihydroxybenzoic acid | 1.05 | 2.5 | 1.05 | 1.9 |
| 2,3,4-Trihydroxybenzoic acid | 2.05 | 2.65 | 1.1 | 2.0 |
| Vanillic acid | 0.85 | 1.95 | 0.85 | — |
| Syringic acid | 0.8 | 1.95 | 0.85 | — |
| Chlorogenic acid | 1.6 | 3.9 | 1.35 | — |

using water as solvent. Fig. 4 shows the separation on a small analytical column of alkaline degradation products of a flavonoid mixture.

Finally, in Tables V and VI $K_D$ values are given for cinnamic acid derivatives and larger phenols with two benzene rings. In caffeic acid, the two hydroxyl groups imparted quite strong binding properties. These were reduced by methylating one of them and reduced still further by the introduction of an additional methoxyl group, probably because of steric hindrance. Diphenyl and naphthalene derivatives were generally strongly adsorbed, especially in water.

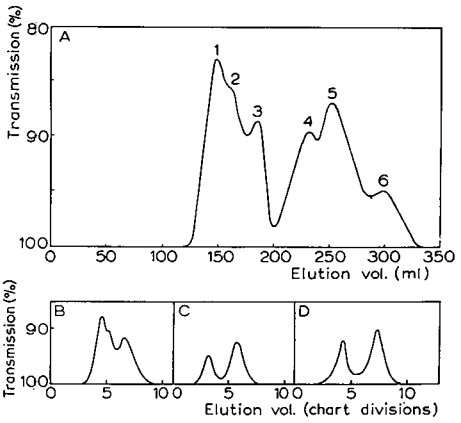


Fig. 3. Separation of phenolic acid mixtures on G25 Sephadex columns. (A) Fractionation of the phenolic acids reported to be present in worts on a 2.5 × 35 cm column eluted with 0.1 *M* sodium chloride. 1 = Vanillic + syringic; 2 = 3,4-dihydroxybenzoic; 3 = gallic; 4 = ferulic; 5 = sinapic; 6 = chlorogenic + caffeic. (B) Rechromatography of fractions 1 + 2 on a 0.9 × 25 cm column of Sephadex using water as eluant. (C) Separation of a ferulic–sinapic acid mixture under the same conditions. (D) Resolution of caffeic and chlorogenic acids from fraction 6 under the same conditions.

*Flavonoids*

Behaviour of the flavonoids in the solvents previously used for simple phenols was investigated first. 1 ml of the appropriate solvent containing approximately 200 $\mu$g of each flavonoid (or a saturated solution if the solubility was very low) was loaded on to the column and elution carried out with either water, 0.5 *M* sodium chloride or 0.1 *M* ammonia. It can be seen from Table VII that apart from the parent chalcone and the single hydroxylated 3-hydroxyflavone, only the glycosides rutin and quercitrin could be eluted with water. Even these were relatively very strongly adsorbed. Binding was even stronger in dilute electrolyte solution. In ammonia, all the compounds could be eluted as sharp peaks with very different $K_D$ values. Naringenin and hesperetin were still strongly adsorbed as was rutin. Quercitrin, however, with

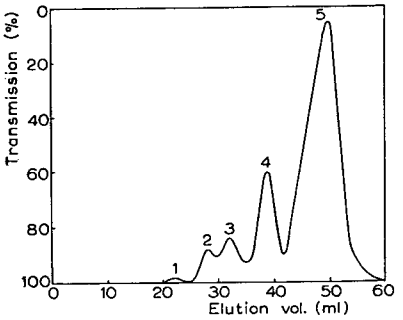


Fig. 4. Separation of alkaline degradation products of a barley flavonoid extract on a 0.9 × 25 cm column of G25 Sephadex using 0.1 *M* sodium chloride as eluant. 1 = *p*-Hydroxybenzoic acid; 2 = vanillic acid; 3 = 3,4-dihydroxybenzoic acid; 4 = 2,4-dihydroxybenzoic acid; 5 = phloroglucinol.

TABLE V

| | *Eluting medium* | | | |
|---|---|---|---|---|
| | *Water* | *NaCl* | $NH_4OH$ | $MoO_4$ |
| *p*-Coumaric acid | 1.45 | 1.3 | 1.2 | — |
| Caffeic acid | 1.4 | 4.0 | 0.95 | 2.8 |
| Ferulic acid | 1.0 | 3.1 | 1.1 | — |
| Sinapic acid | 0.8 | 3.25 | 1.15 | — |

TABLE VI

| | *Eluting medium* | | | |
|---|---|---|---|---|
| | *Water* | *NaCl* | $NH_4OH$ | $MoO_4$ |
| 2,2′-Dihydroxydiphenyl | 2.85 | 3.4 | 2.35 | — |
| 4,4′-Dihydroxydiphenyl | 0.8 | — | 2.4 | — |
| 2-Naphthol | 6.45 | — | 1.7 | — |
| 2,7-Dihydroxynaphthalene | — | — | 2.2 | — |
| 2,3-Dihydroxynaphthalene | 7.4 | — | 2.9 | 7.2 |
| 1,3-Dihydroxynaphthalene | 9.1 | — | 3.05 | — |

TABLE VII

$K_D$ VALUES OF SOME FLAVONOIDS AND RELATED COMPOUNDS ON G25 SEPHADEX COLUMNS

| *Flavonoid* | *Eluting medium* | | |
|---|---|---|---|
| | *Water* | *0.05 M NaCl* | *$NH_4OH$* |
| Chalcone | 3.1 | 3.1 | — |
| 3-Hydroxyflavone | 4.2 | 5.0 | 4.0 |
| Rutin | 5.6 | 6.5 | 3.9 |
| Quercitrin | 11.4 | * | 1.85 |
| Robinetin | * | * | 0.60 |
| Quercetin | * | * | 0.65 |
| Hesperidin | * | * | 0.8 |
| Morin | * | * | 1.2 |
| Catechin | * | * | 1.3 |
| Dihydroquercetin | * | * | 1.6 |
| Naringenin | * | * | 5.6 |
| Hesperetin | * | * | 6.25 |
| Fisetin | * | * | — |

* Irreversibly adsorbed.

similar structure, was eluted much earlier and in a similar position to dihydroquercetin. Quercetin and robinetin were apparently not adsorbed at all and washed from the column very early.

On this evidence elution and fractionation can only be achieved with ammonia. Fig. 5 shows the elution pattern for a synthetic mixture where resolution is obtained except for the group of compounds with $K_D$ 1.2–1.8.

Although separation in ammonia is useful it has the drawback already mentioned, namely that some compounds are not stable under these conditions. Accordingly, the behaviour of molybdate complexes was studied. A column of Sephadex was equilibrated with 0.001 *M* sodium molybdate and elution carried out with this

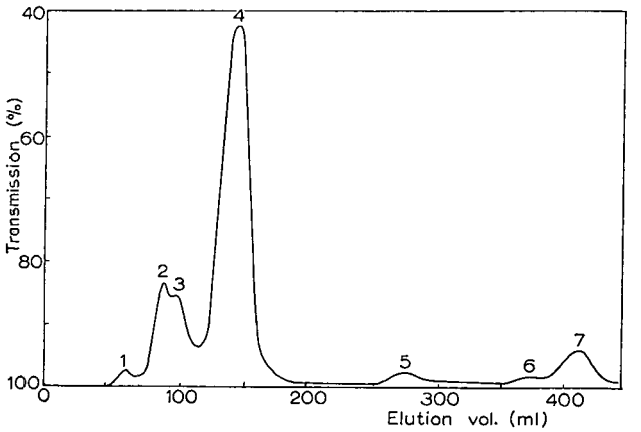


Fig. 5. Separation of a synthetic flavonoid mixture on a 2.5 × 35 cm column of G25 Sephadex using 0.1 *M* ammonium hydroxide as eluant. 1 = High molecular weight impurity; 2 = robinetin + quercetin; 3 = hesperetin; 4 = dihydroquercetin + morin + quercitrin + catechin; 5 = 3-hydroxyflavone + rutin; 6 = naringenin; 7 = hesperidin.

TABLE VIII

ELUTION CHARACTERISTICS OF THE MOLYBDATE COMPLEXES OF SOME FLAVONOIDS ON G25 SEPHADEX COLUMNS

| *Flavonoid* | $K_D$ (*calculated*) | $V_t/V_0$ |
|---|---|---|
| Quercitrin | 0.24 | 1.31 |
| Rutin | 0.26 | 1.35 |
| Catechin | 0.34 | 1.45 |
| 3-Hydroxyflavone | 0.5 | 1.66 |
| Dihydroquercetin | 1.03 | 2.37 |
| Hesperetin | 1.3 | 2.72 |
| Hesperidin | 1.95 | 3.6 |
| Morin | 2.16 | 3.95 |
| Fisetin | 3.33 | 5.42 |
| Robinetin | 4.62 | 7.14 |
| Naringenin | 4.9 | 7.55 |
| Quercetin | — | 10 |

medium. Table VIII show how some representative flavonoids were eluted. In this case the smaller column was used and values of $V/V_0$ ($V$ = elution volume, $V_0$ = void volume) calculated. This is equally characteristic of the phenol under the standard conditions and is open to fewer errors since the value of $V_t$, the inner volume, need not be determined.

There is, however, a simple relationship between the $V/V_0$ ratio and the $K_D$ value,

$$V/V_0 = 1 + \alpha K_D \text{ where } \alpha = V_t/V_0$$

and this has been used to calculate the $K_D$ values for comparison with those obtained on a larger column of G25 fine crushed Sephadex.

$V/V_0$ ratios were very reproducible and were different for many of the flavonoids

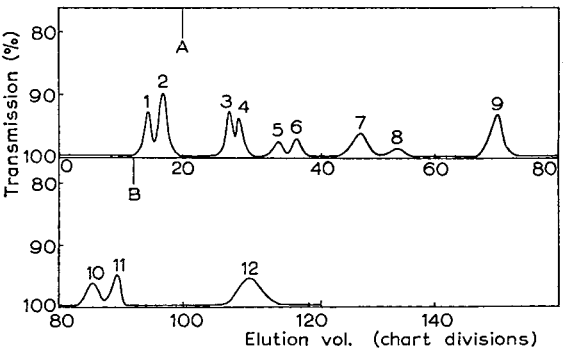


Fig. 6. Fractionation of a complex flavonoid mixture on a 0.9 × 30 cm column of G25 (fine bead) Sephadex. Phenol content of the eluate continuously recorded by the diazonium coupling reaction on an AutoAnalyser assembly. Elution with water proceeded up to point A when 1 ml of 1 *M* sodium molybdate was layered on top of the column. Elution of complex was continued with water to point B where 1 ml 0.1 *M* sodium hydroxide was placed on top of the column. Quercetin was then eluted with water. 1 = 3-Hydroxyflavone; 2 = rutin; 3 = quercitrin; 4 = catechin; 5 = dihydroquercetin; 6 = hesperitin; 7 = hesperidin; 8 = morin; 9 = fisetin; 10 = robinetin; 11 = naringenin; 12 = quercetin.

studied. Flavone glycosides and catechin were relatively loosely bound to the gel matrix but aglycones were strongly adsorbed.

Inspection of the values of $V/V_o$ again indicates that separation of flavonoids in a mixture could be achieved. Whilst quercetin, rutin, catechin and 3-hydroxyflavone have similar elution volumes as molybdate complexes, in water the $K_D$ values are well separated. A complete elution scheme is shown in Fig. 6 in which preliminary elution is carried out with water and the remaining flavonoids are eluted as their molybdate complexes. Quercetin differs from the others in that it requires alkaline conditions for desorption.

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SUMMARY

It has been demonstrated that mixtures of a wide range of phenols and flavonoids can be fractionated on Sephadex columns by making use of the adsorptive properties. $K_D$ values for a number of phenols and derivatives have been determined, using several aqueous elution solvents which have permitted the selection of the optimum conditions for any particular separation. Flavonoids are generally more strongly adsorbed but quite complex mixtures may be fractionated by first eluting with water, then converting the remaining flavonoids to molybdate complexes. This decreases the strength of binding to the column but allows fractionation.

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# THE PURIFICATION OF HUMAN CHORIONIC GONADOTROPIN ON DEAE-SEPHADEX

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A number of methods have been reported for the purification of human chorionic gonadotropin (HCG)[6-8,11,16,17]. These methods are either laborious, time consuming or limited to preparation of relatively small quantities. A simple procedure for the purification of moderate quantities of HCG on DEAE-Sephadex, the immunoelectrophoretic properties of the purified material obtained and a quantitative immunoassay of HCG, form the subject of this report.

## MATERIALS AND METHODS

### *Human chorionic gonadotropin*

Commercial HCG, with a specific activity of 3000 I.U./mg, was purchased from Organon Inc., West Orange, N.J.

### *HCG antiserum*

Albino rabbits of both sexes, weighing 3.5 kg, were used. Animals were bled from the marginal ear vein. Both the commercial HCG preparation and the highly purified immunoelectrophoretically homogeneous HCG was used for the immunization of rabbits. The immunization regimen was as follows:

1. An initial dose of HCG (500 I.U.) dissolved in saline (0.25 ml) and emulsified with an equal volume of Freund's Complete Adjuvant, was injected into the toe pads of the hind feet.

2. Booster doses of HCG (2000 I.U.), dissolved in saline (0.25 ml) and emulsified with an equal volume of Freund's Complete Adjuvant, were injected intramuscularly (hind legs) three weeks after the initial doses and every two weeks subsequently.

### *Fractionation*

DEAE-Sephadex A-50 medium from Pharmacia, Uppsala, Sweden, was treated according to the manufacturer's instructions and then suspended in 0.005 $M$ phosphate buffer, pH 6.0, overnight. The column (1.5 cm diameter) was prepared according to the procedure described by FLODIN[5]. The HCG sample (aqueous solution, 80 mg/ml) was then placed on the top of the column (6 cm column section per 10 mg of protein). Elutions were made either with a stepwise increasing concentration of NaCl (0.00, 0.04, 0.08, 0.12 and 0.16 $M$) in 0.005 $M$ phosphate buffer, pH 6.0, or with continuous gradient elution according to LAKSHMANAN AND LIEBERMAN[9], with $V_0$ = 2000 ml, 0.005 $M$ phosphate buffer, pH 6.0; $C_0$ = 1.5 $M$ NaCl in 0.005 $M$ phosphate buffer, pH 6.0; $R_1$ = 1.0 ml/min; $R_2$ = 2.4 ml/min.

*Protein measurement*

Protein concentration in the fractions from the column was determined by U.V. absorbancy at 280 mμ using a Beckman Model DU spectrophotometer.

*Double diffusion*

Double diffusion was carried out according to the OUCHTERLONY[14] technique. Agar solution (1 %) was prepared in barbital buffer (sodium barbital (5.88 g), sodium acetate (3.84 g), and sodium merthiolate (0.044 g) were dissolved in water (440 ml) and adjusted to pH 8.4 with 3*N* HCl. Stock solution was diluted 1:4 with water before use. Agar solution (12 ml) was pipetted into a petri dish (diameter 8.5) and allowed to solidify. Wells were then cut into the agar, the distance between the center well and the wells surrounding it being 3 mm. Antiserum was introduced into the center well and 10 λ of the sample was introduced into the surrounding wells. The plate was then incubated at 25° for 16 h.

*Immunoelectrophoresis*

Immunoelectrophoresis was carried out employing LKB 6800A immunoelectrophoresis apparatus following the standard immuno-processing technique recommended by the manufacturer. In our hands, the following set of conditions were optimal: 15 mA/tray; 5–6 V/cm; duration 1 h. The plates were incubated in a humid chamber at 25° for a sufficient period of time (2–3 days) for the precipitation bands to occur. The plate was then washed in 1% sodium chloride solution for 16 h, dried and finally stained with amido black.

*Immunodiffusion in agar*

Antiserum (1 ml) was added to agar solution (25 ml) at 50°, and mixed thoroughly. The mixture (10 ml) was placed in a trough (26.8 cm × 2.6 cm) and allowed to solidify. Wells (2.5 mm in diameter) were then cut into the agar at 2.5 cm apart. 6 λ of HCG solution was introduced in each well and the plate incubated in a humid chamber at 25° for 2 days for the precipitation zone to form. The diameter of the precipitation zone was measured under a B & L stereomicroscope with a sealed lens. The diameters of the precipitation zones of HCG solutions of unknown concentrations were compared with those of HCG solutions of known concentration.

## RESULTS

The distribution of serologic activity in a series of fractions obtained by chromatography of commercial HCG, employing a stepwise increasing NaCl gradient, is shown in Fig. 1. Serologic activity was determined by immunodiffusion assay using homogeneous rabbit-HCG-antiserum. In peaks 1 and 2, there was a complete absence of serologic activity as determined by immunoelectrophoresis and immunodiffusion. In peaks 3 and 4, there were traces of serologic activity. Immunoelectrophoresis showed the presence of three antigens, in small amounts, in the α- and β-globulin regions. Most of the serologic activity was found in peak 5. Immunoelectrophoresis showed the presence of two antigens, one in the $\alpha_2$-globulin region and one in the $\alpha_1$–$\alpha_2$-globulin region (Fig. 2b). These fractions (No. 115–145) were combined, lyophylized, and desalted. The material thus obtained was rechromatographed employing a linear

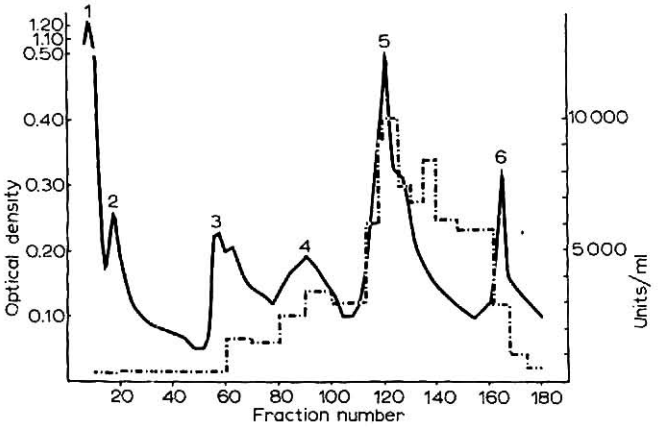


Fig. 1. Chromatography of HCG on DEAE-Sephadex, stepwise increasing concentration of NaCl (0.00, 0.04, 0.08, 0.12 and 0.16 *M*) in 0.005 *M* phosphate buffer, pH 6.0. (———) Protein measurement; (–·–·–) HCG activity (immunological).

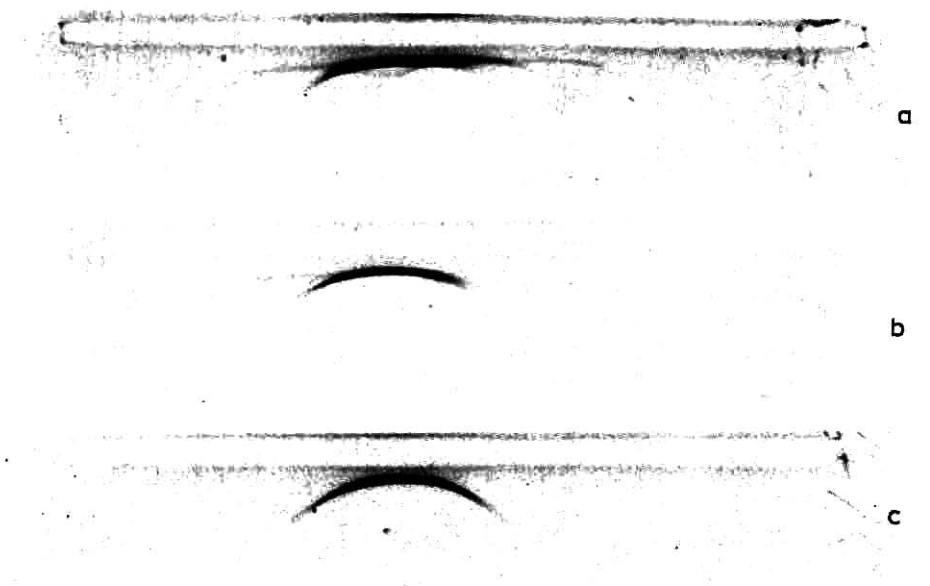


Fig. 2. Electrophorogram of Organon HCG (a), partially purified HCG (see text) (b), and final purified HCG (see text) (c), against antiserum to Organon HCG.

gradient of NaCl. The distribution of serologic activity in a series of fractions obtained from the second chromatography is shown in Fig. 3. Fractions 98 to 123 were combined, lyophylized, and desalted. The final product was serologically homogeneous as shown by double diffusion and by immunoelectrophoresis (Fig. 2c). The specific immunologic activity of this material was 11,000 I.U./mg. The overall recovery of immunological activity was 19%. The biological activity was not assessed.

The heterogeneity of the antiserum produced by the rabbit immunized with commercial HCG is shown in Fig. 4a. A solution of commercial HCG (200 I.U./ml)

was subjected to electrophoresis, developed with homologous antiserum (*i.e.* antiserum produced by a rabbit immunized with commercial HCG) and stained with amido black. Six precipitation bands were observed: one in the $\alpha_1$ region, two in the $\alpha_2$ region, two in the $\beta$ region and one in the $\gamma$ region. The same experiment, carried out with the specific antiserum obtained from rabbit immunized with immunoelectrophoretically homogeneous HCG, showed a single precipitation line located in the $\alpha_2$ region (Fig. 4b). The homogeneity of the antiserum produced in rabbits immunized with immunoelectrophoretically homogeneous HCG was further confirmed by a series of double-diffusion tests according to the OUCHTERLONY[14] technique carried out with the heterogeneous antiserum or homogeneous antiserum in the center wells and human plasma, normal female urine concentrate, pregnancy urine concentrate, commercial HCG and highly purified HCG in the wells surrounding the antisera. With the antisera produced by rabbit immunized with commercial HCG, there were five precipitation lines between commercial HCG and the antiserum, one line between urine concentrate from normal women and antiserum, two lines between pregnancy urine concentrate

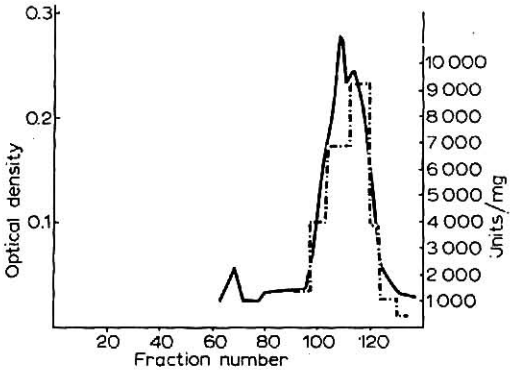


Fig. 3. Chromatography of HCG on DEAE-Sephadex, continuous gradient elution. (———) Protein measurement; (–·–·–) HCG activity (immunological).

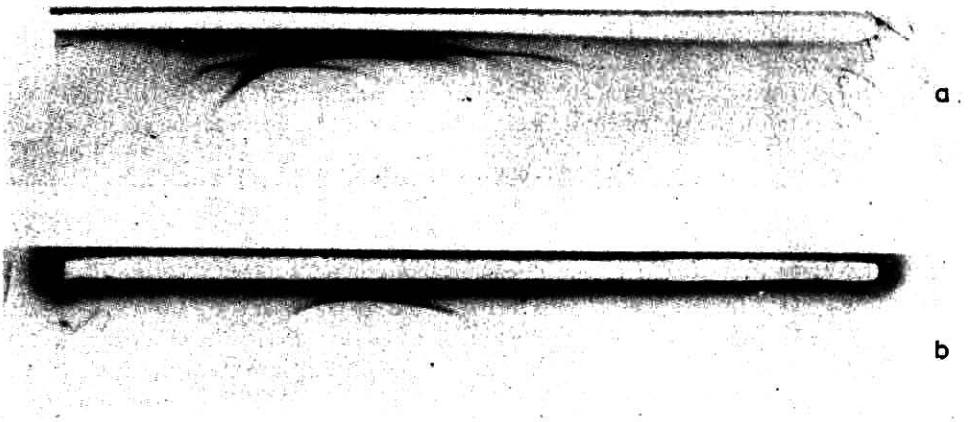


Fig. 4. Electrophorogram of Organon HCG against antiserum to Organon HCG (a) and antiserum to highly purified HCG (b).

and antiserum, and one line between highly purified HCG and the antiserum. With the antiserum produced by rabbit immunized with immunoelectrophoretically homogeneous HCG, there was no precipitation line between the wells containing either human serum or the urine concentrate from normal women and the antiserum. Only a single line was observed between commercial HCG, purified HCG and pregnancy urine concentrate and the antiserum.

The specific rabbit-HCG-antiserum (*i.e.* antiserum produced by a rabbit immunized with electrophoretically homogeneous HCG) was utilized in the quantitative assay of various HCG preparations. An actual diffusion plate is shown in Fig. 5. In this experiment, twelve dilutions were made from a known solution of biologically standardized HCG and 6 $\lambda$ of each solution was placed in a well in the agar plate impregnated with HCG antiserum. The plate was stained with amido black.

A standard curve was constructed by carrying out fourteen similar experiments each involving six dilutions of a standard sample. The mean diameter of precipitation areas and the mean square deviation of the individual values from the mean were calculated for each HCG concentration. Plotting the resulting mean values $\bar{d}$, $\bar{d} + \sigma$ and $\bar{d} - \sigma$ as a function of logarithm of the HCG concentrations, three calibration lines

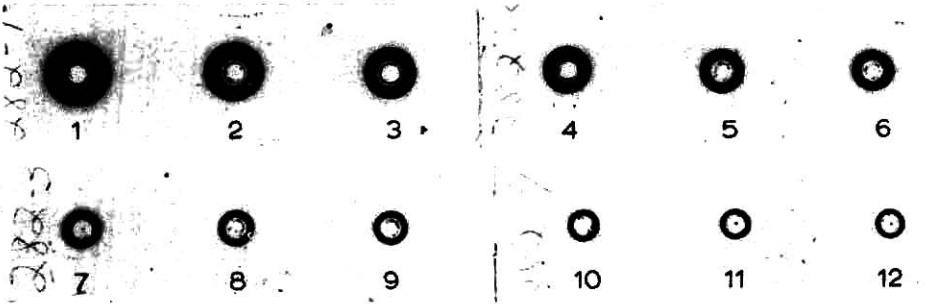


Fig. 5. Quantitative diffusion plate. (1) 19.05 I.U.; (2) 15.12 I.U.; (3) 12.00 I.U.; (4) 9.52 I.U.; (5) 7.56 I.U.; (6) 6.00 I.U.; (7) 4.76 I.U.; (8) 3.78 I.U.; (9) 3.00 I.U.; (10) 2.38 I.U.; (11) 1.89 I.U.; (12) 1.50 I.U.

were obtained (Fig. 6); of these lines, the middle (solid) line is considered the most probable calibration line and the other two lines may be interpreted as the corresponding $\sigma$ confidence limits. To determine the concentration of an unknown HCG solution, four to six tests were made to determine the mean diameter $\bar{d}$ of the precipitation areas and its $\sigma$ confidence limits $\bar{d} + \sigma$ and $\bar{d} - \sigma$; these are then plotted as the ordinates of the calibration graph. Fig. 6 shows how these values, determined for an unknown solution, enabled a graphical determination of the corresponding mean HCG concentration $C_m$ and its confidence limits $C_l$ and $C_u$. These confidence limits correspond approximately to the $\sigma$ limits.

In assays where a high degree of precision was required, three serial dilutions of the standard and three serial dilutions of the unknown were tested and the results treated statistically ($3 \times 3$ factorial assay). An evaluation of this assay ($N = 84$) showed that the concentration of a test solution (1008 I.U./ml) calculated from the data provided by this assay was 1001 I.U./ml. The fiducial limits, $P = 0.95$, were 857.8 to 1184.4 I.U./ml. The calculated slope, $b$, was 89.8. The value for $\lambda$, the index of precision, was 0.1308.

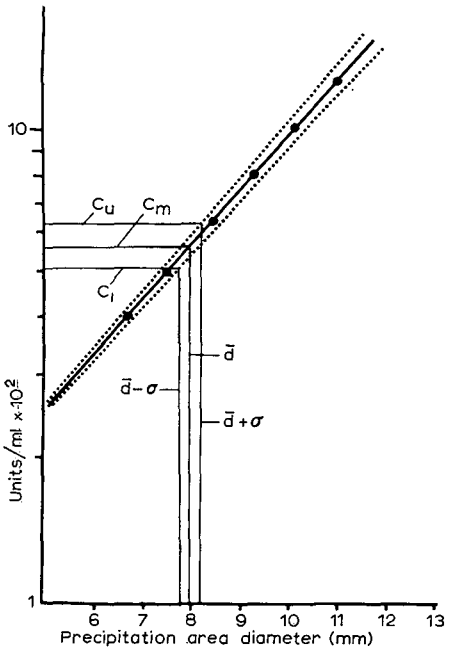


Fig. 6. Standard curve.

## DISCUSSION

Although homogeneous samples of HCG have been isolated[6,7], repeated attempts have been made[12] to develop more convenient and less time consuming methods for the purification of HCG on a preparative scale. Crude HCG in gram quantities can easily be purified by the method here described. The simplicity of the method and economy in time and labor present further advantages. The purified material obtained by this method was shown to be immunoelectrophoretically homogeneous. The specificity of the HCG thus obtained was further demonstrated by the production of specific antiserum to HCG by rabbits immunized with this material.

Until recently only biological tests were used for the assessment of HCG in body fluids. These bioassay techniques for gonadotropin activity were developed on the basis of acceleration of gonadal growth and development. As such, the tests lack specificity, and they are inadequate to test the efficacy of separation procedures for isolating different gonadotropic hormones, or to assess the HCG activity in a sample containing other gonadotropic hormones. Furthermore, existing bioassays yielding quantitative information are laborious and time-consuming. Recently serological methods for the determination of HCG were introduced by BRODY AND CARLSTRÖM[1,2] and LUNENFELD *et al.*[10]. These methods were based on the finding that HCG is able to elicit antibody production in rabbits[1,15], and that the hormone can be detected and assayed by the technique of complement fixation[1–3] or by the inhibition of the agglutination reaction of HCG-coated blood cells[19] or HCG-coated latex particles[8]. Precision and specificity can be achieved with these methods, but each has its limitations. Haemagglutination, for example, could not be performed directly

on untreated serum or amniotic fluids[13,18], and the endpoint was often hard to read.

To measure a particular antigen in a mixture of antigens by any serologic technique, it is necessary to employ a specific antiserum. The antigen employed in our work to produce antiserum to HCG was serologically homogeneous. The specificity of the antiserum obtained was established by immunoelectrophoresis and double-diffusion (Fig. 4a and 4b).

The immunodiffusion method reported here is a modification of Hyland's immunodiffusion plates. It is highly specific, quantitative, sensitive, and extremely simple in operation. An evaluation of this assay ($N = 84$) showed that the concentration of a test solution (1008 I.U./ml) calculated from the data provided by this assay was 1001 I.U./ml (95 % confidence limits 857.8–1184.4). The calculated slope, $b$, was 89.8. The value for $\lambda$, the index of precision, was 0.1308. The sensitivity of the method depends upon the titer of the antiserum employed. With an antiserum having a titer of 1/600, 1 I.U. of HCG can be measured. Greater sensitivity can be achieved by employing antiserum of higher titer. For routine estimation, a standard curve may be set up and the concentrations of an unknown HCG solution may be read off directly from the standard curve. The method can be applied to the determination of HCG in urine and serum without any prior treatment of the sample.

ACKNOWLEDGEMENTS

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SUMMARY

A simple procedure for the purification of commercial human chorionic gonadotropin (HCG) by chromatography on DEAE-Sephadex is described. The method is reproducible and suitable for application on a preparative scale. The HCG thus purified had a specific immunological activity of 11,000 I.U./mg and was homogeneous by immunoelectrophoresis. The overall recovery of immunological activity was 19 %. A simple, specific quantitative assay for HCG employing the technique of immunodiffusion is described. The precision, accuracy, and advantages of the method are discussed.

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# SEPARATION OF PORPHYRINS ON SEPHADEX DEXTRAN GELS

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Separation and characterization of the porphyrins by paper chromatography was first introduced by NICHOLAS AND RIMINGTON[1] and was followed by a systematic study of the behaviour of their methyl esters upon solid adsorption columns[2]. Several modifications of both techniques have since been described; see FALK[3,4]. A disadvantage of both methods is that they are applicable to only relatively small quantities and are thus not well suited for preparative purposes; for the column separation of unesterified porphyrins the only practicable method is that employing cellulose powder and the lutidine–water system as described by ERIKSEN[5].

During the course of a study, shortly to be published, of naturally occurring porphyrin–peptide complexes, the need arose for a method of separating and purifying them without esterification. Ion exchange columns had only limited applicability and we have therefore studied the behaviour of porphyrins upon Sephadex dextran gels. With many materials these gels function as molecular sieves, separation taking place according to molecular size, but in the case of aromatic and highly conjugated planar molecules adsorption phenomena play a prominent role[6]. This proved to be true of the porphyrins but by suitable choice of gel, buffer and particularly of buffer concentration, useful separation techniques have been evolved. With the aid of an automatic fraction collector, mixtures may be resolved on a preparative scale with the minimum of attention, once the sample has been placed on the column.

## MATERIALS AND METHODS

Sephadex dextran gels were obtained from Pharmacia Ltd., London, W.13. Chromatographic columns were constructed from 8 mm internal diameter glass tubing. A plug of glass wool surmounted by some glass beads (height 2 cm) was introduced to support the gel which when packed formed a column 60 cm high. The void volumes of such columns, measured with Sephadex blue, were about 18 ml. Buffer was fed into the top of the tube from a reservoir placed on a shelf above it and through a hypodermic needle piercing a rubber stopper. Outflow from the bottom of the tube was controlled by a screw clip placed on polythene tubing of narrow bore. Operation was at room temperature. The fraction collector was a Shandon model CA-100 holding 200 tubes. In most experiments it was set to collect 2 ml samples gravimetrically. The receivers were 10 cm × 1 cm test tubes. Uroporphyrin I, coproporphyrin III, protoporphyrin IX, deuteroporphyrin IX and haematoporphyrin IX dihydrochloride were carefully purified materials from the laboratory stock; porphyrin c was a gift from

Dr. S. SANO, Kyoto. For application to the column porphyrins were dissolved in a minimal quantity (0.5–1 ml) of 0.2 *M* sodium borate buffer pH 8.6. To each 2 ml fraction was added 2 ml of 3 *N* HCl and the mixture further diluted, if necessary, by 1.5 *N* HCl and optical absorption at the Soret peak was measured on a Unicam SP 500 spectrophotometer using cells of 1 cm light path.

## EXPERIMENTAL AND RESULTS

Since the molecular weights of the porphyrins range from about 500 to 800, it seemed possible that they might be separable on a Sephadex G-10 column. It was immediately evident, however, that factors other than molecular size played a dominant role. Even G-25 and G-100 gels adsorbed the porhyrins strongly in dilute acid or alkaline media or in barbital and phosphate buffers. On the assumption that there might be hydrogen bonding between the porphyrin carboxyl groups and the hydroxyls of the dextran, a change was made to borate buffers. Marked improvement followed and after some experimentation a sodium borate buffer of pH 8.6 containing 0.5 m*M* EDTA and 2–3 drops/l of phenol was selected as most suitable and a column of Sephadex G-25.

*Effect of buffer strength*

Initially, a buffer strength of 0.2 *M* was used but although uroporphyrin was rapidly eluted from the column, porphyrins with fewer carboxyl groups were more firmly retained and tended to spread; deuteroporphyrin was only eluted very slowly and occupied 100 tubes or more (Fig. 1). Porphyrin c (4–COOH groups) could be separated from coproporphyrin on this system but its rate of elution was also very slow. Decreasing the concentration of borate to 0.001 *M* accelerated elution to such an extent that coproporphyrin and deuteroporphyrin came off together in a single peak closely following uroporphyrin. The problem was therefore to find a satisfactory intermediate concentration. With 0.002 *M* buffer, coproporphyrin and deuteroporphyrin were separable with a slight overlap (Fig. 2) and separation was still better when using 0.005 *M* but the optimal concentration proved to be 0.01 *M*. This separated uroporphyrin, coproporphyrin and deuteroporphyrin very satisfactorily although the deuteroporphyrin band was sometimes slightly irregular (Fig. 3). The identity of the fractions was proved by measurement of the Soret maxima and by lutidine paper chromatography[7]. Recoveries were complete.

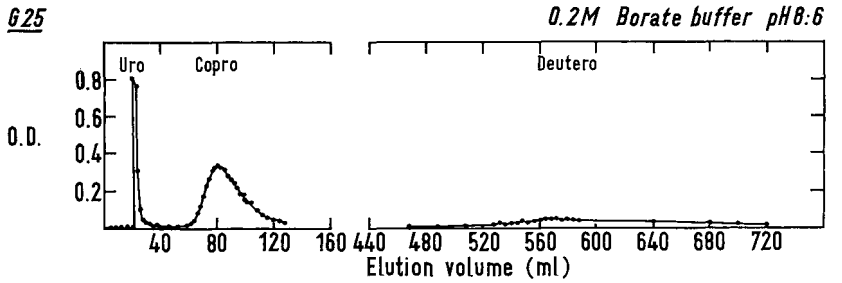


Fig. 1. Elution of uroporphyrin, coproporphyrin and deuteroporphyrin from Sephadex G-25 by 0.2 *M* borate buffer, pH 8.6.

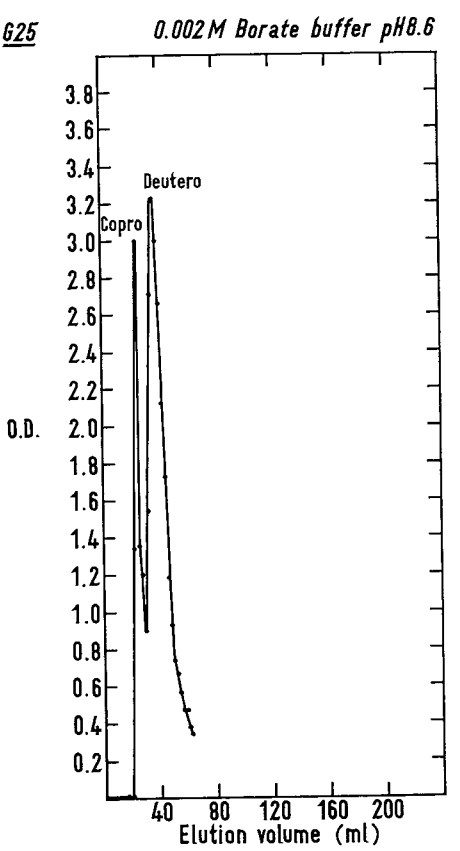


Fig. 2. Elution of coproporphyrin and deuteroporphyrin from Sephadex G-25 by 0.002 *M* borate buffer, pH 8.6.

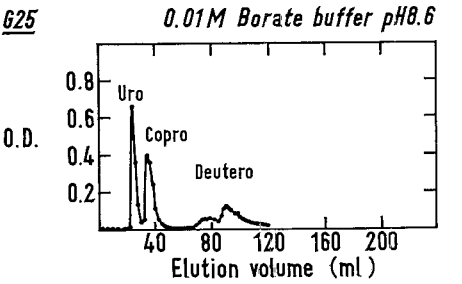


Fig. 3. Elution of uroporphyrin, coproporphyrin and deuteroporphyrin from Sephadex G-25 by 0.01 *M* borate buffer, pH 8.6.

*Separation of coproporphyrin and porphyrin c*

Attention was next turned to the separation of porphyrin c (2,4-dicysteinyl-deuteroporphyrin IX) from coproporphyrin, both of which possess 4–COOH groups. With 0.2 *M* buffer, retention of porphyrin c was nearly as strong as that of the dicarboxylic porphyrins and its band spread out considerably. It would appear that the amino acid carboxyl groups are not comparable, in the effect they exert, to the carboxylic side chains of the porphyrin ring system. 0.01 *M* buffer gave fairly good resolution but the most satisfactory separation of coproporphyrin and porphyrin c was achieved with 0.05 *M* buffer concentration. A small impurity in the porphyrin c even came off immediately in front of the coproporphyrin peak (Fig. 4).

*Separation of porphyrin c and haematoporphyrin IX*

Particular attention has been paid to this separation since during the working up of natural materials structurally resembling porphyrin c, some fission of the thioether linkages is apt to occur giving rise to haematoporphyrin. Although this porphyrin is ether-soluble, in contradistinction to the c type porphyrin, it is also hydrophilic and a chromatographic separation would be very useful. The best result was again obtained by using a 0.05 *M* buffer, very sharp peaks of the two porphyrins

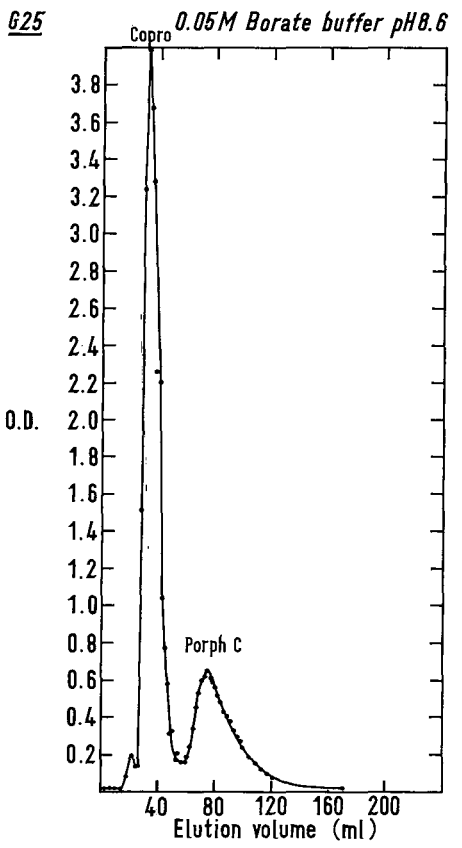


Fig. 4. Separation of coproporphyrin and porphyrin c by elution from Sephadex G-25 with 0.05 *M* borate buffer, pH 8.6.

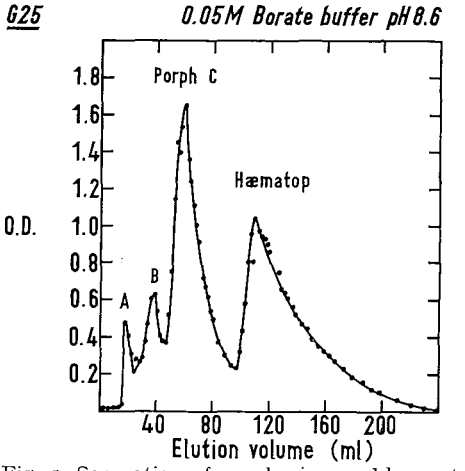


Fig. 5. Separation of porphyrin c and haematoporphyrin by elution from Sephadex G-25 with 0.05 *M* borate buffer pH 8.6. A and B are impurities in the materials.

being obtained. As expected, porphyrin c with its 4 COOH groups preceded the dicarboxylic porphyrin (Fig. 5); minor impurities (A and B) were once more detectable.

*Separation of dicarboxylic porphyrins*

Although a sharp separation of the dicarboxylic porphyrins, deuteroporphyrin and haematoporphyrin, seemed unlikely in view of their closely related chemical structure and their behaviour in lutidine paper chromatography[7], the hydroxylic functions of haematoporphyrin should nevertheless influence to some extent its behaviour on dextran gels. Protoporphyrin which possesses two vinyl side chains should be considerably more firmly retained than the other two porphyrins. These expectations were realized in practice.

A mixture of deuteroporphyrin, haematoporphyrin and freshly prepared protoporphyrin were placed on a G-25 column in 0.05 *M* sodium borate buffer pH 8.6 and developed with the latter. A small red fluorescent impurity left the column rapidly (this is usually seen with protoporphyrin preparations) and was followed by a large unsymmetrical band appearing between 116 ml and 360 ml of eluate. After 400 ml of eluate had passed, the fraction collector was stopped and all porphyrin remaining on

the column allowed to collect in a single overnight fraction. Spectrophotometric analysis and paper chromatography of the esterified porphyrins by the method of CHU, GREEN AND CHU[8] showed that haematoporphyrin was eluted first but that there was some overlapping with deuteroporphyrin. The large band from 116–360 ml did, in fact, exhibit two peaks with different Soret band absorption. The final overnight fraction consisted of dicarboxylic porphyrin with Soret maximum in 5 % HCl of 407–8 m$\mu$ and was therefore protoporphyrin. There is no doubt that repeated filtration on Sephadex of the first, composite band, would effectively separate the haematoporphyrin and deuteroporphyrin which it contained.

## DISCUSSION

Separation techniques employing Sephadex dextran gels and an automatic fraction collecting device have the advantages of requiring minimum attention, having high flow rates, and of being easily reproducible. They may be used on a preparative scale, the quantity of material handled being virtually limited only by the size of the column and the volume of solvent necessary to dissolve the applied mixture. Recoveries of material, moreover, are complete or very nearly so. In the case of proteins, separation generally occurs according to molecule size, the process being one of molecular sieving, but with aromatic substances and highly conjugated planar molecules, adsorption effects become increasingly evident and may predominate. The effects of pH and of buffer concentration then become important[9].

In the present study, physical interaction between porphyrins and the dextran gel were found to be very marked. Only by the use of a borate buffer, pH 8.6, containing some EDTA to remove traces of metals, and careful selection of the appropriate buffer strength could good resolution be achieved without serious tailing. Recoveries were then quantitative. Sephadex G-25 proved to be the most satisfactory gel and, in general, rate of elution increased with increasing number of carboxyl groups in the molecule. Thus uroporphyrin suffers little or no retention whether the buffer concentration be 0.2 *M* or 0.001 *M*. In the weaker buffer, coproporphyrin and deuteroporphyrin also move fairly rapidly and are eluted together but as the buffer concentration is increased their retention on the column is also increased and their bands are broadened. The interval between them becomes greater. For general purposes a 0.01 *M* borate buffer was found optimal in that it gave clear separation in well defined bands.

The behaviour of porphyrin c is particularly interesting. It has 4 carboxyl groups, 2 being present in the cysteinyl groups and 2 in the propionic acid side chains at positions 6 and 7 of the porphyrin ring. On lutidine paper chromatography, porphyrin c runs level with the tetracarboxylic coproporphyrin. On Sephadex G-25, however, it is somewhat more firmly retained than coproporphyrin but less firmly than dicarboxylic porphyrins. It would appear, therefore, that the carboxylic functions of the cysteinyl groups exert a weakened de-adsorptive effect due, no doubt, to the adjacent $\alpha$-amino groups. For good separation from coproporphyrin, a 0.05 *M* was found preferable to a 0.01 *M* buffer. The fact that haematoporphyrin and porphyrin c may be readily separated under these same conditions is exceedingly important since the thioether linkage of this latter porphyrin easily suffers fission during chemical manipulations with resulting formation of some haematoporphyrin.

The methods described in this paper have already been applied successfully to the separation of complex mixtures of porphyrins derived from natural sources. Obviously, however, different problems may demand differing treatment and it is one of the virtues of the Sephadex gel system, as revealed by this study, that one can vary the behaviour of porphyrins in it in a predictable manner. It may be useful, at times, to carry out a preliminary separation at one buffer concentration and then to re-run some fractions in a weaker or stronger buffer.

The use of Sephadex LH 20 for the separation of porphyrin esters or of the free porphyrins was examined with various solvent systems but it was found to be distinctly inferior to the G-25 gel.

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SUMMARY

Porphyrins can be separated on columns of Sephadex G-25 gel by sodium borate buffer, pH 8.6. Alteration in buffer molarity influences separation in a predictable manner; for general purposes 0.01 *M* is optimal but for special separations appropriate concentrations should be used. Recovery is quantitative and the technique is suitable for preparative purposes.

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# CHROMATOGRAPHIC BEHAVIOUR OF INORGANIC IONS ON CRYSTALLINE TITANIUM PHOSPHATE OR ZIRCONIUM PHOSPHATE THIN LAYERS

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## INTRODUCTION

Insoluble salts of polybasic acids with tetravalent metals such as phosphates or arsenates of zirconium, titanium and cerium are generally obtained as amorphous or semicrystalline materials which are well known inorganic ion exchangers[1]. Recently a crystalline form of zirconium phosphate[2] (ZP) and a crystalline form of titanium phosphate[3] (TP) have been obtained whose ion exchange properties are very different from those of the corresponding amorphous materials. While all alkali metal ions are easily exchanged on amorphous ZP and TP (the selectivity increasing from $Li^+$ to $Cs^+$) the uptake of $Rb^+$ and $Cs^+$ by crystalline ZP and the uptake of $K^+$, $Rb^+$ and $Cs^+$ on crystalline TP is negligible in acid media[2,3]. On the other hand $Li^+$, $Na^+$ and $K^+$ on crystalline ZP and $Li^+$ and $Na^+$ on crystalline TP are easily exchanged with very high ion exchange capacity (about 7 mequiv./g). The large difference in the ion exchange capacity suggested that the crystalline form of ZP and TP could be employed in the separation of alkali metal ions. For this reason the chromatographic behavior of alkali metal ions on crystalline titanium or zirconium phosphate thin layers has been examined. Owing to the lack of information on the exchange properties of these crystalline ion exchangers towards inorganic ions other than alkali metal ions, the chromatographic behavior of alkaline earths and thallium(I) has also been examined and the chromatographic results obtained with crystalline and amorphous materials are compared.

## EXPERIMENTAL

### *Materials and eluents*

The amorphous ZP was ZP-1 of Bio-Rad Laboratories. Crystalline ZP was prepared from ZP-1 Bio-Rad according to the procedure of CLEARFIELD AND STYNES[2]. Amorphous TP was prepared according to GAL AND GAL[4]. Crystalline TP was prepared by refluxing amorphous TP with 10 *M* $H_3PO_4$ for 50 h[3]. Cellex N-1 cellulose (Bio-Rad) was employed for thin-layer preparations.

Tetramethylammonium acetate or ammonium acetate buffers were prepared by adding conc. acetic acid to 1.1 *M* tetramethylammonium hydroxide or to 1.1 *M* ammonium hydroxide respectively, until pH 5 was reached.

*Preparation of thin layers*

For the preparation of cellulose–ion exchanger thin layers a slurry of one part of cellulose, 3.5 parts of water, and the proper amount of ion exchanger (200–400 mesh) was applied to smooth glass plates (60 $cm^2/g$ cellulose) and air dried. When buffered layers had to be used the layers were sprayed with the eluent and again air dried. The amount of ion exchanger to be mixed with cellulose is related to the mequiv. of ions to be separated, and this will be discussed later.

*Chromatographic procedure*

The solutions of metal ions were prepared by dissolving the nitrates in the eluent.

The lower portion (2 cm) of the thin layer was coated with Whatman 3 MM paper to avoid breakage of the layer. 2–10 $\mu$l of samples were spotted 3 cm from the lower end of the layer. The plates were allowed to develop by ascending chromatography.

$Na^+$ and $Cs^+$ ions were detected radiometrically using $^{22}Na$ and $^{137}Cs$ as tracers.

$Li^+$ and $K^+$ ions were located by flame photometry, after cutting the layers in portions (1 cm in length) and eluting with 0.1 *N* HCl.

Other ions were detected by suitable spot test reactions.

TABLE I

$R_F$ VALUES OF SOME INORGANIC IONS ON CELLULOSE AND ON INORGANIC ION EXCHANGER THIN LAYERS

Eluent: 0.1 *M* $HClO_4$.

| *Ion* | *Thin-layer material* | | | | |
|---|---|---|---|---|---|
| | *Cellex* | *Amorphous ZP* | *Crystalline ZP* | *Amorphous TP* | *Crystalline TP* |
| Li | 1.0 | 0.9 | 1.0 | 0.9 | 0.9 |
| Na | 0.9 | 0.8 | 0.9 | 0.9 | 0.9 |
| K | 0.9 | 0.3 | 0.9 | 0.8 | 0.9 |
| Cs | 0.9 | 0.2 | 0.9 | 0.7 | 0.8 |
| Tl(I) | 0.9 | 0.2 | at L.F. with tail | 0.3 | 0.8 |
| Ca | 1.0 | at L.F.* with tail | 0.8** | 0.9 | 0.8** |
| Ba | 1.0 | at L.F. with tail | 0.9** | 1.0 | 0.9** |
| Sr | 1.0 | at L.F. with tail | 0.9** | 1.0 | 0.9** |

* L.F. = Liquid front.
** Elongated spot.

RESULTS AND DISCUSSION

Table I shows the chromatographic behavior of various inorganic ions when eluted with 0.1 *M* $HClO_4$ and applied to cellulose thin layers and cellulose thin layers containing amorphous ZP, crystalline ZP, amorphous TP and crystalline TP, respectively. The results show that some uptake of K(I), Cs(I) and Tl(I) ions occurs on thin layers containing amorphous exchangers, whereas all the ions examined behave in nearly the same way on either cellulose, crystalline ZP or crystalline TP. In strongly acid media a negligible ion exchange capacity was found on crystalline ZP and TP for all the ions examined.

Eluent solutions at pH $> 3$ were found necessary to exchange the hydrogen ion with other inorganic ions. When such eluents are used the exchanged hydrogen ions may give rise to the formation of an acid zone around the point of application. This acid zone will move with the front of the eluent, not being neutralized during the chromatographic development, even though buffered eluents are used. As a result, a fraction of the ions spotted at the application point migrate with the acid zone.

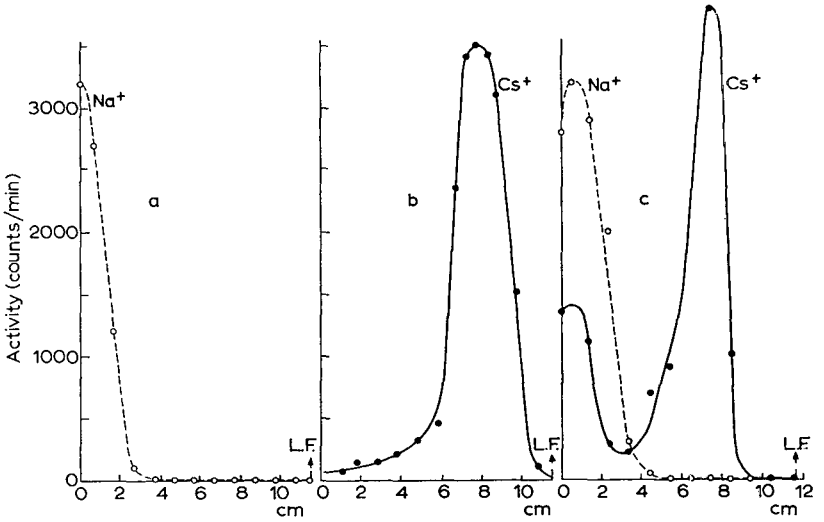


Fig. 1. Chromatographic behavior of $Na^+$ (a), $Cs^+$ (b) and $Na^+$–$Cs^+$ mixture (c) on crystalline ZP thin layers (4.2 mg ZP/cm$^2$). Amount spotted of each ion: $7.5 \cdot 10^{-3}$ mequiv. Eluent: tetramethylammonium acetate–acetic acid buffer at pH 5. Ordinates: activity in c.p.m.; abscissae: length of the chromatogram in cm; L.F. = liquid front.

In such a case the acid front can be completely neutralized by using a thin layer previously moistened with the eluent and air dried. The chromatographic behavior of the alkali metal ions on crystalline ZP has been examined, using this technique with the tetramethylammonium acetate–acetic acid buffer at pH 5. The tetramethylammonium ion was employed since its large ionic size avoids ion exchange by crystalline ZP in $H^+$ form at this pH value. When eluted with tetramethylammonium acetate–acetic acid buffer at pH 5, $Li^+$ and $Na^+$ ions are strongly retained at the point of application while $Cs^+$ ion, in good agreement with CLEARFIELD's data[2] are not retained. The chromatographic behavior on crystalline ZP of $Na^+$ (Fig. 1a) and $Cs^+$ (Fig. 1b) would suggest a good separation of these ions. However, when $Na^+$ and $Cs^+$ ions are both present in a mixture, a large fraction of $Cs^+$ is retained together with $Na^+$ at the application point (Fig. 1c). This result suggests that while $Cs^+$ ion is negligibly retained by crystalline ZP in $H^+$ form, it can be retained by the $Na^+$ form of the exchanger, probably owing to the characteristic layer-structure of crystalline ZP[5]. Batch experiments carried out in this laboratory[3] showed that it is possible to convert even the crystalline ZP into the $Cs^+$ form by percolating 0.1 *M* CsCl over the $Na^+$ form of the exchanger. Thus, crystalline ZP seems to be of little use in the separation of alkali metal ions or other inorganic ions.

Attention was then mainly devoted to crystalline TP, since preliminary ex-

periments showed that the amount of $Cs^+$ retained by the $Na^+$-form of this exchanger was negligible when compared with the amount retained by crystalline ZP under the same experimental conditions.

TABLE II

$R_F$ VALUES OF SOME INORGANIC IONS ON CELLULOSE AND ON AMORPHOUS TP OR CRYSTALLINE TP THIN LAYERS

Eluent: ammonium acetate–acetic acid buffer at pH 5.

| *Ion* | *Thin-layer material* | | |
|---|---|---|---|
| | *Cellulose* | *Amorphous TP* | *Crystalline TP* |
| Li | 0.8 | 0.8 | 0.0 |
| Na | 0.8 | 0.7 | 0.0 |
| K | 0.8 | 0.6 | 0.8 |
| Cs | 0.7 | 0.5 | 0.7 |
| Tl | 0.8 | 0.0 | 0.4 |
| Ca | 0.8 | long tail | at L.F.* with tail |
| Ba | 0.8 | long tail | at L.F. with tail |
| Sr | 0.8 | long tail | at L.F. with tail |

*L.F. = Liquid front.

In our opinion, the ion exchange rate of ions of large size, such as $K^+$ or $Cs^+$, is very low on crystalline TP. During the chromatographic development, the relatively short contact-time permits these ions to exchange only at the surface or on the outer layers of the exchanger. Thus, very small amounts of ions of large size can be exchanged by crystalline TP. Nevertheless, when the amounts of the ions to be separated

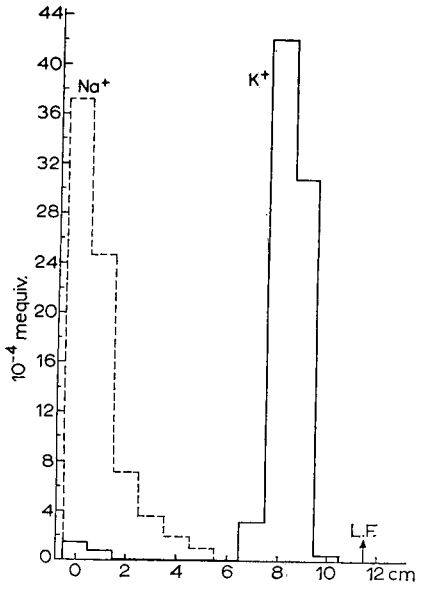


Fig. 2. Separation of $Na^+$–$K^+$ on crystalline TP thin layer (8.3 mg TP/cm²). Amount spotted of each ion: $7.5 \cdot 10^{-3}$ mequiv. Eluent: ammonium acetate–acetic acid buffer at pH 5. Ordinates: $10^{-4}$ mequiv.; abscissae: length of the chromatogram in cm; L.F. = liquid front.

are very small (*e.g.* carrier free solutions), the extent of ion exchange on the crystalline TP may be considerable. For this reason, as already pointed out, the amounts of crystalline TP per $cm^2$ of thin layer must be related to the amounts of ions to be separated. In this work it has been found convenient to employ thin layers containing 0.5–1.0 mg of crystalline TP per $cm^2$ and per $\mu$equiv. of ion spotted at the application point.

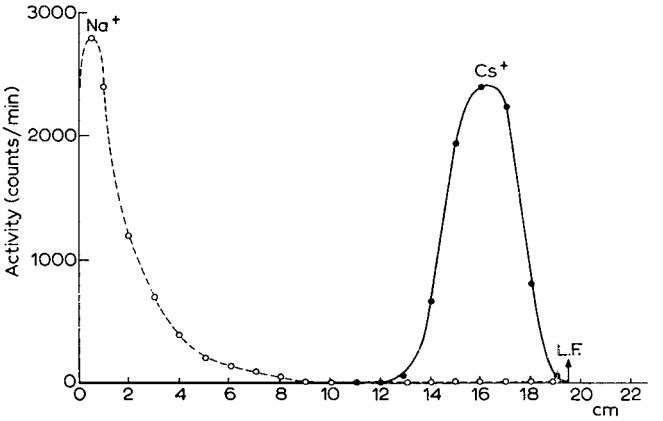


Fig. 3. Separation of $Na^+$–$Cs^+$ on crystalline TP thin layer (8.3 mg TP/$cm^2$). Amount spotted of each ion: $7.5 \cdot 10^{-3}$ mequiv. Eluent: ammonium phosphate buffer at pH 7. Ordinates: activity in c.p.m.; abscissae: length of the chromatogram in cm; L.F. = liquid front.

Furthermore it has been found that not only $N(CH_3)_4^+$ but even $NH_4^+$ ion is poorly exchanged in acid or weak acid media by crystalline TP. It was therefore possible to employ ammonium acetate–acetic acid buffer as eluent. In Table II the chromatographic results obtained with this eluent at pH 5 are reported. In the same table the chromatographic results obtained with amorphous TP are reported by comparison, showing the drastic changes of the ion exchange properties caused by the crystallization process. $Li^+$ and $Na^+$ ions are in fact retained more by crystalline than by amorphous TP. The opposite behavior shown for $K^+$ and $Cs^+$ ions is in good agreement with the data reported in a previous paper[3]. The alkaline earth metal ions form somewhat elongated spots due (in our opinion) mainly to the formation of precipitates between these ions and the phosphate ion produced by hydrolysis of the exchanger, rather than to an effective ion exchange process. Beside $Li^+$ and $Na^+$ ions, Tl(I) is the only ion which is partially retained by crystalline TP. Considering the large ionic size of the Tl(I) ion, this deserves further investigation. The high selectivity of amorphous TP for Tl(I) must also be emphasized.

The chromatographic experiments confirm thus that crystalline TP acts as an ion exchanger with high selectivity for $Li^+$ and $Na^+$ ions only.

The data of Table II can be used to predict some separations of alkali metal ions. Fig. 2 shows, as an example, the chromatographic separation of $Na^+$ and $K^+$ ions, using as eluent an ammonium acetate–acetic acid buffer solution at pH 5. A phosphate buffer at pH 7 can be successfully employed in the absence of ions which give insoluble phosphates (Fig. 3). The separation Na–Cs was also performed with tetramethylammonium acetate–acetic acid buffer solution at pH 5 (Fig. 4). The better performance

of crystalline TP, as compared to crystalline ZP, for $Na^+$–$Cs^+$ separations under the same experimental conditions, can be seen by comparing Fig. 4 and Fig. 1.

It appears that crystalline TP can be successfully employed in the separation of some alkali metal ions. The large difference in the chromatographic behavior of $Na^+$ and $K^+$ ions, is particularly interesting.

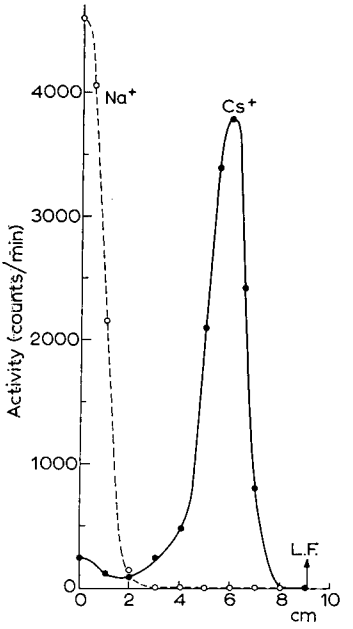


Fig. 4. Separation of $Na^+$–$Cs^+$ on crystalline TP thin layer (8.3 mg TP/cm$^2$). Amount spotted of each ion: $7.5 \cdot 10^{-3}$ mequiv. Eluent: tetramethylammonium acetate–acetic acid buffer at pH 5. Ordinates: activity in c.p.m.; abscissae: length of the chromatogram in cm; L.F. = liquid front.

ACKNOWLEDGEMENT

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SUMMARY

The chromatographic behaviour of alkali metal ions, alkaline earths and Tl(I) on crystalline and amorphous zirconium phosphate and titanium phosphate thin layers has been examined. Large differences in the ion exchange properties of amorphous and crystalline materials were found. Crystalline titanium phosphate has been successfully employed in the separation of some alkali metal ions.

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## Notes

### Wedge-compressed membrane filters: their formation and application to small-scale filtration chromatography

Nitrocellulose membrane filters (UFS), either intact or impregnated with non-ionic detergents or proteins, were shown recently to be a convenient medium for rapid electrophoretic and chromatographic operations, deproteinization, and also for special determinations of very small amounts of proteins[1-8]. A regular microporous structure of the membranes[9] and the narrow distribution of pore sizes were important conditions for successful experiments with this material.

In the present investigation we wanted to probe the idea of forming a continuous pore-size gradient by pressing the membrane structure into a wedge between two plane blocks, under given conditions, and then fractionating molecules of different sizes by passing them from the larger pores towards the smaller ones (Fig. 1). It was assumed that during this sieving–chromatographic process the high-molecular weight substances would move more slowly than those having a lower molecular weight, the retardation being due mostly to the higher frequency of collisions with the membrane structure. The larger particles would be finally intercepted and immobilized in zones where the mean pore size begins to be smaller than the effective diffusion volumes of the given particles. The smallest particles could pass even through the line $h_2$ of maximum compression. Thus a separation of different particle size populations could possibly be achieved under simple experimental conditions and using very small amounts of material (*cf.* refs. 1–8). However, first of all it was necessary to find suitable materials and conditions for the realization of this idea.

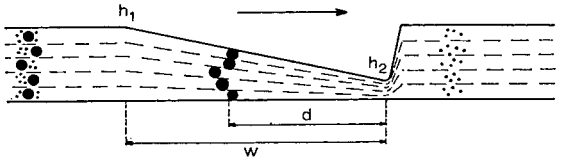


Fig. 1. Scheme of the separation of particles on a wedge-compressed membrane (longitudinal section). $h_1$ and $h_2$ = Lines of minimal and maximal compression of the membrane structure (*cf.* Fig. 3); $d$ = part of the wedge unpenetrable for the greater particles; $w$ = wedge length. Dashed lines indicate schematically the deformation of the pore sizes. Direction of flow indicated by the arrow.

The proposed microtechnique is in a certain sense the reverse of the principle of gel filtration on dextran gels[10,11] and differs also from methods using discontinuous pore-size gradients, *e.g.* stacked layers of gels of growing concentrations[12,13] or cascades of membranes having different permeability[14,15].

Nitrocellulose membranes VUFS* (produced by VCHZ Synthesia, Uhříněves,

* New name: Synpore 8, distributed by Chemapol, Prague.

Czechoslovakia), 0.05 mm thick, pore size 0.1–0.3 $\mu$m, in strips usually 3–4 cm long, were impregnated with 2 % Tween 60 as was described previously[1], to minimize the unwanted adsorption of particles[1,5,6]. The membranes were stored in the buffer to be used for chromatography (0.1 *M* TRIS–1 *M* NaCl, pH 7.9 adjusted by HCl).

The arrangement of the pressing device was relatively simple (Fig. 2) but sufficient to ensure reproducible conditions for the production of a standard series of wedge-compressed membranes. Best results were achieved when the blocks A and B, used to press the membranes, were made of hard steel: lathe knives "Poldi Radeco" 2.5 × 2.5 × 16 cm. (Softer steel was not suitable since it was easily and irreversibly deformed during wedge-pressing. Glass blocks even 2.0 cm thick were broken at the pressures necessary here.) The two operational sides of the steel blocks were first carefully plane-ground and then fine-polished to brightness on a mirror glass, using appropriate carborundum and sapphire polishing powders suspended in mineral oil. Special care must be paid to the pressing edge of the upper block A; no flaws discernable at 40 × magnification should be present. The edge must not be strictly sharp but somewhat curved (*cf.* Fig. 1), otherwise the membrane would be cut through during pressing.

The wet membrane with sufficient excess buffer was laid with its glossy side down on the block B (*cf.* Fig. 2), the pressing block A was placed above, supported at one side by two suitable steel supports (blades of 0.08 mm thickness were used). Then a steel or brass string of 3–4 mm diameter was laid on the upper block A at a given distance *a* from the edge and the whole system was compressed for 5–10 sec in a hydraulic press at laboratory temperature, using suitable controlled pressures in the region of 15–100 atm. The constancy of the parameters *P*, *a*, *b* and *c* was very important for the reproducibility of the experiments. The calculation of the angles and of the actual pressures acting on the membrane was not necessary here.

The membrane was then transferred with forceps from the press into the buffer and could be stored there for 1–2 days without any observable macroscopic change of the wedge-compressed area. This relative "irreversibility" of the deformation of the membrane for a fairly long time is very important and convenient from the practical aspect of the method. In most experiments, however, chromatography was usually begun within 1 h of pressing.

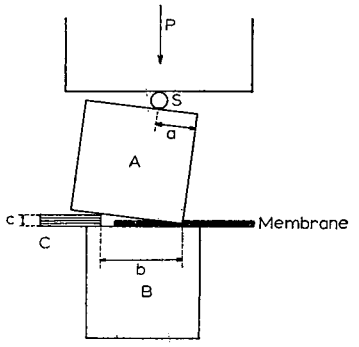


Fig. 2. Scheme of the pressing device. P = Head of the hydraulic press; S = steel string; A and B = polished pressing blocks of hard steel; C = flat steel supports; *a*, *b* and *c* = distances important for reproducible compression.

The pore size gradient was easy to observe, since the intact membrane was not transparent, whereas after compression a continuously increasing transparency could be seen along the membrane, reaching its maximum at the line $h_2$. When the membrane was dried slowly at laboratory temperature even the line $h_1$ (see Figs. 1 and 3) could be detected and marked very accurately, since the compressed area remained wet and dark for a longer time than the quickly drying uncompressed area.

Horizontal chromatography was performed as described before[6,7]. To ensure a more regular suction of the buffer through the membrane a junction consisting of a wet PUFS* membrane (pore size 4 $\mu$m) was placed between the paper wicks and the chromatographic strip. For the detection of proteins, the still wet membranes were dipped into solutions of Ponceau S or amido black 10B in 3–5 % trichloracetic acid; this method was most suitable[16].

It was also found convenient to use an ordinary indelible pencil to make the necessary marks (the position of the start, of line $h_1$ etc.) on the wet membrane, because this pigment adhered very firmly to nitrocellulose during the necessary operations.

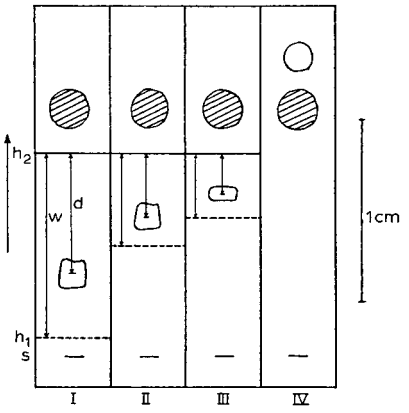


Fig. 3. Sieving-chromatography on wedge-compressed nitrocellulose membranes VUFS impregnated with Tween 60. Light spots: a high molecular protein fraction of modified serum; dark spots: phenolsulphophthalein. s = Start; $h_1$ = line of minimal compression (*cf.* Fig. 1); $h_2$ = line of maximal compression; $w$ and $d$, *cf.* Fig. 1. Membranes No. I, II and III were compressed at a pressure of 25 atm, with $a$ = 8 mm and $b$ = 15 mm (*cf.* Fig. 2); $c$ was 0.08 mm for No. I; 0.16 mm for No. II; 0.24 mm for No. III; membrane No. IV was not compressed. Buffer: 0.1 *M* TRIS–HCl + 1 *M* NaCl, pH 7.9; separation for 4.5 h; about $10^{-4}$ ml of 1 % samples were applied.

The separation of phenolsulphophthalein and the high-molecular protein fraction (mol. wt. of the order of $10^6$), separated by gel filtration on pearl-condensed agar[17] from a blood volume expander "Resorba" made of modified bovine serum, was chosen as a model in order to investigate whether the proposed technique really operates. The results of several experiments are shown schematically in Fig. 3. The wedge lengths $w$ corresponding to the given angles of compression were in very good agreement with the expected calculated values. This means that the eventual deformation of the steel blocks can be neglected here.

The ratios $d/w$ or $1-d/w$ remained constant (within the experimental error) on

* Now Synpore 1.

all three membranes (I, II and III) and were independent of the distance between the start and the line $h_1$. It would seem that these ratios possibly have a similar meaning to $R_F$ values in chromatography. It may be also concluded from our results that the particles did not escape the sieving process by streaming on the surface of the membrane. The sieving effect of the wedge-compressed membrane structure becomes most evident when comparing strips No. I, II and III with the uncompressed strip No. IV.

It may be summarized that the proposed microtechnique seems to offer the possibility of separating, in a relatively short time, very small amounts ($10^{-4}$ to $10^{-5}$ ml samples) of substances differing in molecular size. The manipulation and detection is simple and the membranes store well for documentation. For further details on the method, its applications and limitations, see ref. 18.

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## Column fractionation of polymers

### XI. Constant volume pulseless pump for gel permeation chromatography

The technique of gel permeation chromatography (GPC) requires a constant volume flow of solvent to prevent loss in resolution from fluctuating flow and to facilitate the rapid comparison of chromatograms[1–4].

A common solution to this problem has been the use of a variable stroke piston-type pump[5]. Because of the pressure fluctuations, a buffering system is required—otherwise, the pressure fluctuations cause a high noise level in the very sensitive differential refractometer detector frequently employed.

A pulseless constant volume pump has been in use for some time in this laboratory with a gel permeation chromatograph. Fig. 1 is a schematic representation of the pump. The instrument achieves constant volume flow by the use of commercially available constant volume pumps (Zenith Products Company, West Newton, Mass.

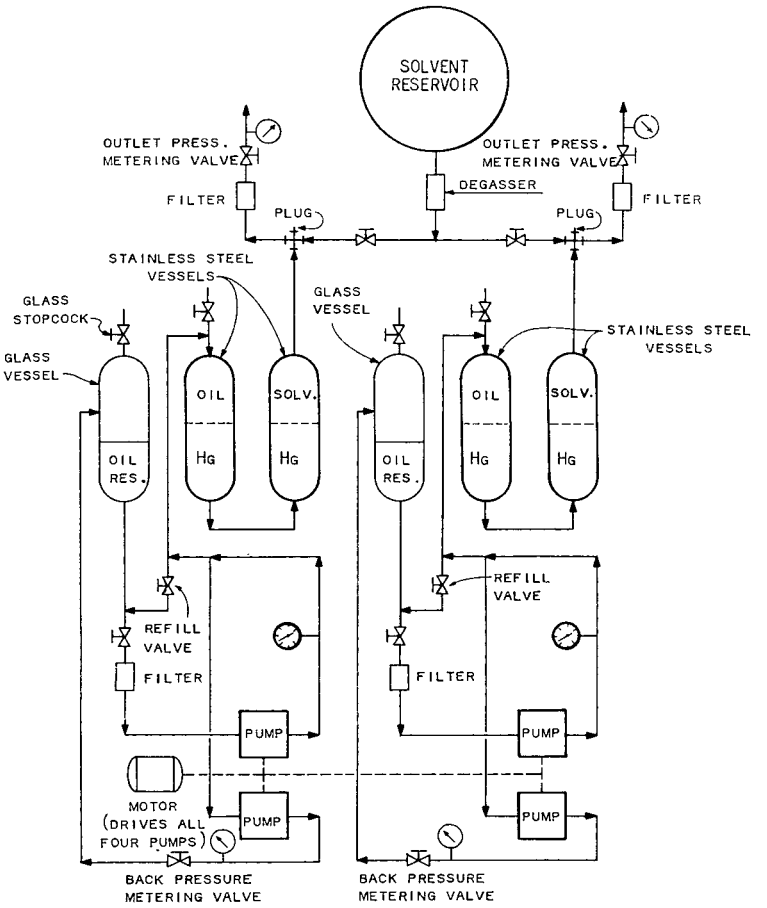


Fig. 1. Dual constant volume pumping unit, schematic.

No. 1/2 B-4391 with Teflon U-seals). To obtain the necessary low flow rates, two pumps are operated differentially. In differential operation one constant volume pump delivers a volume, V-1, as the source for the second pump which delivers a slightly smaller volume, V-2. The excess V-1 minus V-2 from a T-connection between the two pumps is the flow delivered to the chromatograph. To achieve constant volume flow, these gear-type pumps must operate on a fluid with considerable viscosity. This precludes their delivering directly the low viscosity solvents employed in GPC. Therefore, the pumps are operated on a mineral oil with an approximate viscosity at room temperature of about 200 centistokes. The reservoir containing the oil is made of glass. The mineral oil enters a stainless steel chamber (high pressure cylinder 1800 p.s.i. No. 8HD1000, Hoke, Inc., One Tenakill Park, Creskill, N.J.) where it displaces mercury into another stainless steel chamber. There the mercury displaces the solvent to the chromatograph. The output of the second pump, V-2, returns to the mineral oil reservoir. The pump is, therefore, not a continuous one but a batch type. The solvent capacity, however, is over 500 ml, which is more than sufficient for a fractionation on the usual GPC analytical scale.

The pump is driven by a synchronous motor operating at 16 2/3 r.p.m. (Superior Electric Slo-Syn SS 250 PI 115V, 1 phase, Superior Electric Co., Bristol, Conn.). A common gear drives the two metering pumps. The gears from the pumps that engage the common gear vary in the number of teeth to provide for the differential flow rate. By changing the pump gears, which is a simple operation, various flow rates can be attained. Use of different constant speed motors, other sizes of pumps, and so on, could provide many other flow rates. The pump and pressure vessels are operable to 1000 p.s.i. —pressures far in excess of those required by the conventional GPC, which are usually under 200 p.s.i.

It is highly undesirable to get mercury into the system. Two safety features have been provided. First, the oil in the reservoir is put in volumetrically so that there is insufficient oil to displace mercury into the system. Additionally, the pump is controlled by a timer which can be preset to shut it off after a selected time interval.

Conventionally, gel permeation chromatographs use differential detectors. Solvent flows through a reference column and through the fractionating column. Therefore, the pump is a dual-type pump with identical arrangements for the reference and fractionating side. The two pairs of metering pumps are driven from the same motor.

Although the viscosity of the oil being pumped is relatively high, there is some backward flow through the pump. The amount of this flow depends on the backpressure. Therefore, a backpressure metering valve is installed between the pump furnishing volume, V-2, and the oil reservoir. This permits adjusting the backpressure to any desired value. If the pressure drop across each pump is the same, the leakage rate across the pumps should be the same. This occurs when the backpressure on pump V-2 is twice that of the outlet pressure as the pressure drop across pump V-1 is the outlet pressure and that on V-2 is the pressure at the metering valve minus that of the outlet pressure. Fig. 2 illustrates the performance of the pump under these conditions. The flow rate is constant within experimental error regardless of outlet pressure.

The pressure drop across pump V-2 may be varied to attain a variety of flow rates without changing gears. When the pressure drop is less than that of the outlet

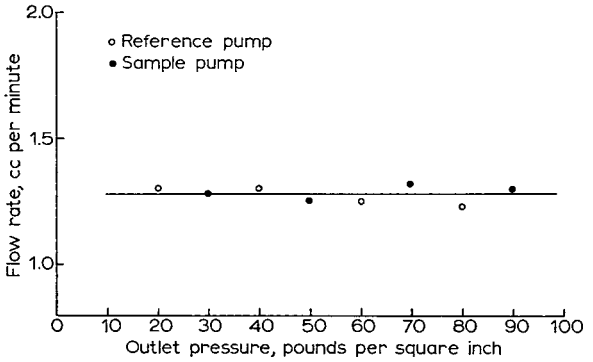


Fig. 2. Flow rate when back pressure is twice outlet pressure.

pressure, the leakage through pump V-2 is less than through pump V-1, and the differential flow rate decreases. Increased flow rate is achieved by use of a higher pressure drop across pump V-2 than across pump V-1.

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## Isolation and separation of mono- and digalactosyldiglycerides from spinach leaves with Sephadex LH-20

The usual methods for the isolation and separation of monogalactosyldiglyceride (GDG) and digalactosyldiglyceride (GGDG) are chromatography on Florisil, DEAE-cellulose and silicic acid[1] and treatment with acetone followed by chromatography on a silicic acid column as described by WEENINK[2-4], and ROUSER *et al.*[5]

This note describes the isolation and separation on a column of Sephadex LH-20 of GDG and GGDG from the supernatant remaining after removing the majority of the phospholipids by acetone precipitation of the total lipid extract.

*Methods and materials*

*Extraction of total lipids.* About 1 kg of spinach leaves (*Spinacia oleracea*) were washed with water and kept one minute at 95° to denature the enzymes. After cooling, the leaves were homogenized in a Waring blendor with 96 % ethanol and the residue was filtered off on a Büchner funnel. The residue was then washed with 96 % ethanol until colorless. The total filtrate was dried *in vacuo* to yield 14 g of solid material. This was dissolved in 600 ml diethyl ether and was washed with water. The ether phase was dried *in vacuo* to give a yield of 10.9 g. This residue was then dissolved in 220 ml *n*-heptane pre-equilibrated with 95 % methanol and was distributed between *n*-heptane pre-equilibrated with 95 % MeOH and 95 % MeOH pre-equilibrated with *n*-heptane as described by CARTER, MCCLUER AND SLIFER[6]. After drying the MeOH-phase *in vacuo*, the phospholipids from the MeOH phase were removed by precipitation with acetone at −10°. The precipitate was filtered off and the filtrate was used after drying for Sephadex column chromatography.

*Sephadex LH-20 chromatography.* 100 g Sephadex LH-20 were allowed to swell for 2 h in chloroform and was then packed into a column with inner dimensions of 50 × 3 cm. The column was washed with 500 ml chloroform. The acetone soluble lipids dissolved in 4 ml chloroform were applied to the column. Successive elutions were made with 800 ml $CHCl_3$, 1000 ml $CHCl_3$–MeOH (10:1, v/v), and 575 ml MeOH. Fractions of 5 ml were collected.

*Results and discussion*

It was observed that not all phospholipids were removed from the acetone-soluble lipids by repeated precipitation. When the acetone-soluble lipids were chromatographed on TLC plates of silica gel with $CHCl_3$–MeOH (10:1, v/v) as solvent, a blue spot was found after colouring with the molybdenum blue reagent of DITTMER AND LESTER[7]. These remaining phospholipids came out of the column only after elution with 260 ml $CHCl_3$. The glycolipid GDG was eluted directly after the phospholipids in 200 ml. After elution with 400 ml $CHCl_3$–MeOH (10:1, v/v) the second glycolipid (GGDG) came through in the following 150 ml. The phospholipid GDG- and GGDG-fractions were concentrated and examined by TLC with $CHCl_3$–MeOH (10:1, v/v) and $CHCl_3$–MeOH–$H_2O$ (65:25:4, v/v) as solvent systems. Detection was with rhodamine B–2′,7′-dichlorofluorescein[8]; anisaldehyde reagent[9] and molybdenum blue reagent.[7]

The GDG- and GGDG-fractions were hydrolysed with 0.1 *N* KOH in MeOH for 15 min at 37°. The reaction mixtures were neutralized with Amberlite IR 120 (H-form), filtered and the filtrate was applied to a paper chromatogram (Whatman No.

3MM). Elution was with phenol–water (3:1, w/w). After colouring with the periodate–Schiff reagent we found a single spot in each fraction. In the GDG-fraction, a spot with $R_F$ 0.68 (= monogalactosyglycerol) was found and in the GGDG-fraction the spot had $R_F$ 0.51 (= digalactosylglycerol).

This demonstrated that:

(1) The phospholipid fraction contained no glycolipids.

(2) The GDG-fraction contained no phospholipids or GGDG.

(3) The GGDG-fraction was free of GDG and phospholipids.

Most of the plant pigments were removed by this method. However, the glycolipid fractions contained some yellow-brown pigments.

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## Partial resolution of mandelic acid with Sephadex gels

Racemic mandelic acid and other racemic substances have been resolved, at least partially, with optically inactive eluents and with starch[1–3] and cellulose[4–8] as the stationary phase. Since Sephadex is a crosslinked polymer of glucose, it seemed likely that it could also serve as the immobile phase in chromatographic resolutions.

*Experimental*

Sephadex G-10 and G-25 were obtained from Pharmacia Fine Chemicals and pretreated according to the company literature. Research grade DL-mandelic acid (Aldrich Chemical Co., Inc., Milwaukee 10, Wisc.) was found to be optically inactive and was used without further purification. De-ionized water was used. The other chemicals were of reagent grade and were used without further treatment.

The column of Sephadex G-10 was 306 cm × 0.785 $cm^2$ and had an interstitial volume of 88.1 ml. The column of G-25 was 310 cm × 0.785 $cm^2$ with an interstitial volume of 94.8 ml. Eluent was forced through the columns with a Minipump (Milton Roy Co., 1300 E. Mermaid Lane, Philadelphia, Pa.) at a rate of 0.092 cm/min. The effluent was monitored spectrophotometrically at 257 m$\mu$ with a 1-cm flow cell. Fractions of 5.54 ml of eluate were collected automatically, and the rotation of each was measured with either a Rudolph Model 80 or a Perkin Elmer Model 141 automatic polarimeter after acidification with hydrochloric acid. Ultraviolet and infrared spectra of some fractions were determined. For the latter, the solute was extracted with ether and mulled with Nujol.

*Results and discussion*

A preliminary elution of 0.68 mmole of racemic mandelic acid with water as eluent and Sephadex G-10 as stationary phase gave an elution graph (absorbance *vs.* volume of eluate) with two peaks. The first peak was about $^1/_{10}$ as high as the second, had a maximum at 180 ml, and tailed badly. These fractions had no detectable rotation but showed the absorption spectra of mandelic acid. The second peak with a maximum at 510 ml was approximately Gaussian in shape. These fractions also had the absorption spectra of mandelic acid. The fractions on the ascending part of this peak had distinct positive rotations, the largest being +0.028° in a 2-dm tube; those on the descending portion had negative rotations, the largest being —0.079°. The sum of the negative rotations was 2.6 times the sum of the positive rotations. The authors are unable to offer a satisfactory explanation of the first peak. They suggest that the excess of laevo rotations is due to the presence in the Sephadex of bacteria that destroy the (+)-mandelic acid faster than the (—)-isomer.

Several changes were made in subsequent elutions: (1) Aqueous sodium chloride was used as the eluent instead of pure water. This increased not only the sorption of both isomers but also the specificity of sorption[9], thus improving the separation at the expense of time. (2) In order to compensate in part for the greater adsorption of mandelic acid in the presence of sodium chloride, a gel of lesser crosslinking (G-25) was used. (3) The column was pretreated with aqueous ethanol to decrease the bacterial population. (4) The eluent was passed through membrane filters (0.45 and/or 0.20 $\mu$) to eliminate or impede the entrance of bacteria with the eluent. The last two precautions were partly successful; in one elution, they decreased to 1.3 the ratio of the cumulative negative rotations to the cumulative positive rotations. On the other hand, no method of eliminating the small, first peak was found.

The data of the second peak of an elution are given in Table I. This was performed after pretreatment of the column with ethanol. The sample was 2.00 ml of 0.355 *M* racemic mandelic acid in 3.0 *M* sodium chloride. In this and in all other elutions, the sign of the rotation changed at the peak. The data of columns 2 and 3 were used to calculate the figures in the last two columns. The elution graphs of each isomer were drawn from these data. Although these curves departed rather markedly from the ideal Gaussian shape, they were used to calculate[10] the number of plates per cm of column (9.0 and 9.4 for the (+) and (—)-isomers, respectively) and the length of column required for a quantitative separation ($6.7 \cdot 10^5$ cm).

Although these data indicate that a quantitative resolution of mandelic acid with Sephadex G-25 is not practicable, modest resolutions were obtained with very

TABLE I

PARTIAL CHROMATOGRAPHIC RESOLUTION OF MANDELIC ACID WITH SEPHADEX G-25

| *Volume (ml)* | *Total concn. (mmole/ml)* | *Rotation* × 1000 (degrees)* | *Concn. of enantiomers × 1000 (mmole/ml)* | |
|---|---|---|---|---|
| | | | (+) | (—) |
| 310.7 | 2.2 | +10 | 1.2 | 1.0 |
| 316.2 | 2.1 | +12 | 1.2 | 0.9 |
| 321.8 | 1.8 | + 9 | 1.0 | 0.8 |
| 327.3 | 8.5 | +61 | 4.9 | 3.6 |
| 332.8 | 20.0 | +119 | 11.3 | 8.7 |
| 338.4 | 37.6 | +90 | 19.8 | 17.8 |
| 343.9 | 52.2 | —202 | 24.0 | 28.2 |
| 349.5 | 18.1 | —142 | 7.5 | 10.6 |
| 355.0 | 5.4 | —40 | 2.3 | 3.1 |
| 360.6 | 2.5 | —12 | 1.1 | 1.4 |

* In a 2-dm tube.

little difficulty, and surely other racemic substances may be more easily separable by this method.

*Conclusion*

Partial resolution of racemic acid has been accomplished by elution through Sephadex with water or preferably aqueous sodium chloride. The elution graphs (concentration of mandelic acid *vs.* volume) have two peaks. The first peak is much smaller, shows no rotation and tails badly. The second peak is approximately Gaussian. The fractions on the ascending slope have positive rotations; those on the descending slope, negative. An impracticably long column would be required for quantitative resolution of this particular compound.

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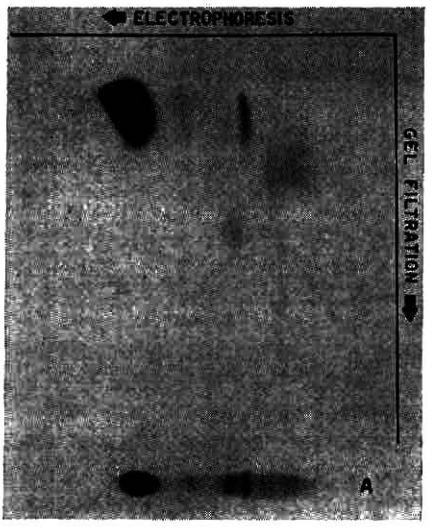
## Electrogel filtration: A combination of thin-layer gel filtration on Sephadex G 200 superfine and electrophoresis on cellulose acetate

A fractionation method combining thin-layer gel filtration on Sephadex G 200 superfine with subsequent electrophoresis in a direction perpendicular to that of the filtration has proved of special interest for qualitative studies of serum proteins. It allows the characterization of serum proteins on the basis of their molecular size and electrophoretic mobility.

A recent attempt by JOHANSSON AND RYMO[1] along these lines, using Sephadex G 200, has shown, however, that with this technique the electrophoretic migration of some protein fractions of larger molecular dimensions is considerably delayed, presumably due to the Sephadex frictional effect. This phenomenon is most evident for the $\alpha_2$M-globulins, which fall into electrophoretic position $\beta$. Even using veronal–lactate buffer, of which it is well known that it improves the fractionation of $\alpha_2$-globulins, we have failed to obtain satisfactory results with JOHANSSON AND RYMO's technique.

In the method described in this paper the serum proteins fractionated by thin-layer gel filtration on Sephadex are transferred by imprinting on a cellulose acetate membrane. The electrophoretic fractionation is then carried out perpendicularly to the direction of the gel filtration, on the cellulose acetate membrane, so as to avoid the Sephadex frictional retardation.



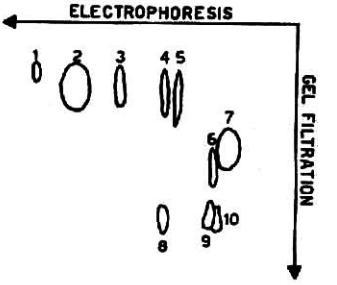




Fig. 1. Electrochromatographic pattern of a normal serum. The electrophoresis is carried out on a cellulose acetate membrane to which the serum proteins are transferred by imprinting after Sephadex gel filtration. A = Reference electrophoresis of the same serum.

Fig. 2. Schematic representation of the normal serum proteins in the electrochromatogram. 1 = Prealbumin; 2 = albumin; 3 = $\alpha_1$-glycoprotein; 4 = haptoglobin (?); 5 = hemopexin, transferrin; 6 = IgA; 7 = IgG; 8 = $\alpha_2$-M; 9 = $\beta$-lipoprotein; 10 = IgM.

## Gel permeation properties of decrystallized cotton cellulose

Cotton cellulose, decrystallized by ball milling[1], has been found to have gel permeation properties comparable to those of the most highly crosslinked dextran and polyacrylamide gels[2]. It is permeable to compounds of molecular weights below approximately 1500, and measurements of the retention of known compounds in this range are being used to obtain information regarding the structure of the amorphous polymer gel. The lower part of the range, below *circa* 700 molecular weight, has been explored with sugars that can be determined quantitatively from polarizations of fractions of the column eluate measured by the sensitive ETL-NPL automatic polarimeter*. Partial separation of two sugars may be detected readily by pairing a dextrorotatory sugar of high molecular weight, such as raffinose or stachyose, with either fructose or erythrose, which are levorotatory. Elution in the order of decreasing molecular weights is evident from the dextrorotations of the initial fractions collected, followed by levorotatory fractions containing a preponderance of the lower molecular weight sugar. Dependence of retention by the cellulose upon molecular weight was determined by experiments with individual sugars, for which the peak elution volumes were found to be inversely proportional to their molecular weights.

Relative elution volumes have been determined individually for the following sugars: erythrose (mol. wt. 120), fructose (mol. wt. 180), maltose monohydrate (mol. wt. 360), raffinose pentahydrate (mol. wt. 594), and stachyose tetrahydrate (mol. wt. 738). Measurements for each of the five sugars were made on comparable columns containing unmodified, decrystallized cellulose, and a sample of the same material that had been crosslinked to 5.2 % $CH_2O$ content, by treatment with formaldehyde[3]. The effect of crosslinking was similar to that produced in other gels, the limit of permeability being reduced. Relative retentions do not differ sufficiently to make these materials efficient for separating sugars, but the results obtained with these columns, eluted at very slow rates of flow permitting local equilibrium to be approached closely, are of general interest in regard to the mechanism of separations effected by other gels having similar properties.

Experimental details will be described elsewhere[4] and only those results pertinent generally to techniques of gel permeation chromatography are reported here. The volumes characterizing each column, and the elution volumes of each of the five sugars on the unmodified cellulose and on the crosslinked cellulose column are assembled in Table I. These characteristic column volumes are defined as: $V_t$, total volume of column; $V_0$, void volume measured by the peak elution volume of blue dextran, mol. wt. *ca.* $2 \cdot 10^6$; $V_s$, volume occupied by dry solid, estimated from its weight and density; $V_r$, total internal volume, or regain, obtained by difference $V_t - (V_0 + V_s)$; and $V_i$, effective internal solvent volume determined by extrapolation of the curves of Fig. 1. The density of the solid was assumed to be 1.59, the theoretical value calculated from the unit-cell dimensions of crystalline cellulose[5]. Densities of the amorphous materials probably are slightly lower, and that of cross-

* ETL-NPL Automatic Polarimeter, Type 143A, manufactured by Bendix-Ericcson, U.K., Ltd., distributed in U.S. by Bendix Corp., Cincinnati Div., Cincinnati, Ohio. Mention of a company and/or product by the Department does not imply approval or recommendation of the product to the exclusion of others which may also be suitable.

TABLE I

CHARACTERISTIC VOLUMES OF CELLULOSE COLUMNS AND ELUTION VOLUMES OF SUGARS

| *Column volumes* | *Celluloses* | | | |
|---|---|---|---|---|
| | *Unmodified* | | *Crosslinked* | |
| | *Total* | *Per g* | *Total* | *Per g* |
| Total volume, $V_t$ (ml) | 46.60 | 3.330 | 47.10 | 3.438 |
| Void volume, $V_0$ (ml) | 23.00 | 1.642 | 22.40 | 1.635 |
| Solid volume, $V_s$ (ml) | 8.80 | 0.629 | 8.62 | 0.629 |
| Regain volume, $V_r$ (ml) | 14.80 | 1.059 | 16.08 | 1.176 |
| Internal volume, $V_i$ (ml) | 6.93 | 0.500 | 9.94 | 0.760 |
| Weight of solid (g) | 14.0 | — | 13.7 | — |
| *Sugars (mol.wt.)* | *Elution volumes, $V_e$* | | | |
| | *Ratio* | *ml/g* | *Ratio* | *ml/g* |
| Dextran (reference) | (1.00) | 1.642 | (1.00) | 1.636 |
| Stachyose·4 $H_2O$ (738) | 1.18 | 1.937 | 1.23 | 2.012 |
| Raffinose·5 $H_2O$ (594) | 1.20 | 1.970 | 1.27 | 2.079 |
| Maltose·1 $H_2O$ (360) | 1.25 | 2.052 | 1.36 | 2.225 |
| Fructose (180) | 1.27 | 2.086 | 1.40 | 2.290 |
| Erythrose (120) | 1.27 | 2.086 | 1.43 | 2.340 |

linked cellulose may differ somewhat from the density of the unmodified sample. The probable magnitude of the difference in density is too small to account for the considerably larger effective internal volume found for the crosslinked cellulose for which $V_i$ is about 50 % greater than that of the unmodified cellulose.

The elution volumes are plotted in Fig. 1 which shows that a simple inverse

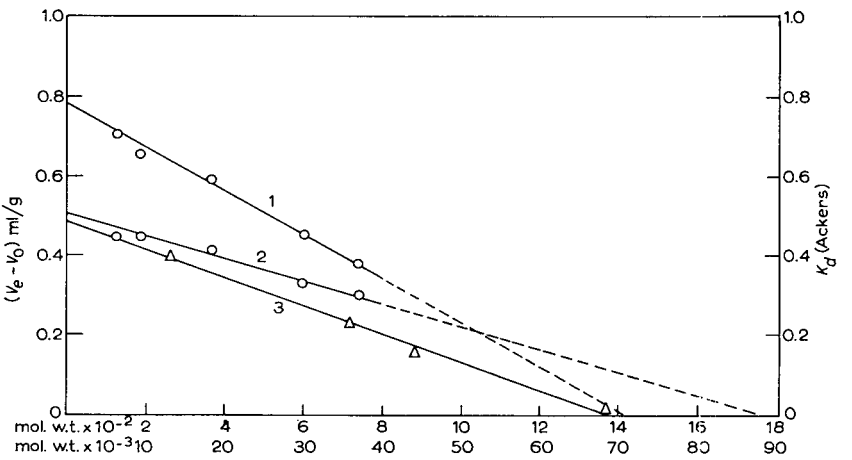



Fig. 1. Relation of elution volume, or distribution coefficient, to molecular weight. (1) Sugars on crosslinked cellulose; (2) sugars on unmodified cellulose (refer to left ordinate and upper abscissa scales); (3) ACKERS' data for cytochrome-*c*, β-lactoglobulin, ovalbumin and hemoglobin in equilibrium with Sephadex G-75 (refer to right ordinate and lower abscissa scales).

linear relationship to molecular weight is approximated closely, if molecular weights are taken to be those of the characteristic stable crystalline hydrates of the dextrorotatory sugars, maltose·1 $H_2O$, raffinose·5 $H_2O$, and stachyose·4 $H_2O$. Experiments with compounds of higher molecular weight will be necessary to determine whether linearity holds up to the limit of permeability; if extrapolation is justified, this limit is indicated to be approximately molecular weight 1750 for the unmodified cellulose, and approximately 1400 for the crosslinked sample. Extrapolation of these lines to their intercepts with the ordinate appears to provide the soundest basis for estimating the correct effective internal volumes of the columns. The total internal volumes, $V_r$, given in Table I are seen to be considerably larger than those obtained from Fig. 1 by extrapolation of curves 1 and 2. Binding of part of the total internal water by hydrophilic gel materials, which reduces the effective internal solvent volume, is known to occur[6]. Bound and free water have been distinguished in completely swollen amorphous regions of cotton fiber[7]. In the unmodified cellulose sample, approximately 53 % of the internal water appears to be bound. Substitution of hydroxyl groups by reaction with formaldehyde in the treated sample makes this material less hydrophilic, and only 38 % of the total water is bound, resulting in a larger effective internal solvent volume, $V_i$, for this column.

Solvent flow rates of only 1.0–1.5 ml/h/cm$^2$ were used in developing these columns. Rates above 4 ml/h/cm$^2$ were not possible because of the high resistance to flow of the finely divided cellulose. Chromatography of macromolecules on gel columns that can be eluted at relatively high flow rates has given excellent correlations of elution volumes with the logarithm of molecular weight in many cases[8]. Conditions are such that local equilibrium is never attained, and probably is not approached closely; the extent of permeation of the internal solvent volume depends largely upon the relative rates of diffusion of the solutes. Deviations from the logarithmic relationship have been reported, and may be accounted for by intermediate rate conditions under which permeation is controlled by partial equilibration as well as diffusion. MORRIS[9] found the logarithmic relationship to hold for a number of proteins on a column of Sephadex G-200, but results for the same proteins showed a deviation therefrom on a column of G-100 gel operated at the same flow rate, evidently because equilibrium could be approached more closely in the G-100 gel which is less permeable to molecules of a given size.

ACKERS[10] determined the distribution coefficients for a number of proteins, both in columns eluted by the usual procedures, and by equilibration of their solutions with the gels Sephadex G-75, G-100 and G-200. The coefficients, $k_d$, were calculated as $(V_e - V_0)/V_i$, in which the internal volume, $V_i$, was taken to be the elution volume of tritrium water (THO). Tritium is known to exchange readily with bound water and with hydrogens of the hydroxyl groups in cellulose, and undoubtedly exchanges with that in dextran also, resulting in a value at least equal to that of the total internal water rather than the effective internal solvent volume. Results of ACKERS' equilibrium measurements for four proteins with Sephadex G-75 are plotted as curve 3 of Fig. 1, showing a linear dependence of distribution coefficient on molecular weight which, by extrapolation, yields a value for the effective internal volume much smaller than that which he obtained with THO. Equilibrium measurements of distribution coefficients of a group of proteins with Sephadex G-200 deviated widely from those obtained by elution of the proteins from a column of

the same gel. A graph of data for the column experiment shows a smooth curve and, as closely as the coefficients can be estimated from this plot, they are related to the logarithm of the molecular weights of the proteins. Sephadex G-200 is a highly permeable gel with a large internal volume, and the difference between effective solvent volume and the total volume measured by THO is less than for the more highly crosslinked dextran gel. One report[11] of work in which the internal volume was determined with THO records values for the internal and external volumes, $V_i$ and $V_0$, the sum of which exceeds the total volume calculated from dimensions given for one of the columns used. The empirical correlation of elution volumes with the logarithm of molecular weights, without reference to an estimated or independently determined internal volume, provides a sound basis for comparing the molecular weights of macromolecules by gel permeation through columns eluted at sufficiently high rates of flow. High rates of development, rather than equilibration with the internal solution, also should be more effective for chromatographic separations.

This work is being extended to develop a technique that can be used to provide a quantitative measure of differences in the cellulose polymer structure produced by chemical modifications[4].

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## Eine vielseitig anwendbare Methode der zyklischen Sephadexgelfiltration durch Verwendung von Kapillarnebengleisen

Bei der zyklischen Sephadexgelfiltration[1-3], die zur Auftrennung von Substanzen unterschiedlicher Molekülgrösse herangezogen wird, besteht manchmal die Notwendigkeit, einzelne Fraktionen aus dem Zyklus zu eliminieren, um ein Wiedervermischen von bereits getrennten Fraktionen durch Überholen auf der Säule zu vermeiden. Dies geschieht normalerweise dadurch, dass man die unerwünschte Fraktion in einem Fraktionensammler auffängt. Beim Auffangen auch kleiner Einzelmengen geschieht immer eine Wiedervermischung der eliminierten Fraktion im Reagenzglas.

Die hier beschriebene Methode gibt die Möglichkeit, beliebig grosse Fraktionen aus dem Zyklus herauszunehmen, indem man sie in Kapillarschläuche einlaufen lässt, die als "Nebengleise" in das System der zyklischen Sephadexgelfiltration eingeschaltet sind. Diese Kapillarschläuche kann man entweder im Nebenschluss belassen, um sie nach Belieben wieder in den Zyklus einzuschalten, oder man kann die an beiden Enden durch Quetschhähne verschlossenen Kapillarschläuche herausnehmen und im Kühlschrank beliebig lange aufbewahren. Eine Vermischung der Fraktionen im Kapillarschlauch ist praktisch nicht möglich.

Fig. 1 zeigt das Schaltschema der Methode.

Um die notwendige Anschaffung einer Vielzahl sehr teurer handelsüblicher Mikroventile zu umgehen, haben wir selbst einfache Mikroventile entworfen, die in der Werkstatt unseres Institutes angefertigt wurden. Die Fig. 2 und 3 zeigen eines der verwendeten 3-Wegeventile.

An Hand des Schaltschemas (Fig. 1) können die folgenden Schaltungen für die einzelnen Arbeitsvorgänge verfolgt werden.

(1) *Ansaugen der aufzutrennenden Probe*

*Weg*: Messzylinder, Ventile 2, 3, Pumpe, Säule, Durchflussphotometer, Ventile 4, 5, 6, Pumpe, Fraktionensammler.

*Hähne*: offen: f, d, g, i, k, m, n; geschlossen: c, e, h, j, l, $\beta$.

(2) *Zyklisches Chromatographieren*

(*a*) *Weg*: Ventile 2, 3, Pumpe, Säule, Durchflussphotometer, Ventile 4, 7, 21, 2 usw.

*Hähne*: offen: d, g, i, j, $\gamma$, e; geschlossen: f, c, h, k, o, $\alpha$.

Zur Kontrolle der geförderten Menge schaltet man parallel über die gleiche Pumpe folgendermassen:

(*b*) *Weg*: Vorratsgefäss, Ventile 1, 20, 6, Pumpe, Fraktionensammler.

*Hähne*: offen: a, b, $\beta$, n; geschlossen: c, $\alpha$, m.

(3) *Abschieben einer Fraktion in ein Kapillarnebengleis*

Soll z.B. eine Fraktion von *ca.* 10 ml aus dem Zyklus herausgenommen werden, so wartet man, bis der Beginn der entsprechenden Fraktion vor dem Ventil 7 ist (abzulesen am Punktschreiber, der die Messung des Durchflussphotometers aufzeichnet) und schaltet folgendermassen:

*Weg*: Vorratsgefäss, Ventile 1, 20, 21, 2, 3, Pumpe, Säule, Durchflussphotometer, Ventile 4, 7, 8, 10, 11, 13, 15, 17, 18, 19, 5, 6, Pumpe, Fraktionensammler.

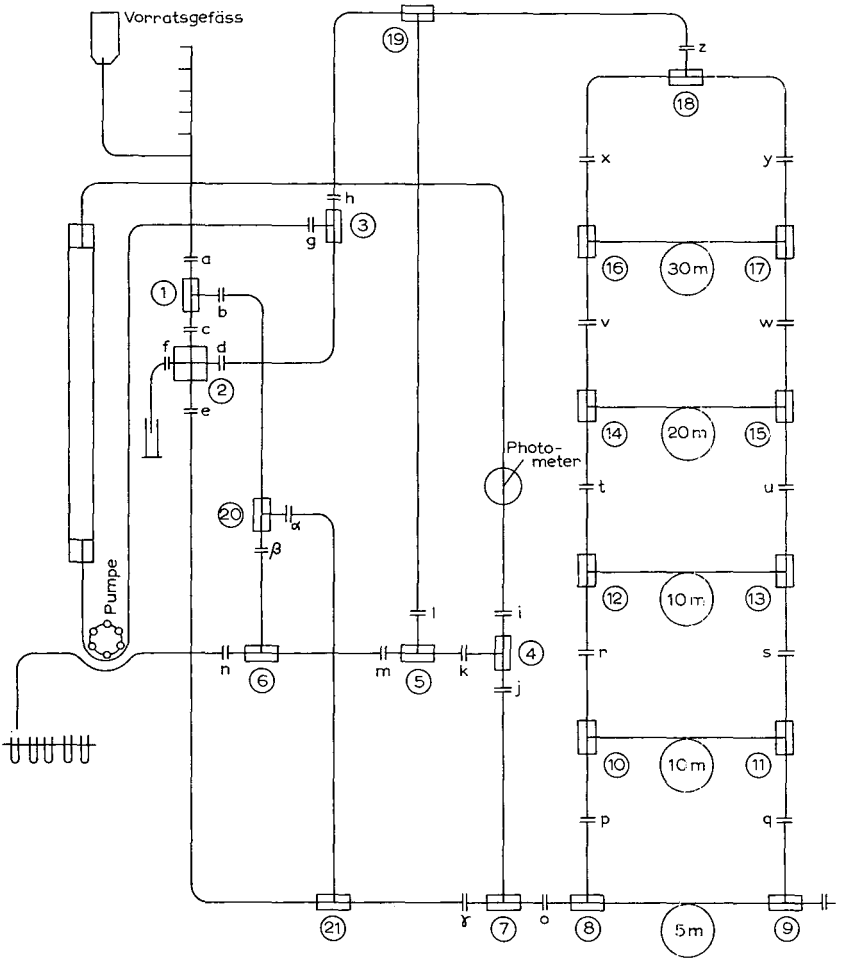


Fig. 1. Schaltschema. Als Vorratsgefässe wurden normale Infusionsflaschen verwendet, die mit handelsüblichen Infusionsbestecken an das Sammelrohr angeschlossen wurden.

3-Wegmikroventil;

4-Wegmikroventil;

Quetschhahn (a, b, ....);

Messzylinder, aus dem die aufzutrennende Substanz angesaugt wird;

Fraktionensammler;

Durchflussphotometer mit Punktschreiber;

Kapillarschlauch, aufgerollt, in der angegebenen Länge;

Sammelrohr zum Anschluss weiterer Vorratsgefässe.

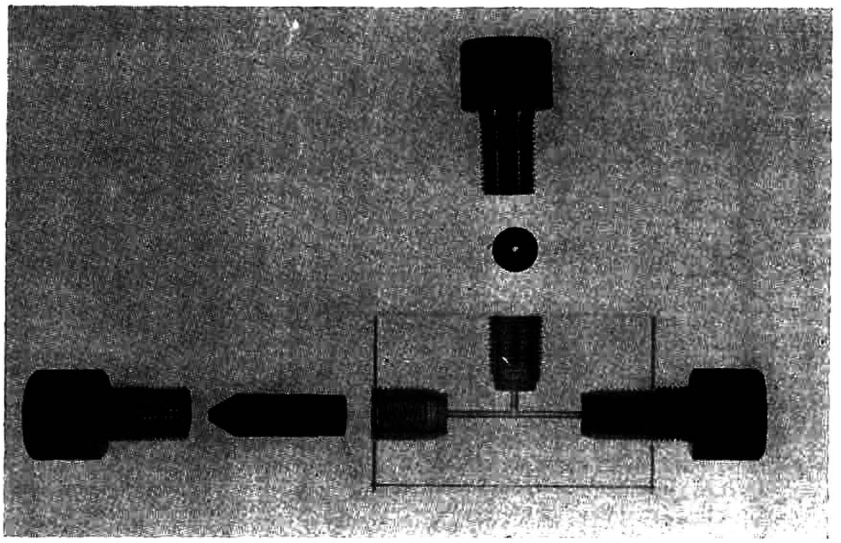

Fig. 2. Abbildung eines Mikroventils.

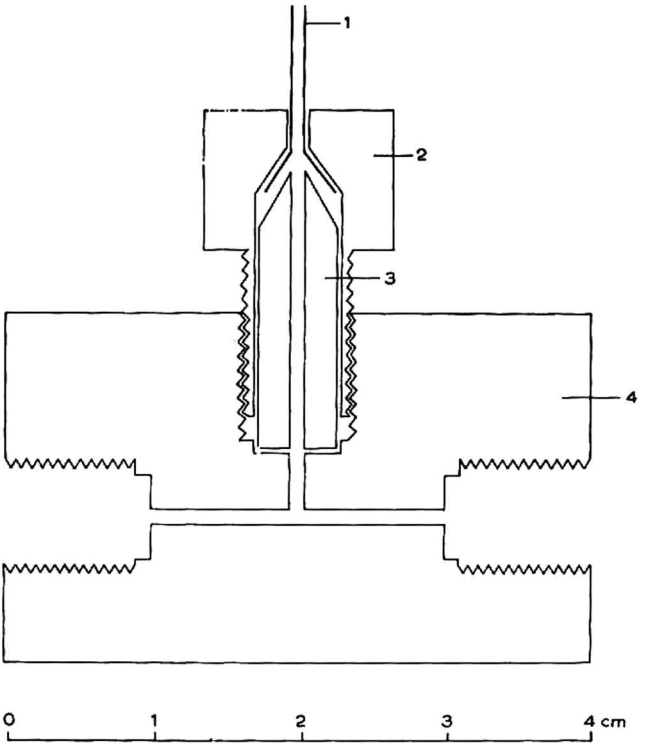


Fig. 3. Schnittzeichnung des in Fig. 2 gezeigten Mikroventils. 1 = Kapillarschlauch; 2 = Überfallmutter aus Messing; 3 = Konus aus Plastik; 4 = 3-Wegehahn mit Kapillarbohrungen aus Plexiglas.

*Hähne*: offen: a, b, α, e, d, g, i, j, o, p, s, u, w, y, z, l, m, n; geschlossen: c, β, γ, f, h, k, q, r, t, v, x.

(4) *Einschleusen der abgestellten Fraktion zur weiteren zyklischen Chromatographie*

*Weg*: Vorratsgefäss, Ventile 1, 2, 21, 7, 8, 10, 11, 13, 15, 17, 18, 19, 3, Pumpe, Säule, Durchflussphotometer, Ventile 4, 5, 6, Pumpe, Fraktionensammler.

*Hähne*: offen: a, c, e, γ, o, p, s, u, w, y, z, h, g, i, k, m, n; geschlossen: b, d, f, α, j, q, r, t, v, x, l, β.

Sowie die Fraktion in der Säule ist, weiter chromatographieren wie unter (2).

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HANS-JÜRGEN GERMANN**

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2 J. KILLANDER, *Biochim. Biophys. Acta*, 93 (1964) 1.
3 J. PORATH UND H. BENNICH, *Arch. Biochem. Biophys., Suppl. 1* (1962) 152.

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* Direktor: Prof. Dr. R.-E. BADER.
** Anschrift des Verfassers: Medizinische Universitäts-Poliklinik, 355 Marburg, Deutschland.



## Separation of myoglobin and hemoglobin on Sephadex thin layers

Sephadex gels have proved very convenient for the separation of substances differing in molecular weight. In chemical pathology it is sometimes necessary to differentiate Mb from Hb* by a simple method.

Although we have succeeded by using conventional paper electrophoretic separation[1,2] we decided to try also Sephadex thin layers (TLC) for this purpose.

TLC was performed on glass plates 18 cm × 4 cm. A suspension of Sephadex was prepared by shaking swollen Sephadex beads by means of an agitator in an 0.1 *M* phosphate buffer, pH 7.4, or in a mixture of this buffer with the same volume of 0.9% NaCl. Sephadex G-50 Superfine, G-75 Superfine and G-200 Superfine were used. The volume ratio of gel solution was chosen according to the instructions of the manufacturer[3]. Layers 0.9 mm thick were spread by means of a glass rod provided with cuffs of adhesive tape (Isolepa). Descending chromatography was performed on glass plates inclined at an angle of 12°, connected with the solvent trough by a bridge of Whatman 3 paper and placed in a closed glass tank. Detection was performed either by the use of reactions specific for the heme groups of Hb and Mb, with benzidine[4] or *o*-dianisidine[5], respectively; or by staining for proteins with bromphenol blue[6].

Fig. 1 shows the separation of Hb from Mb on Sephadex G-50 Superfine in

* Abbreviations: Mb = myoglobin; Hb = hemoglobin.

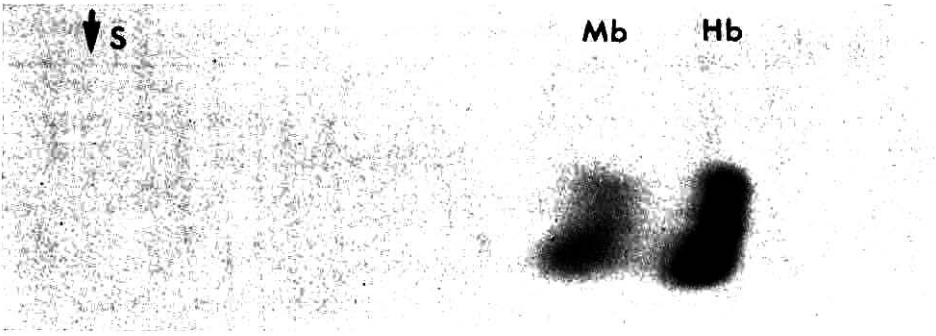


Fig. 1. TLC on Sephadex G-50 of muscle extract and added Hb. Chromatography was performed with phosphate buffer for 40 min.

phosphate buffer, pH 7.4, after 40 min development. Detection with benzidine–$H_2O_2$. Similar results were obtained with Sephadex G-75 and with a mixture of the phosphate buffer and 0.9 % NaCl (1:1). The solution of NaCl alone (with any kind of Sephadex) was not suitable. With Sephadex G-200 the chromatography proceeded very slowly and even after 16 h separation of Mb and Hb was not achieved. The minimum detectable amount of Hb by the benzidine–$H_2O_2$ reaction after chromatography was about 15 $\mu$g when a 2 cm long line had been spotted.

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Z. STRÁNSKÝ
M. SRCH

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2 Z. STRÁNSKÝ AND M. SRCH, *Česk. Fysiol.*, 16 (1967), in press.
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6 Č. MICHALEC, J. KOŘÍNEK, J. MUSIL AND J. RŮŽIČKA, *Elektroforesa na Papíře a v Jiných Nosičích*, ČSAV, Prague, 1959.

Received January 2nd, 1967

## Book Reviews

*Standard Methods of Chemical Analysis*. Vol. III. *Instrumental Methods of Analysis* (comprising two books, Part A and Part B), edited by F. J. WELCHER, 6th Ed., Van Nostrand, London, 1966, 2,048 pp., price £18.18.0.

These volumes constitute the third part of the classical work on chemical analysis, which has been so far the most reliable source of information in the chemical laboratory. Volume III is an addition to previous editions; it is a collection of the more important instrumental techniques and the applications of these techniques to the analysis of special materials. The aim of this volume is to provide the analytical chemist, who faces a challenging variety of new and increasingly complex materials and has to determine a diversity of constituents, which may be present in traces, information on the large number of instrumental methods which may be used for a particular analysis. The contents of this volume may appear to go beyond the title of the work as few instrumental methods, strictly speaking, may be defined as standard in the sense that they have been standardized by a particular group or have been accepted as such as a result of extensive use. Nevertheless the inclusion of the material has been sagacious because the wide coverage of these methods enables the analytical chemist to select the one most suitable for a given purpose.

The volume is divided into 74 chapters. Each of the first 41 chapters is devoted to a single instrumental method; a uniform scheme is followed: first the basic principles with the necessary equations, then the instrumentation and finally the descriptions of representative applications. Less common topics such as particle analysis and sedimentation analysis are also included.

The remaining chapters are devoted either to specific fields of analysis or to a particular field such as alloys, air pollutants, foods, clinical medicine, glasses, plastics, etc. In each chapter the selected procedures making use of instrumental methods, which have proved most useful in practical and industrial work, are described with sufficient details. It is difficult to keep a volume of this size up to date but it is worth noting that all chapters cover literature references up to 1963 and in some cases up to 1964.

From a practical stand point the volume is to be regarded as the most complete and the most useful text available today on instrumental analysis.

The features described above render this volume an invaluable working tool for analytical chemists, especially if engaged in industrial work. The high cost of the volume may prevent a chemist from buying his personal copy but the volume should be available for reference in every library.

A. LIBERTI (Naples)

*Laboratory Handbook of Chromatographic Methods*, by O. MIKEŠ, Van Nostrand, London, 1966, 434 pp., price 75s.

The book is a rewritten edition of the original Czech which first appeared in 1961. It is perhaps the best attempt in recent years to write a text dealing with all chromatographic methods without specialising in biochemistry or another field. The chapters are written by experts and deal with the following: (1) Fundamental types of chromatography; (2) partition chromatography; (3) adsorption chromatography; (4) thin-layer chromatography; (5) ion exchange chromatography; (6) gel filtration; (7) mechanisation and automation of column chromatography; (8) electrophoretic methods of separation; (9) gas chromatography.

It may strike the reader that some chapters deal with a mechanism and others with a technique, however the reviewer agrees that these are the best divisions in which all aspects of chromatography can be treated correctly. Naturally there is much emphasis on some and little on other methods (*e.g.* ion exchange papers). The sections dealing with applications are almost always sketchy, citing only typical examples, and in some cases (for example immunoelectrophoresis) only an obscure Czech paper is cited when reference to the standard texts would be indicated. On the whole, however, all separation methods receive a mention. The reviewer would recommend it rather as a textbook than as a handbook, and rather to the general analyst or the student than to the chromatographer.

MICHAEL LEDERER (Rome)

*Thin-Layer Chromatography*, par K. RANDERATH, 2ème édition, Academic Press, New-York et Londres, 1966, xiv + 285 pp., prix U.S. $ 9.50.

La première édition du livre de RANDERATH, parue en 1963, a connu en France un succès certain, dû en grande partie à l'existence de la traduction en langue anglaise.

La seconde édition (1966) représente une augmentation de volume de 14 % par rapport à l'édition originale. Ce nouvel rapport n'est qu'un reflet de l'accumulation considérable des publications en ce domaine entre 1963 et 1966. L'auteur a su conserver à l'ouvrage son abord facile, et ses aspects pratiques. Ce livre est à la fois une source réelle de renseignements, et un instrument de travail.

Après une introduction technique qui utilise environ le tiers de l'ouvrage, l'auteur décrit l'application de la chromatographie sur couche mince aux principales familles de substances naturelles, ainsi qu'à quelques composés non naturels: alcaloides, acides aminés, sucres, stéroides, lipides, pigments, vitamines, produits médicaux, insecticides, colorants, etc. La dernière partie, consacrée à la séparation des ions minéraux, paraît considérablement abrégée (4 pages).

M. BARBIER (Paris)

*Gas Cromatografia*, by MARCO TARAMASSO, Franco Angeli Editore, Milan, 1966, 187 pp., price Lit 4000.

This is the first Italian book covering this interesting subject. The author is well known for his research work on gas chromatography, especially on preparative techniques.

This book deals with all the more important aspects of gas chromatography. The theory is developed in a very simple way, yet includes all fundamentals and definitions. Preparation of both packed and capillary columns is described in detail, and the more common types of detectors are described, with various illustrations.

All the qualitative and quantitative techniques of analysis are discussed with many selected examples and applications. Many useful suggestions are given for the appropriate choice of the columns and the different working conditions.

The book will be a practical guide for everybody using gas chromatography as an analytical tool or for research. Many references and useful tables complete this valuable text.

G. P. CARTONI (Rome)

*Gas Chromatographic Retention Data*, by W. O. MCREYNOLDS, Preston Technical Abstracts Co., Evanston, Ill., 1966, 335 pp., price $ 25.00.

Zu den Hauptkriterien für die Identifikation von Substanzen gehört die Messung der Retentionsgrössen in einem chromatographischen System. Das bedeutet aber keinesfalls, dass das Problem der Retentionsdaten schon völlig gelöst ist, besonders in Bezug auf die Reproduzierbarkeit. Bisher konnten die veröffentlichten Daten nur als ein annäherndes Mass der Beweglichkeit einer Substanz in dem chromatographischen System verwendet werden, eventuell als Leitfaden für die Auswahl der experimentellen Bedingungen. Ausser den in der Literatur veröffentlichten Daten existiert eine Publikation von J. S. LEWIS, der aus der Literatur die Retentionsdaten für etwa 2,000 Substanzen ausgesucht hat. Für ihre Zusammenstellung hat er ein System benutzt, das vom Gas Chromatography Subcommittee of the Tennessee Eastman Company Analytical Committee entwickelt wurde, und zwar unter Verwendung von Lochkarten des IBM-Systems zur Aufbewahrung der Daten. Für die Beweglichkeit der Substanzen benutzte er die relative Retention. Da es sich aber um Daten handelte, die von verschiedenen Laboratorien veröffentlicht wurden, waren die Ergebnisse nicht genau vergleichbar.

Die besten Resultate in Bezug auf die Ausnützung für die Identifikation wurden bisher dann erzielt, wenn die veröffentlichten Daten auf einer Arbeitsstelle gewonnen wurden. Eine diesbezügliche Arbeit wurde von MCREYNOLDS durchgeführt, der für etwa 350 ausgesuchte Stoffe die Retentionsdaten auf 77 stationären Phasen festgestellt hat. Für die Messung der Daten konstruierte der Author ein spezielles Gerät, das

eine simultane Chromatographie der Probe auf 12 Kolonnen mit verschiedenen stationären Phasen ermöglicht; das Gerät wurde mit einem Detektor-block mit 12 Detektoren versehen unter Anwendung von einem 12-Kanal-Registrierungsgerät. McREYNOLDS arbeitete an jeder stationären Phase bei zwei Temperaturen (meistens 20° oder 40° voneinander entfernt) in einer Temperaturspanne von 80–160°. Die Daten wurden als spezifische Retentionsvolumina $V_g$ und weiter in der Form von KOVÁTS Retentionsindices $I$ ausgedrückt, die aber indirekt aus Retentionsvolumina errechnet wurden. Die Angaben sind in dem vorliegenden Buch je nach der stationären Phase für die meisten Substanzen eingeordnet, und zwar in solcher Weise, dass in der ersten Hälfte bei den einzelnen stationären Phasen die Substanzen nach den Funktionsgruppen und nach steigernden Molekulargewicht eingereiht sind, wobei in der zweiten Hälfte die Einreihung nach steigernden Retentionsvolumina folgt. Dieses System ermöglicht relativ schnelle Identifikation einer unbekannten Bande (unter Voraussetzung, dass es sich um eine Substanz handelt, für welche die Retentionsdaten vorhanden sind), und weiterhin eine schnelle Auffindung der optimalen experimentellen Bedingungen für die bekannte zu analysierende Substanzen.

Man könnte gewiss mit dem Author über alle gewählten experimentellen Bedingungen diskutieren, über die Auswahl des Trägermaterials (es wurde Celite 545 gewählt, bei den meisten stationären Phasen mit Zugabe von Polytergent J-300), über die Auswahl der stationären Phasen usw. Meiner Ansicht nach dient jedoch die Auswahl von womöglich einfachen Bedingungen mit einer geringen Anzahl von Varianten dem ganzen Problem der chromatographischen Daten nur zum Nutzen. Ich würde sogar für geeigneter halten die Anzahl von stationären Phasen zu reduzieren und lieber die Anzahl der Stoffe zu erhöhen, für welche die chromatographischen Daten veröffentlicht wurden. Es bliebe zu wünschen übrig, dass der Author diese Arbeit mit einer weiteren Gruppe von Substanzen fortsetzen würde.

K. MACEK (Prag)

*Liquid Chromatography Handbook*, Phoenix Precision Instrument Company, Philadelphia, Pa., 1966, price $ 10.

This is not a handbook nor does it deal with liquid chromatography, but the instructions for operating the Phoenix automatic amino acid analyser. As such it is well-prepared with all technical details and numerous clinical examples. For the price of $ 10 one expects, though, a book and not 60 odd pages in a loose leaf holder dealing with only one apparatus.

MICHAEL LEDERER (Rome)

*Guide to the Analysis of Pesticide Residues*, by H. P. BURCHFIELD AND DONALD E. JOHNSON, with the assistance of ELEANOR E. STORRS, Superintendant of Documents, U.S. Government Printing Office, Washington, D.C., 1966, price $12.75 in set of 2 vols. only.

The two manuals were compiled to provide standardized methods for the U.S. Public Health Service. The authors have been quite successful in accomplishing this objective.

Multidetection and specific methods capable of analyzing a large number of diverse biological extracts such as those of soil, water, plant and animal tissues are presented in detail for many pesticide residues. This guide, assembled in sections, outlines and discusses the intricacies of sample preparation, extraction and clean-up techniques, determinative procedures, status of the method, quantitative measurement, and identification of residues. Laboratory equipment, organization and protocol are discussed also.

In addition, a list of pesticides and their properties, the composition of a typical diet and the fat and water content of several foods is included.

The organization of material has been given considerable attention by the authors. Each section is divided into sub-sections and within each sub-section there is a further division according to the subject matter. Comprehensive index and subject coding provide ready access to the desired material. An additional feature makes it possible to extract and assemble loose leaf pages on a particular subject into a separate, working notebook.

Information is meagre or non-existent in certain areas, such as air analysis, bioassay techniques and the sources, handling, storage conditions, stability, etc. of pesticide reference standards. The omissions are not the fault of the authors but are due to the lack of research and published information.

These manuals co-ordinate information on selected methods, and focus attention on their adequacy and limitations. Many of the methods have not been fully evaluated for the types of samples specified, nor can they be considered applicable to other similar types of samples. The analyst is cautioned to satisfy himself, by critical evaluation of the methods in his laboratory, that reliable analytical data will be obtained. In general, the compilation of information is such that those areas which require intensive research effort on method development are easily recognized.

H. MCLEOD (Ottawa)

*Research on Steroids, Transactions of the Second Meeting of the International Study Group for Steroid Hormones*, edited by C. CASSANO, published by Il Pensiero Scientifico, Rome, 1966, 553 pp.

The Proceedings of the meeting, including a verbatim report of all discussions, cover 534 pages. Obviously only a fraction of all the reported material can be men-

tioned by the reviewer. There were three main topics on the agenda: Estrogens in Pregnancy (120 pages), Methodology in Steroid Estimation (210 pages) and Steroidal Antifertility Agents (Round-Table Conference) (202 pages). For topics 1 and 2 four main lecturers had been appointed, communications related to the subject followed each main lecture. BAULIEU lectured on the mechanism of action of estradiol, FRANDSEN on the site of production of oestrogenic hormones in pregnancy, DICZFALUSY AND BENAGAINO on oestriol metabolism in midpregnancy and KLOPPER on the assay of urinary oestriol as a measure of placental function. Rapid methods for oestriol determinations in urine or plasma really seem to represent a fundamental contribution in the assessment of the functional state of the foeto-placental unit, and therefore are a big advancement in the otherwise scanty laboratory means for diagnosis in obstetrics.

In the course of the day dedicated to methodology, HORNING AND GARDINER lectured on the estimation of human steroids by gas–liquid chromatographic methods, GALLETTI on the identification and estimation of urinary metabolites of gonadal steroids by thin-layer chromatography, DE HERTOGH on isotopic methods in the study of steroid hormone metabolism *in vivo*, and DUVIVIER on the methodology of corticosteroid biosynthesis (a review). Quite a number of communications dealt with the assay of testosterone in urine or blood plasma, which only shows how much interest and work is dedicated at present to the solution of the problems concerning the role of testosterone in the physiology and pathology of the human female.

Apart from the foreword and opening and closing remarks, 30 speakers contributed to the topic "Steroidal antifertility agents". Rather than a Round-Table the meeting had the character of a symposium. Only 7 papers were concerned with the mechanism of action of compounds used as oral contraceptives. The rest discussed the effect of these compounds on the ovary, on the adrenal cortex function, on the menstrual cycle, on the urinary levels of testosterone, on vaginal cytology, on the endometrium, on the liver function, on the thyroid, and on blood coagulation, etc. One communication dealt with fertility after stopping oral medication and with the health of children born to these mothers. The last 15 pages of the book contain an appendix, prepared by A. ERCOLI, which is a very complete and useful "List of Antifertility Steroids and their Combinations", and of great value for reference purposes.

The editor is to be complimented for having brought out these Proceedings just a few months after the meeting. The field of endocrinology moves so fast that what is said today is very often old tomorrow. In this case what was said at the Rome Meeting reaches the reader in record time. Some of the reports bring new data, some summarize or review not too well-known subjects, some just critically analyze known facts as happens at every other meeting of this kind. The reader will pick and choose the details or the information he is interested in, and will be pleased to find most information up to date to the end of 1965.

G. HECHT-LUCARI (Darmstadt)

*Handbook of Physical Constants*, revised edition, edited by SYDNEY P. CLARK, JR., Memoir 97, Geological Society of America, New York, 1966, pp. 587, price U.S. $ 8.75.

The appearance of the revised edition after almost a quarter-century must be very welcome to many "earth" scientists. The twenty-seven sections bring together an extremely valuable body of up-to-date data on the physical constants of "earth" materials. The contribution of the editor in this achievement is noteworthy. The preponderance of North American data in some sections is understandable but will be regretted by scientists of other continents. Pseudoviscous flow and secondary flow (p. 227) are not synonymous. More data on igneous and sedimentary rocks are needed before the first sentence on p. 235 becomes convincing. The glide plane $01\bar{1}2$ for calcite is omitted (p. 241). Data on rock mechanics without reference to B. SANDER and on magnetic properties without reference to E. IRVING seem to lack some authority. Properties such as reflectivity, micro-hardness and some "engineering properties" are omissions worth mentioning for consideration in the next edition. The binding, paper and print are well up to the usual high standards of American publishing. The Society, the contributors to its Memoir Fund, the editor and his contributors have rendered a great service to "earth" scientists.

ARTHUR S. RITCHIE (Newcastle, Australia)

*Whatman Technical Bulletin C4, "Paper Chromedia"*, H. Reeve Angel & Co. Ltd., London, 1965, 51 pp.

Simultaneously with the phenomenal expansion of thin-layer chromatography, paper chromatography has developed in various directions, and some new techniques have perhaps not received the attention due to them (for example see the possibilities of silica gel impregnated paper, G. V. MARINETTI, in A. T. JAMES AND L. J. MORRIS (Editors), *New Biochemical Separations*, Van Nostrand, London, 1964, pp. 339–377).

The Whatman Technical Bulletin C4, which gives a good discussion of the modified papers produced by Whatman, will thus prove interesting reading for chemists who have to work out new separations.

The following papers are discussed: Silicone treated papers (14 references); adsorbent loaded papers, silica gel and aluminium hydroxide (18 references); glass fibre papers, there are now three qualities (23 references); ion exchange celluloses (68 references). These are not complete bibliographies, but the scope of each of the products is clearly illustrated.

M. LEDERER (Rome)

*A Course of Lectures on Chromatography (Ciclo di Seminari sulla Cromatografia)*, organised at the Istituto Superiore di Sanità, Rome, April 5th to June 3rd, 1965.

This course of eight postgraduate lectures has now appeared as a special issue of the "*Annali dell' Istituto Superiore di Sanità*, Vol. II, fascicolo speziale 1, 1966".

It contains summaries only of the lectures by A. LIBERTI and J. JANÁK and the complete texts of the following lectures:

K. SAKODINSKY—Gas cromatografia preparativa (in Italian)

K. MACEK—Identifizierung von Substanzen mittels Papier- und Dünnschicht-chromatographie (in German)

M. LEDERER—Ion exchange paper chromatography of inorganic ions (in English)

G. VIVALDI—Separazione cromatografica su colonna di proteine eterogenee (in Italian)

L. TENTORI—La determinazione quantitativa dei dinitrofenil-derivati degli amminoacidi mediante cromatografia automatica su colonna di gel di silice (in Italian)

G. B. MARINI-BETTÒLO—Applicazioni biochimiche della cromatografia su gel di destrano

Although only a limited number of topics is treated in these lectures it is the first attempt to review the progress in chromatography for Italian readers. All lectures are well documented and illustrated and will interest both analysts and biochemists.

*Vorträge des V. Symposiums über Gas Chromatographie in Berlin, Mai 1965*, herausgegeben von H. G. STRUPPE AND D. OBST, Akademie Verlag G.m.b.H., Berlin, 1965, 606 pp., Nachtrag 141 pp.

The main volume of the symposium contains 43 papers and the "Nachtrag" another 10. There is a preponderance of contributions from Eastern countries but most authors' names usually figure also on the lists of symposia held in Western countries. Giving the titles of the papers here would only be duplication of the bibliography section of this journal.

The book is an offset print of type-written manuscripts and bound in soft covers. However the print is readily legible and the figures well reproduced. The reviewer asks himself how well the many symposium volumes, such as the present one or those of the Gas Chromatography Discussion Group, are really available in libraries, say five years after their appearance, and how much is lost to many readers by not using scientific journals for the publication of original work.

## News

### Third International Symposium on Reproducibility in Paper and Thin-Layer Chromatography

Organized by the Chromatography Group of the Czechoslovak Chemical Society, at Liblice, October 2–5, 1967.

*Preliminary Information*

Symposia organized by the Chromatography Group of the Czechoslovak Chemical Society have been devoted to a limited field of general problems connected with chromatographic techniques: Relationships between chemical structure and chromatographic behaviour and Systematic analysis (1961) and Stationary phase in paper and thin-layer chromatography (1964). The 3rd Symposium will deal with *reproducibility of the position of spots and zones* and *reproducibility of the quantitative methods.*

As in the preceding Symposia, the programme will consist of introductory papers (ranging from a brief presentation of the questions to be discussed to review of the problem), reports on personal experience bearing on the problem, and ample discussion time. Time for presentation of the papers will be limited.

The bulk of the attendance will be represented by invited specialists. The discussion will be limited to English and German, yet papers in other languages will also be accepted and translated provided their manuscript reaches the secretary before September 15th.

The registration fee for active members will approximate 500 Kčs ($ 35) and will cover board and lodging in the Liblice castle for three days as well as coach transport from and to Prague, a half-day excursion (Prague), an evening concert and a picnic.

*Tentative Programme*

*Reproducibility of the position of spots and zones on the chromatogram*

I. Influence of materials used in PC and TLC (paper, sorbents, preparation of plates, precoated plates and foils, humidity, etc.)

II. Influence of systems and development (systems, impregnations, tanks and development techniques)
Introduced by F. Geiss (Ispra)

III. Comparison of various techniques (especially PC and TLC)
Introduced by K. Macek (Prague)

IV. Standardization of chromatographic procedure
Introduced by E. Stahl (Saarbrücken)

V. Publication of chromatographic data
Introduced by M. Lederer (Rome)

*Reproducibility of quantitative analysis*

VI. Quantitative analysis with main reference to the *in situ* methods
Introduced by R. Klaus (Darmstadt)

## Apparatus

A routine-type *NMR-instrument* with 0.3 c.p.s. resolution has been announced by Japan Electron Optics Laboratory Co., Ltd., capable of detecting 0.002 moles/l using slow sweep and 0.007 moles/l at normal scanning speed. The spin decoupler is built in and provides both frequency sweep and field sweep decoupling. A widely variable sample temperature range is available (—100°C to +200°C) and a stability of ±0.2 c.p.s. is guaranteed. F19, B11, P31 resonance can be observed with the aid of accessories which employ a newly developed technique called nuclear single side-band method. This method eliminates the extra side bands generated by modulation which may overlap and interfere with the signals under observation.

A new Instrumentation Data Sheet PKU-1066, describing the *PKU Micro-Analyzer* is available through Phoenix Precision Instrument Co., Philadelphia, Pa. 19140. This analyzer allows the rapid qualitative and quantitative analysis of various amino acids in a 10 × 0.6 cm column in 15 min. Specimen chromatograms are given and suggestions made for the use of the analyzer in clinical laboratories for the determination of *e.g.* valine, isoleucine, leucine or tyrosine and phenylalanine in diagnosis.

Perkin-Elmer have announced the new *RMU-6E Mass Spectrometer* which is based on a building block concept of versatile high performance components. A number of these combinations include facilities for combining the mass spectrometer with a gas chromatograph. The Hitachi Perkin-Elmer instrument is particularly suitable for this work, as it offers very fast scans—3 sec for a full 12 to 500 mass scan—and has inlet systems specially designed for link-up, especially to an F11 gas chromatograph.

Infotronics Corp., 7800 Westglen, Houston, Texas have announced the Infotronics CRS 100 *Electronic Digital Integrator* for gas chromatography.

A new instrumentation data sheet on the Phoenix *Amino Acid Micro-Analyzer* is now available from Phoenix Precision Instrumentation Company, Philadelphia, Pennsylvania.

The new data sheet contains detailed information and specification about the M-7800 Micro-Analyzer, a low-cost companion to the Phoenix Amino Acid Analyzer line, which is designed primarily for the small laboratory or for routine analyses.

For a copy of the instrumentation data sheet, MA-566, write to: Phoenix Precision Instrument Company, 3805-North Fifth Street, Philadelphia, Pa., 19140, U.S.A.

A new attractively styled *Mid-size Multipoint Recorder* is now available from the Barber-Colman Industrial Instruments Division. This new compact instrument, which is capable of measuring 2, 3, 4, 6 or 12 inputs, has a full six and one-half inch scale. The face area of the instrument is approximately two and a half times less than a conventional twelve-point instrument which uses a twelve inch chart. Two Barber-Colman Series RD5 Multipoint Recorders can be flush mounted side by side in a standard 19-inch panel for relay rack installation.

The print head will clearly print three different printout configurations: dots with a printed number every 25th dot, all numbered dots or dots without numbers—all at the user's choice. A simple screwdriver adjustment makes it easy to change from one type of printout to another.

A removable flip-out chart transport is used for convenience and time saving. By using a spare transport, a new chart can be preloaded for immediate use. Instrument operation is hardly interrupted. Less than 15 seconds is required to exchange chart transport. An automatic print head lifter eliminates tearing of charts. The chart transport doubles as a writing platen. It can be set at a 45° angle during recorder operation for making chart notations.

The convenient slide-out chassis of the multipoint recorder has solid state circuitry for reliable and trouble-free operation. A Zener diode constant voltage reference power supply is used for continuous reference. The fully shielded and guarded measuring circuit eliminates stray signal pickup and provides precision measurement of the input variable. Common mode and series mode rejection is high.

For additional information, contact Barber-Colman Company, Industrial Instruments Division, Rockford, Ill. 61101, U.S.A. and ask for bulletin 1221.5 DB 3.

A new three-function *Intermediate Controller for Proportional Current Control Systems* has been announced by Barber-Colman Company, Industrial Instruments Division.

The Series 683, a compact all electronic transistorized unit, provides proportional plus rate plus reset action for processes requiring stable control in spite of severe and frequent load changes. It can be used to drive silicon rectifier power packs, pilot amplifiers of saturable reactors of electro-pneumatic converters, as well as other compatible electric systems.

In addition to the basic three-function control action, two important extracts are incorporated—reset wind-up inhibition and Load Line-out. Both are standard features in the Series 683 Controllers. Reset wind-up inhibition is particularly desirable in processes where large step increases or decreases occur. It insures that reset action will always be fully effective in the area of the control set point. Load Line-out is an exclusive feature which effectively prevents overshoot in batch processes. It is achieved by superimposing a manually set signal on the automatic reset action, varying the reset reference voltage.

Bulletin 1312 DB 4 gives complete information and specifications. Contact the Barber-Colman Company, Industrial Instruments Division, Rockford, Ill. 61101, U.S.A.

The new Series 692 Barber-Colman *Three Function Controller* is a compact all electronic transistorized unit serving as the intermediate station in a proportional-position control system. Producing proportional plus rate plus reset action, it is the ultimate in control for processes requiring a high degree of stability in spite of frequent and severe load changes. It is designed for use with a proportional-type motorized operator, employing a 100 ohm feedback slidewire, to position a fuel valve air, damper or similar device.

In addition to the basic three-function control action, two important extras are incorporated—reset windup inhibition and Load Line-out. Both are standard features in the Series 692 Controllers. Reset windup inhibition is particularly desirable in processes where large step increases or decreases occur. It insures that reset action will always be fully operative in the area of the control set point. Load Line-out is an exclusive feature which effectively prevents overshoot in batch processes. It is achieved by superimposing a manually set signal on the automatic reset action, varying the reset reference voltage.

Bulletin 1312 DB 5 from the Barber-Colman Company, Industrial Instruments Division, Rockford, Ill., U.S.A., contains complete detailed specifications.

New literature on the Barber-Colman Chronotrol, an *Automatic Program Control Unit*, describes the many basic models of the instrument which are available for process and laboratory control applications.

The Chronotrol provides automatic program control, either linear or non-linear, for the regulation of predetermined time-variable cycles. After an initial start, a program is maintained without adjustment or supervision throughout a complete operation cycle.

Ask for new literature 1214 DB 2-2, Barber-Colman Company, Industrial Instruments Division, Rockford, Ill., 61101, U.S.A.

A new eight-page brochure gives detailed illustrations and description of Nester/Faust's Model 850 Prepkromatic *Automatic Preparative Gas Chromatograph.*

Prepkromatic 850 is used to separate and collect pure components for a multitude of scientific requirements. Features such as annular "bi-wall" columns patented by Nester/Faust, automatic injection system, unique peak selection computer, efficient high temperature exit-collection valve and a versatile computer programmer are described at length. Accessories to the Prepkromatic are also listed. Copies of the brochure may be obtained by writing to Nester/Faust, Instruments Products Div., 2401 Ogletown Road, Newark, Dela. 19711, U.S.A.

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See also PC section.

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## Gas chromatography

### 2. FUNDAMENTALS, THEORY AND GENERAL

*2b. Gas–solid systems*

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### 3. TECHNIQUES I

*3a. Detectors*

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*3b. Column performance and filling studies*

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*3c. Apparatus, accessories and materials for GC*

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# ENRICHISSEMENT DES ISOTOPES DU CARBONE ET DU NÉON PAR CHROMATOGRAPHIE EN PHASE GAZEUSE. IÈRE PARTIE

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## INTRODUCTION

En 1933, TAYLOR, GOULD ET BLEAKNEY[1] ont mis en évidence un effet de sélection isotopique dans les phénomènes d'adsorption. Ces auteurs constatent que la teneur en deutérium d'un mélange hydrogène–deutérium est supérieure après adsorption sur du charbon actif à 77°K.

Cette technique de séparation isotopique fut néanmoins laissée dans l'ombre pendant près de vingt ans. Et il faut attendre le développement de la chromatographie analytique, vers 1956, pour que la chromatographie isotopique conduise à quelques résultats positifs.

Depuis, de nombreuses publications rapportent les résultats d'expériences nouvelles en ce domaine. Mais il faut remarquer que la majeure partie des travaux publiés, dont nous citons plus loin les principaux, concerne les isotopes de l'hydrogène sous forme d'élément ou en combinaison dans les hydrocarbures.

La séparation des isotopes de l'hydrogène par chromatographie de déplacement a été étudiée par: GLUECKAUF ET KITT[2–5], SALMON[6], CHADWICK[7], OHKOSHI, TENMA, FUJITA ET KWAN[8], THOMAS ET SMITH[9], PHILLIPS, OWENS ET HAMLIN[10,11], BOTTER, MENES, TISTCHENKO ET DIRIAN[12].

La séparation des isotopes et des isomères de l'hydrogène par chromatographie d'élution a été réalisée par: MOORE ET WARD[13,14], OHKOSHI, TENMA, FUJITA ET KWAN[15,16], SMITH, HUNT ET CARTER[17–22], GANT ET YANG[23], VAN HOOK ET EMMETT[24], FURUYAMA ET KWAN[25–27], BOTTER, DE LA PERRIERE, TISTCHENKO ET CERCY[28–31], ERB[32], BACHMAN, BECHTOLD ET CREMER[33], SHIPMAN[34], MOHNKE ET SAFFERT[35], PHILLIPS ET OWENS[36], KING[37], VENUGOPALAN ET KUTSCHKE[38].

Enfin, WILZBACH ET RIESZ[39], dans le cas du cyclohexane, FALCONER, CVETANOVIČ ET DUNCAN[40,41] dans le cas de l'isopentane et de l'éthylène, LIBERTI, CARTONI ET BRUNER[42,43] pour le benzène, ROOT, LEE ET ROWLAND[44], VAN HOOK et KELLY[45], GANT ET YANG[46] pour différents alcanes, ont réalisé la séparation des hydrocarbures simples et de leurs homologues deutérés. Enfin, très récemment SWEELEY et ses collaborateurs[47] ont mis en évidence la séparation chromatographique partielle de molécules organiques lourdes marquées en deutérium.

La séparation des isotopes d'éléments autres que l'hydrogène a été peu étudiée. Seuls GLUECKAUF, BARKER ET KITT[48] dans le cas du néon et BRUNER, CARTONI ET LIBERTI[49] pour l'oxygène ont abordé le problème. Nous nous sommes proposés d'étudier la séparation par chromatographie d'élution des isotopes 20 et 22 du néon et 12 et

13 du carbone. Pour ce dernier cas, l'oxyde de carbone a été choisi comme phase gazeuse[50,51].

## GRANDEURS MESURÉES ET MODÈLE

*Cas d'un seul constituant*

Au lieu de mesurer des pics de concentration à l'aide d'un détecteur différentiel, on peut considérer les intégrales de ces pics qui sont à débit de gaz vecteur constant, proportionnelles au nombre de molécules introduites dans la colonne. Le principe d'une expérience de chromatographie est simple: l'introduction de l'échantillon dans la

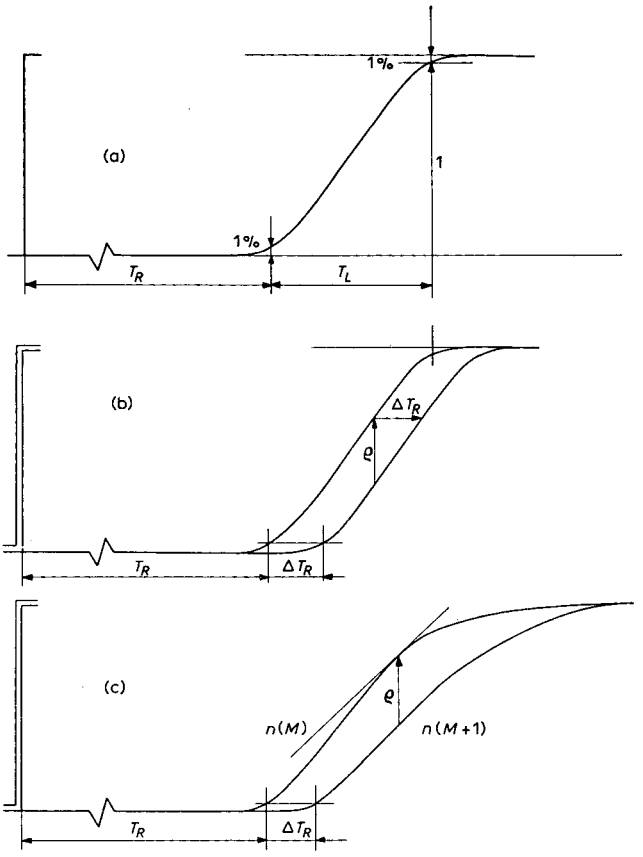


Fig. 1. Pics chromatographiques intégrés: (a) intégrale de pic chromatographique; (b) intégrales de deux isotopes théoriques; (c) intégrales de deux isotopes réels.

colonne est analogue à la création d'un signal rectangulaire que le dispositif chromatographique transmet après un temps $T_R$ (temps de rétention), sous forme d'un signal déformé d'une quantité $T_L$ (temps de déformation) (Fig. 1a). Le paramètre $T_L$ est arbitrairement choisi comme l'intervalle de temps nécessaire au signal pour passer de 1 % à 99 % de sa valeur finale.

Pour mesurer l'efficacité d'une colonne chromatographique, nous définissons une nouvelle grandeur, le facteur de résolution $R$: c'est le rapport du temps de rétention au temps de déformation:

$$R = \frac{T_R}{T_L} \tag{1}$$

Le temps de rétention d'un composé dépend des conditions expérimentales et en particulier du débit du gaz vecteur. On fait alors apparaître une grandeur corrigée $V_R$ que l'on appelle le volume de rétention: c'est le produit du temps de rétention $T_R$ (grandeur se rapportant à l'échantillon) par le débit volumique du gaz vecteur $D$ (grandeur qui ne se rapporte pas à l'échantillon) ramené aux conditions de température et de pression de fonctionnement dans la colonne. L'expression $V_R$ en fonction des grandeurs mesurées seulement est:

$$V_R = T_R \cdot D \cdot \frac{T_c}{T_0} \cdot {}^3/_2 \cdot \frac{(P_1/P_0)^2 - 1}{(P_1/P_0)^3 - 1} \tag{2}$$

Le facteur:

$${}^3/_2 \cdot \frac{(P_1/P_0)^2 - 1}{(P_1/P_0)^3 - 1}$$

est appelé facteur de JAMES ET MARTIN[52].

Les causes de la déformation $T_L$ du pic sont multiples: la diffusion de l'échantillon dans le gaz vecteur entraîne un élargissement du pic, de façon symétrique par rapport à la concentration maximum et égal à

$$\frac{[2\gamma \mathscr{D} T_R(1 + C)]^{\frac{1}{2}}}{\mu}$$

où $\mathscr{D}$ est le coefficient de diffusion de l'échantillon dans le gaz vecteur, $\gamma$ le coefficient d'obstruction, $C$ la constante d'adsorption[53] et $\mu$ la vitesse du gaz vecteur; la courbure des isothermes fait que les zones d'échantillon à forte concentration avancent plus vite que les zones d'échantillon à faible concentration et provoquent ainsi une "traînée" des pics[54-56]. Cette traînée peut être aussi provoquée par le retard à l'établissement de l'équilibre d'adsorption[57]. Enfin, la géométrie de remplissage entraîne une variation de la vitesse d'avancement avec le chemin parcouru[58].

*Cas de deux constituants*

Considérons le cas de deux constituants que le dispositif chromatographique ne sépare que d'une petite quantité $\Delta T_R$ telle que $\Delta T_R/T_R$ soit de l'ordre de quelques pour mille. C'est le cas par exemple de deux isotopes d'un même corps. La Fig. 1b représente le cas théorique où les signaux obtenus à la sortie de la colonne sont deux courbes déduites l'une de l'autre par une translation égale à $\Delta T_R$. A partir de ces deux courbes on peut mesurer une quantité associée à l'idée de séparation isotopique et qui s'introduit de la manière suivante.

Supposons que l'on injecte à l'entrée de la colonne chromatographique un échantillon contenant le même nombre $n(M)$ et $n(M + 1)$ de deux isotopes de masse $M$ et $M + 1$ et que l'on recueille le gaz effluent à la sortie de la colonne chromatographique. La composition isotopique du mélange recueilli dépend du temps $t$ où l'on

fait le prélèvement. A l'apparition du signal $n(M)$, on n'a que des isotopes correspondants à la masse $M$; à partir de l'apparition du signal $n(M + 1)$ on obtient des molécules $n(M + 1)$. Comme l'échantillon initial avait un rapport isotopique 1, aussi longtemps que $dn(M)/dt > dn(M + 1)/dt$ le mélange effluent est plus riche en isotope M que l'échantillon initial. Pour $dn(M)/dt < dn(M + 1)/dt$ au contraire, le mélange effluent est plus pauvre en isotope $M$. Au temps $t$, pour lequel $dn(M)/dt = dn(M + 1)/dt$, le mélange effluent a la même composition que le mélange initial. C'est le temps $t_m$, où la différence des courbes $n(M) - n(M + 1)$ passe par un maximum:

$$\frac{d}{dt}\left[n(M) - n(M + 1)\right]_{t_m} = 0 \tag{3}$$

On appelle facteur d'enrichissement $\rho$ la valeur maximale de la différence $n(M) - n(M + 1)$ des courbes correspondant à chaque isotope. C'est cette grandeur maximale que l'on mesurera par la suite pour caractériser l'efficacité de la séparation chromatographique. Deux cas particuliers sont à noter: d'une part, le cas de la séparation parfaite où l'on obtient deux pics franchement séparés. Alors $\rho$ est maximal et égal à 1; d'autre part, le cas où les deux pics ne sont ni séparés, ni déformés: alors $\rho$ est nul.

Dans le cas où l'échantillon introduit ne contient pas le même nombre de molécules de masse $M$ et $M + 1$, on définit le facteur d'enrichissement par le maximum de la quantité:

$$\rho_t = \frac{1}{n(M)} \int_0^t dn(M) - \frac{1}{n(M + 1)} \int_0^t dn(M + 1) \tag{4}$$

Le facteur $\rho$ représente donc dans tous les cas, le maximum de la différence des fractions des populations des isotopes de masse $M$ et $M + 1$ issues de la colonne.

Dans une expérience de chromatographie isotopique, on cherche à obtenir des échantillons enrichis ou appauvris par rapport à l'un des isotopes. On peut décider de couper le pic effluent en deux parties seulement. Pour cela on constitue deux fractions l'une formée de tout l'effluent collecté jusqu'au temps $t_m$ où la différence des intégrales est maximale, l'autre fraction contenant le reste de l'effluent à partir de $t_m$. On obtient alors facilement les rapports isotopiques $R^-$ et $R^+$ de ces deux fractions à partir de:

$$R^- = R_0 \left(1 - \frac{n(M)}{n^-(M)} \cdot \rho\right)$$

$$R^+ = R_0 \left(1 + \frac{n(M)}{n^+(M)} \cdot \rho\right) \tag{5}$$

où $R_0$ représente le rapport isotopique initial de l'échantillon:

$$R_0 = \frac{n(M + 1)}{n(M)}$$

et $n^-(M)$ et $n^+(M)$, le nombre de molécules sorties dans les intervalles de temps $(0 - t_m)$ et $(t_m - \infty)$.

*Modèle*

Les molécules de l'échantillon sont, dans la colonne, partagées entre la phase

gazeuse et la phase stationnaire. De façon plus précise, si $N_{PS}$ et $N_{PG}$ sont les concentrations des molécules d'échantillon dans la phase stationnaire et dans la phase gazeuse, on caractérise l'efficacité de l'adsorbant par le rapport:

$$C = \frac{N_{PS}}{N_{PG}} \tag{6}$$

la constante $C$ est appelée constante d'adsorption.

Au fur et à mesure de l'avancement de l'échantillon dans la colonne, les molé-

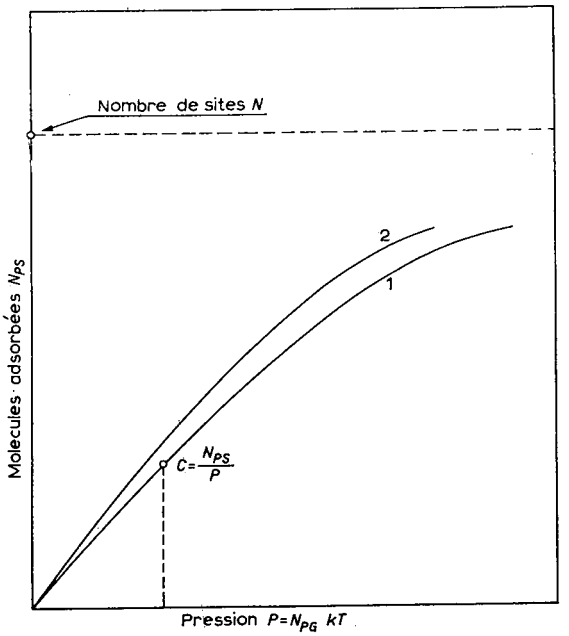


Fig. 2. Schéma de principe des isothermes d'adsorption d'un mélange de molécules d'espèce différente (1 et 2).

cules se trouvent tantôt dans la phase solide tantôt dans la phase gazeuse, mais réparties toujours de facon que le rapport $N_{PS}/N_{PG}$ soit constant, si l'isotherme d'adsorption correspondante est une droite.

On montre que la constante $C$ est liée au volume de rétention $V_R$ par la relation:

$$C = \frac{V_R - V_{PG}}{V_{PS}} \tag{7}$$

en désignant par $V_{PG}$ et $V_{PS}$ les volumes de phase gazeuse et de phase stationnaire. Dans le cas de deux corps voisins, on a en négligeant $V_{PG}$ devant $V_R$:

$$\frac{\Delta C}{C} = \frac{\Delta V_R}{V_R} = \frac{\Delta T_R}{T_R} \tag{8}$$

L'écart relatif des temps de rétention est égal à l'écart relatif des constantes d'adsorption.

On peut relier de façon simple cet écart relatif des constantes d'adsorption au facteur d'enrichissement $\rho$ précédemment défini. On déduit en effet de la Fig. 1b la relation suivante:

$$\frac{\rho}{\Delta T_R} = \frac{1}{T_L}$$

soit:

$$\rho = R \cdot \frac{\Delta T_R}{T_R}$$

qui s'écrit en tenant compte de la formule (8):

$$\rho = R \frac{\Delta C}{C} \tag{9}$$

Ainsi donc, ce modèle simplifié fait apparaître la participation de deux grandeurs à l'enrichissement isotopique, d'une part, la sélectivité isotopique de l'adsorbant $\Delta C/C$, d'autre part, un paramètre lié à l'efficacité analytique de la colonne remplie d'un adsorbant donné, qui est le pouvoir de résolution $R$.

La formule (9) a été établie dans le cas où les deux pics isotopiques sont seulement décalés dans le temps par une translation simple (cas de la Fig. 1b). L'expérience montre que les pics isotopiques sont à la fois décalés et déformés l'un par rapport à l'autre (Fig. 1c). Dans ce cas, nous admettons que la formule (9) reste encore valable en première approximation.

*Adsorption gaz–solide*

A une température donnée, l'équilibre entre un gaz à la pression $P$ et un solide adsorbant est traduit par une isotherme d'adsorption du type représenté sur la Fig. 2, où l'ordonnée est $N_{PS}$, nombre de molécules de gaz adsorbées par unité de volume de phase stationnaire. Pour les fortes valeurs de $P$, $N_{PS}$ tend vers une limite $N$ qui est égale à la concentration de centres adsorbants de la phase stationnaire.

D'après LANGMUIR[59] et FOWLER[60,61], l'isotherme d'adsorption est représentée par l'équation:

$$N_{PS} = N \cdot \frac{P \cdot F(T,M)}{1 + P \cdot F(T,M)} \tag{10}$$

où

$$F(T,M) = \left(\frac{h^2}{2\pi M}\right)^{3/2} \cdot (kT)^{-5/2} \cdot \exp\left(-E/kT\right) \tag{11}$$

$M$ est la masse de la molécule, $T$ la température, $E$ l'énergie d'adsorption, $h$ et $k$ respectivement les constantes de PLANCK et BOLTZMANN. On ne tient pas compte dans les relations précédentes des fonctions de partition correspondant aux mouvements de

vibration et de rotation de la molécule qui sont sensiblement égales à l'état gazeux et à l'état adsorbé dans le domaine de température que l'on considère.

FOWLER a également proposé des équations des isothermes d'adsorption d'un mélange de deux corps. Dans ce cas, si ces corps sont notés 1 et 2, les relations deviennent:

$$N_{PS}^1 = N \cdot \frac{P_1 \cdot F_1}{1 + P_1 \cdot F_1 + P_2 \cdot F_2}$$

$$N_{PS}^2 = N \cdot \frac{P_2 \cdot F_2}{1 + P_1 \cdot F_1 + P_2 \cdot F_2} \tag{12}$$

ou encore:

$$N_{PS}^1 = N \cdot \frac{N_{PG}^1 \cdot f_1}{1 + N_{PG}^1 \cdot f_1 + N_{PG}^2 \cdot f_2} \tag{13}$$

$$N_{PS}^2 = N \cdot \frac{N_{PG}^2 \cdot f_2}{1 + N_{PG}^1 \cdot f_1 + N_{PG}^2 \cdot f_2}$$

avec $f = kTF$.

Dans les équations ci-dessus, la courbure des isothermes résulte de la présence des termes $N_{PG} \cdot f$ au dénominateur, présence qui traduit le fait que les sites déjà occupés n'interviennent pas dans la probabilité de capture d'une molécule gazeuse. Dans le cas de deux molécules, on a ainsi le même facteur au dénominateur, soit: $1 + N_{PG}^1 \cdot f_1 + N_{PG}^2 \cdot f_2$, le nombre de sites occupés intervenant seul et non le type de molécules les occupant.

On considère maintenant le cas de deux isotopes dont les isothermes ne sont pas confondues, c'est-à-dire pour lesquelles $f_2$ est différent de $f_1$ et on cherche à établir une relation simple liant les fonction $f$ à l'écart relatif des constantes $\Delta C/C$.

Si on pose:

$$C_1 = \frac{N_{PS}^1}{N_{PG}^1} \qquad C_2 = \frac{N_{PS}^2}{N_{PG}^2}$$

on déduit la relation suivante:

$$\frac{\Delta C}{C} = \frac{C_2 - C_1}{C_1} = \frac{f_2 - f_1}{f_1} = -\,{}^3/_2 \cdot \frac{M_2 - M_1}{M_1} - \frac{1}{kT}(E_2 - E_1) \tag{14}$$

Ainsi la courbure des isothermes entraîne une variation de la constante d'adsorption avec la pression d'équilibre. La formule (10) montre en effet que la constante d'adsorption diminue quand la pression d'équilibre augmente. Mais pour un mélange de deux corps voisins, des isotopes par exemple, l'écart relatif $\Delta C/C$ reste constant quelle que soit la pression et égal à l'écart que l'on obtiendrait si les deux isothermes étaient linéaires.

La formule (14) fait apparaître deux termes dont le premier est négatif et le second certainement positif. En effet, les énergies d'adsorption $E$ sont négatives, et généralement les particules lourdes sont plus liées que les particules légères.

Dans la pratique, si le terme positif l'emporte sur le terme négatif, les particules lourdes sortiront les dernières de la colonne. Mais il peut se produire une inversion de l'ordre de sortie des pics en fonction de la température, comme l'ont par exemple, remarqué expérimentalement LIBERTI, CARTONI ET BRUNER dans le cas du mélange $CH_4$–$CD_4$[62].

Notons enfin que la séparation isotopique ne fait intervenir les énergies de liaison que par leur différence, mais que la valeur absolue de ces énergies détermine la possibilité même de la chromatographie en phase gazeuse. Une énergie d'adsorption trop grande singifie en effet que les particules piégées ne sont pas reémises de leur piège en des temps raisonnables par rapport au temps de l'expérience.

## APPAREILLAGE ET TECHNIQUE DE MESURE

L'analyse chromatographique avec détecteur classique n'est possible que si les constituants sont franchement séparés par la colonne. Le spectromètre de masse au contraire, permet de doser en continu la répartition des isotopes dans un pic au fur et à mesure de leur apparition. On a donc associé au chromatographe un spectromètre de

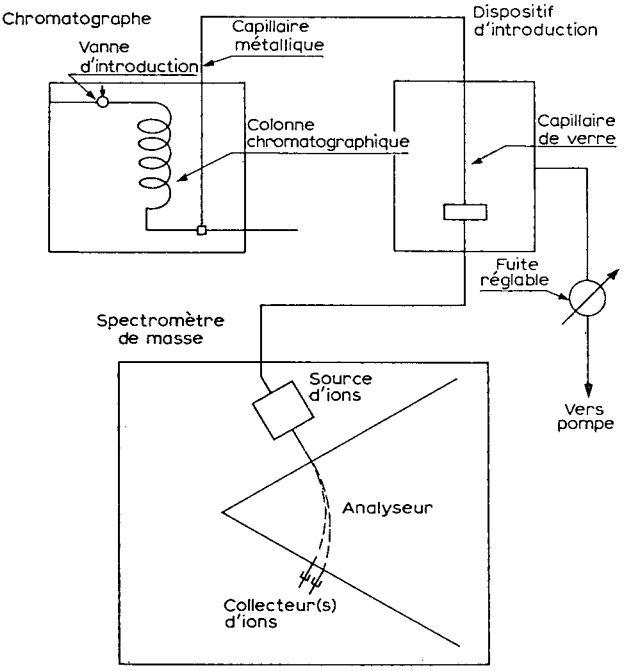


Fig. 3. Schéma de principe de l'appareillage.

masse selon le schéma représenté sur la Fig. 3. Le spectromètre est soit équipé de deux collecteurs d'ions soit d'un seul. Dans le premier cas, le champ magnétique de l'analyseur est réglé pour que les ions de masse $M$ et $M + 1$ soient reçus simultanément par chacun des collecteurs. Dans le second cas, on utilise un dispositif de balayage magnétique qui permet de recevoir alternativement sur le collecteur unique les isotopes que

l'on veut étudier. Ainsi, tout se passe comme si deux détecteurs étaient branchés en parallèle à la sortie de la colonne chromatographique. On peut alors déceler des enrichissements isotopiques qui échappent aux autres moyens de détection.

*Chromatographe*

Les chromatographes utilisés ont été construits aux laboratoires de Chimie Physique de la SNPA. L'introduction des échantillons dans la colonne se fait par une vanne pneumatique à disque de distribution. La colonne est placée dans une enceinte thermostatée et sa température est réglée entre — 80°C et 250°C. Enfin, un catharomètre est branché à la sortie de la colonne en parallèle avec le spectromètre de masse. Le débit moyen de gaz vecteur est de l'ordre de 1 cm³/sec. On le mesure avec un débitmètre à film de savon.

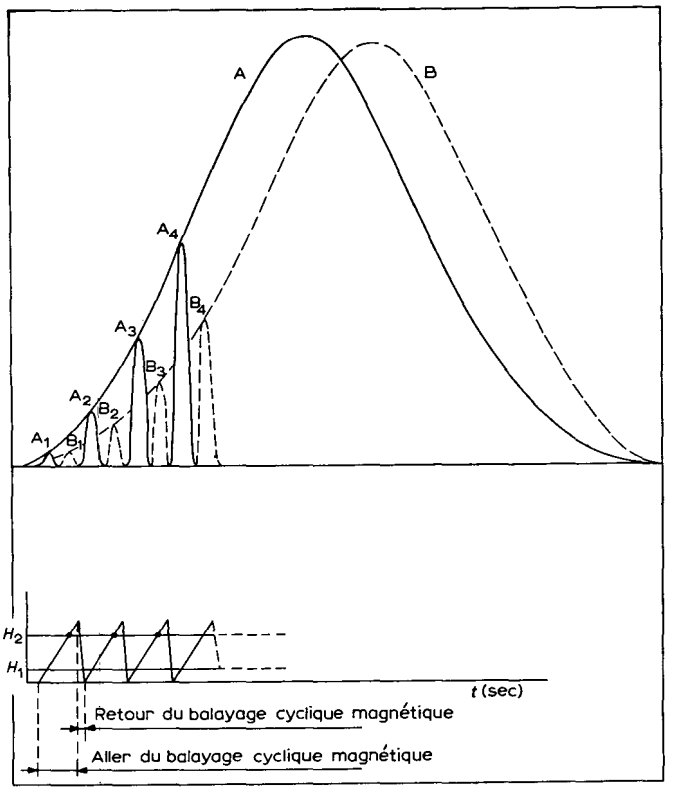


Fig. 4. Représentation schématique des pics chromatographiques donnés par le spectromètre de masse à collecteur unique équipé d'un balayage cyclique magnétique.

*Spectromètre de masse*

On utilise deux spectromètres de masse commerciaux ATLAS CH 4 et C.S.F SM 100. Tous les deux sont équipés de source d'ions classique (type NIER).

L'ATLAS CH 4 travaille en double collection; le champ magnétique analyseur est fixé de telle manière que les faisceaux d'ions correspondants aux deux isotopes

étudiés tombent simultanément sur les deux collecteurs d'ions. Chacun de ces derniers est suivi d'un amplificateur à courant continu.

Le C.S.F. SM 100 travaille en simple collection. Pour procéder aux mesures isotopiques, on fait varier le champ magnétique autour de deux valeurs correspondant aux isotopes étudiés à l'aide d'un balayage cyclique magnétique. De cette façon, on reçoit alternativement les ions de chaque isotope sur le collecteur unique; celui-ci est suivi d'un amplificateur à courant continu et d'un dispositif électronique original de traitement d'information.

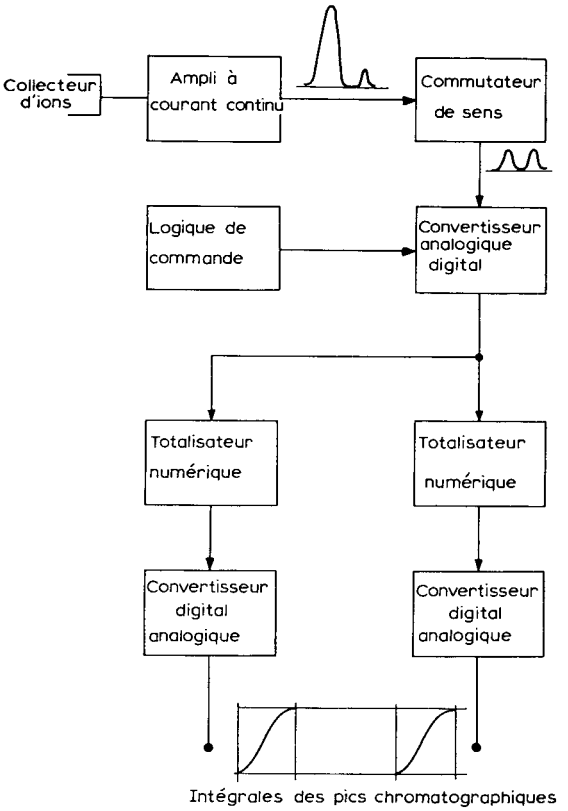


Fig. 5. Montage électronique intégral.

L'information donnée par le spectromètre de masse à collecteur unique équipé d'un balayage cyclique magnétique est schématisée sur la Fig. 4. Le collecteur voit défiler les isotopes A et B et l'on enregistre au cours du premier cycle de balayage par exemple, les signaux $A_1$ et $B_1$. La succession des signaux $A_1$, $A_2$... $A_p$ reproduit la variation d'amplitude du pic chromatographique du constituant A. La somme des amplitudes $A_1 + A_2 + \ldots + A_p$ est proportionnelle à la surface de ce pic, c'est-à-dire au nombre de molécules A qui ont traversé le détecteur. Il en est de même pour le constituant B.

Le schéma de principe du dispositif de traitement d'information que l'on a

réalisé est reproduit sur la Fig. 5. Un commutateur de sensibilités permet de normaliser les intégrales des signaux A et B. Un convertisseur analogique–digital convertit à chaque instant les signaux en nombre d'impulsions proportionnel à leur hauteur. Deux totalisateurs numériques comptent les nombres totaux des impulsions qui représentent les intégrales des pics chromatographiques. Chaque totalisateur est suivi d'un convertisseur digital analogique. L'ensemble du dispositif est commandé par une logique dont les variables sont engendrées soit par les pics eux-mêmes, soit par un signal de synchronisation à partir du courant magnétisant de balayage cyclique[51].

On trouve dans la littérature récente[47] la description d'un montage voisin de notre dispositif qui permet d'enregistrer le maximum de chaque pic isotopique. Toutefois notre montage semble présenter l'avantage de la digitalisation des mesures qui permet de résoudre facilement l'intégration des pics et d'autre part celui d'un balayage continu des masses étudiées qui évite le réglage délicat du spectromètre de masse au maximum de chaque pic isotopique.

Dans la suite de cet exposé, on appellera "montage différentiel" le dispositif utilisant les deux collecteurs d'ions, et "montage intégral" celui qui n'en utilise qu'un seul.

*Liaison entre le chromatographe et le spectromètre de masse*

La sortie de la colonne chromatographique est au voisinage de la pression atmosphérique; le débit gazeux à cet endroit est de l'ordre du centimètre cube par seconde. La source du spectromètre de masse fonctionne à une pression inférieure à $10^{-4}$ torr et le débit du gaz à cette pression est de l'ordre de $10^{3}$ cm$^3$/sec, c'est à dire $10^{-4}$ cm$^3$/sec à la pression atmosphérique. Le dispositif qui relie le chromatographe au spectromètre de masse doit procéder à une détente dans le rapport $10^{7}$ et ne prélever, en fait qu'une fraction de l'ordre de $10^{-4}$ de flux gazeux de la colonne chromatographique. En dernier lieu, dans le type d'expérience qu'on s'est proposé de faire, il convient que le système d'introduction n'apporte pas de perturbations dans les rapports isotopiques.

Différents systèmes ont été proposés par des auteurs[63–67]. Le système original que nous avons construit est représenté schématiquement sur la Fig. 3. Il comporte essentiellement deux étages. Un premier capillaire métallique ($L$ = 1 m, $\varnothing$ = 0.2 mm) est branché sur le chromatographe en amont du détecteur à fils chauds. Il conduit une fraction des effluents à une chambre de détente intermédiaire où débouche un capillaire en verre ($L$ = 30 mm, $\varnothing$ = 0.01 mm) directement raccordé à la source. Un système de pompage relié par une fuite réglable à la chambre de détente intermédiaire permet de modifier la pression dans cette chambre et par suite de régler le débit des gaz qui s'écoulent dans le spectromètre de masse. La Fig. 6 donne le schéma détaillé de la réalisation de ce système d'introduction.

On a vérifié que ce système d'introduction ne modifie pas la forme des pics, dont la surface est une fonction linéaire du volume d'échantillon introduit dans la colonne chromatographique[50]. Enfin, le système n'introduit pas d'erreur dans la mesure des écarts des rapports isotopiques.

*Mesure du facteur d'enrichissement*

La relation (4) exprimant le facteur d'enrichissement $\rho$ peut se mettre sous les deux formes suivantes:

$$\rho_t = \frac{\int_0^t [R_0 \mathrm{d}n(M) - \mathrm{d}n(M+1)]}{\int_{-\infty}^{+\infty} R_0 \mathrm{d}n(M)} \qquad (4 \text{ bis})$$

$$\rho_t = \frac{\int_0^t R_0 \mathrm{d}n(M) - \int_0^t \mathrm{d}n(M+1)}{\int_{-\infty}^{+\infty} R_0 \mathrm{d}n(M)} \qquad (4 \text{ ter})$$

Ainsi, on peut effectuer la mesure de $\rho$ par deux voies différentes: à partir de l'intégrale de la différence instantanée des deux signaux isotopiques (relation 4 bis) ou au moyen de la différence instantanée de deux intégrales (relation 4 ter).

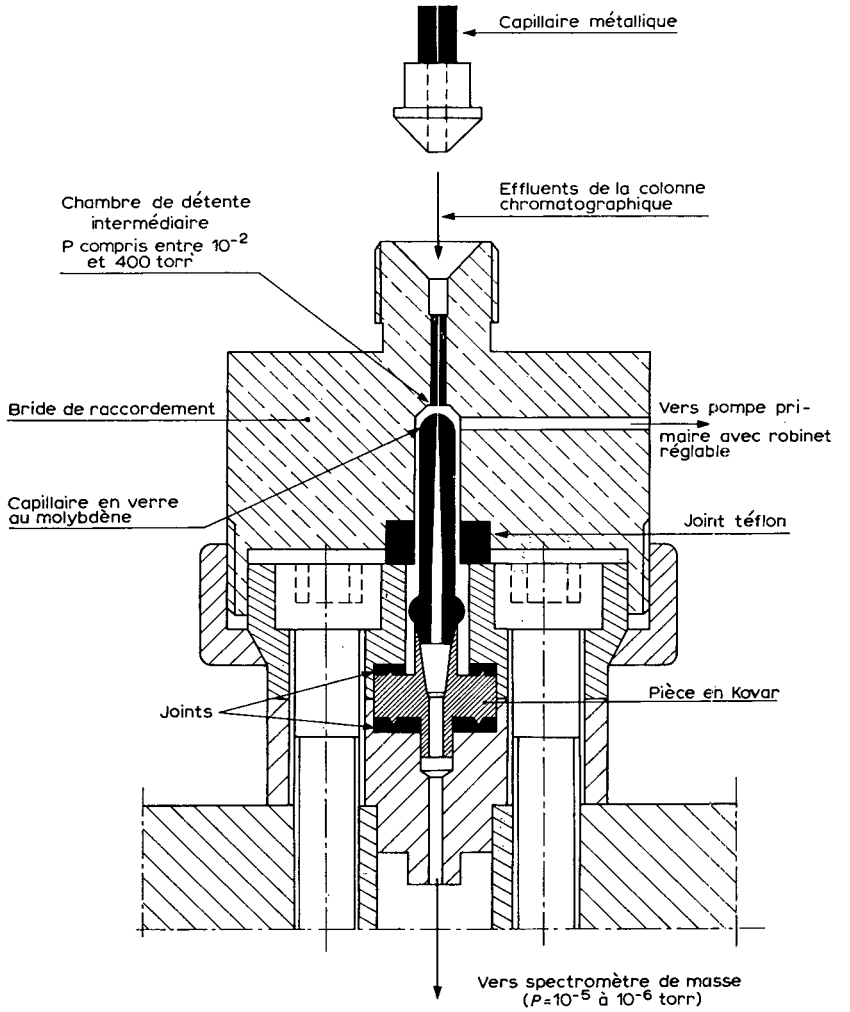


Fig. 6. Schéma détaillé du système d'introduction continue.

On a utilisé les deux méthodes. La première dans le cas du "montage différentiel"; l'autre avec le "montage intégral".

*Montage différentiel.* Dans ce montage, représenté sur la Fig. 7 les deux amplificateurs associés aux collecteurs sont couplés par l'intermédiaire du potentiomètre P et de la résistance $r'(M+1)$. A l'entrée de l'amplificateur A on retranche au courant $I(M+1)$, la fraction:

$$p\,\frac{r(M)}{r'(M+1)}$$

du courant $I(M)$, d'où le signal de sortie $\Delta V$:

$$\Delta V = r(M+1)\left[p\,\frac{r(M)}{r'(M+1)}\,I(M) - I(M+1)\right]$$

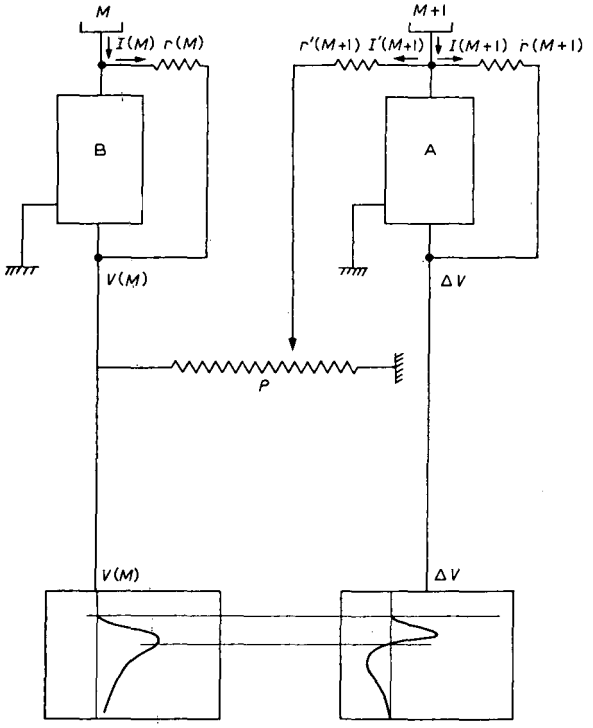


Fig. 7. Montage électronique différentiel.

On règle P sur l'échantillon non enrichi de rapport isotopique $R_0$ de manière que $\Delta V$ soit nul. Dans ces conditions on a avec les notations précédemment définies:

$$R_0 = \frac{I(M+1)}{I(M)} = p\,\frac{r(M)}{r'(M+1)}$$

On enregistre alors pour un échantillon enrichi:

$$\Delta V = r(M+1)\,[R_0 \cdot I(M) - I(M+1)]$$

et simultanément sur l'autre canal:

$$V(M) = r(M) \cdot I(M)$$

Les courants ioniques $I$ étant proportionnels au nombre de molécules instantanées, la formule (4 bis) s'écrit alors, lorsque $r(M + 1) = r'(M + 1)$:

$$\rho = \frac{\int_0^{t_m} \Delta V}{P \int_{-\infty}^{+\infty} V(M)} \tag{15}$$

Pratiquement, les mesures de $\rho$ sont faites à partir de l'intégration manuelle des courbes de la Fig. 7.

*Montage intégral.* Dans ce montage, on oppose les tensions proportionnelles aux intégrales des deux pics isotopiques (Fig. 5) de façon a en faire la différence. On enregistre alors directement cette grandeur qui est proportionnelle au facteur d'enrichissement $\rho$.

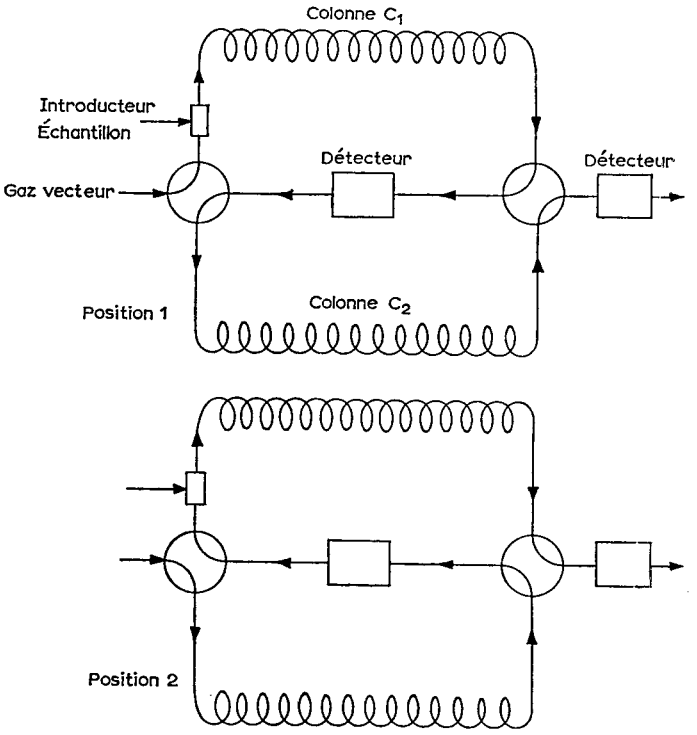


Fig. 8. Schéma de principe de l'appareil de récyclage.

*Appareillage annexe*

Cet appareil construit dans notre laboratoire[68] permet de faire circuler un grand nombre de fois un même échantillon sur deux colonnes de chromatographie identiques.

Son principe est voisin de celui d'autres appareils mis au point par différents auteurs[44]. Au moyen d'une double vanne de recyclage pouvant prendre deux positions, on recycle autant de fois qu'on le veut, un même échantillon sur deux colonnes $C_1$ et $C_2$ (Fig. 8). Selon la position 1, montrée sur cette figure, l'échantillon gazeux va pénétrer dans la colonne $C_2$ quand il sortira de la colonne $C_1$. Selon la position 2 de la vanne de recyclage, à la sortie de la colonne $C_2$, l'échantillon pénétrera dans la colonne $C_1$. L'originalité de l'appareil réside dans le fait que la double vanne de recyclage est construite dans le même bloc d'acier qui contient de plus le détecteur à fils chauds intermédiaires. Les deux vannes sont donc commutées ensemble. De plus, les deux détecteurs possèdent des réglages de zéro indépendants.

## CONCLUSION

L'utilisation d'un spectromètre de masse comme détecteur d'une colonne de chromatographie permet de suivre en continu le rapport isotopique le long d'un pic chromatographique. On a utilisé cette technique pour étudier l'enrichissement des isotopes du carbone et du néon par chromatographie en phase gazeuse. Les résultats et leur comparaison avec le modèle proposé sont décrits dans la IIème partie de ce travail.

## RÉSUMÉ

La chromatographie de l'oxyde de carbone et du néon nous a permis d'obtenir un enrichissement important des isotopes 12 et 13 du carbone et 20 et 22 du néon, sans atteindre toutefois la séparation complète, en une seule étape.

Pour suivre la répartition des isotopes dans le pic chromatographique, on a associé un spectromètre de masse à un chromatographe à gaz. Le spectromètre fournit en continu deux informations correspondant aux concentrations des deux isotopes choisis.

A partir de ces informations, on définit une nouvelle grandeur qui caractérise une expérience de chromatographie isotopique. On montre que cette grandeur est le produit du pouvoir de résolution qui caractérise l'efficacité analytique de la colonne et de l'écart relatif des constantes d'adsorption des deux isotopes.

## SUMMARY

The chromatography of carbon monoxide and neon has enabled us to obtain a considerable enrichment of the isotopes carbon-12 and 13 and neon-20 and 22 without attaining a complete separation in one single stage.

In order to determine the distribution of the isotopes in the chromatographic peak, a mass spectrometer is used in conjunction with the gas chromatograph. The spectrometer gives continuous information concerning the concentrations of the two isotopes.

As a result of this information, a new magnitude, $\rho$, the factor of enrichment, is defined. This factor $\rho$ is the product of the resolving power which characterises the analytical efficacy of the column and the relative difference of adsorption constants of the two isotopes.

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# ENRICHISSEMENT DES ISOTOPES DU CARBONE ET DU NÉON PAR CHROMATOGRAPHIE EN PHASE GAZEUSE. IIe PARTIE

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## ENRICHISSEMENT DES ISOTOPES 12 ET 13 DU CARBONE

L'enrichissement des isotopes du carbone a été obtenu par chromatographie de l'oxyde de carbone sur tamis moléculaire 5A. Celui-ci, fabriqué par Union Carbide, est réduit en poudre de granulométrie homogène, puis déshydraté à 200° sous courant d'hélium pendant 15 h.

*Mise en évidence de l'enrichissement isotopique*

Les conditions chromatographiques sont les suivantes: colonne de longueur 3 mètres, de diamètre intérieur 4 mm et remplie de tamis moléculaire dont les grains ont entre 0.35 mm et 0.42 mm; température 0°; volume d'échantillon 2 cm³ TPN, débit de gaz vecteur (hélium) 1 cm³/sec.

La Fig. 1 est la reproduction photographique de l'enregistrement simultané du signal $V(28)$ correspondant à la molécule $^{12}C^{16}O$ et de la quantité $\Delta V = r(M + 1)[R_0 I(28) - I(29)]$ où $I(28)$ et $I(29)$ sont les courants d'ions correspondant aux masses

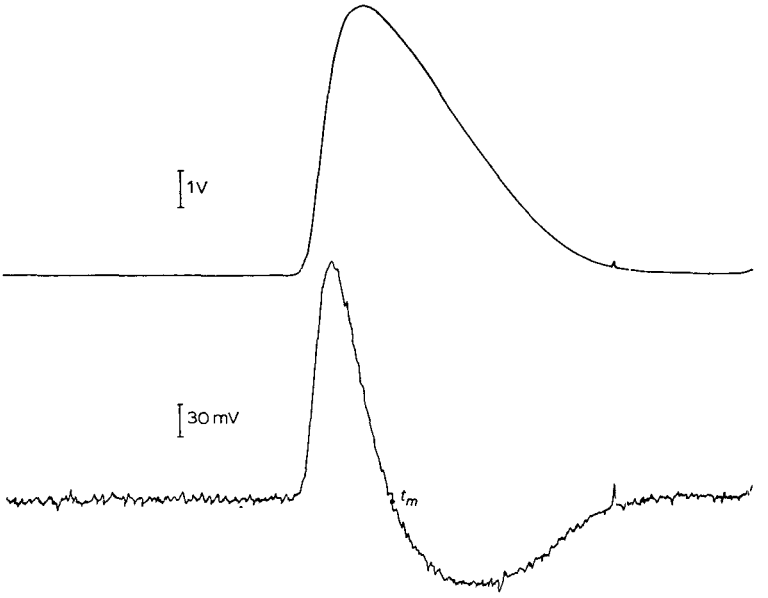


Fig. 1. Reproduction photographique d'un enregistrement simultané de $V(28)$ et $\Delta V$.

28 et 29, $R_0$ le rapport isotopique initial et $r(M+1)$ une constante[1]. L'enregistrement différentiel présente d'abord une partie positive: les molécules légères sortent de la colonne avec une faible avance sur les molécules lourdes. Au temps $t_m$, le rapport isotopique instantané est égal au rapport initial; ensuite les lourds "traînant" plus que les légers, on a un enrichissement en isotopes lourds tout le long de la branche négative de la courbe.

La Fig. 2 donne dans les mêmes conditions expérimentales, la photographie de l'enregistrement donné par le "dispositif intégral" de mesure du facteur d'enrichissement. La partie supérieure montre les intégrales $\int_0^t R_0\,dn\,(28)$ et $\int_0^t dn\,(29)$ qui représentent à chaque instant le nombre total de molécules $^{12}C^{16}O$ et $^{13}C^{16}O$ sortis depuis le début de l'expérience. Ces intégrales partent de zéro et atteignent leurs valeurs maximales rendues identiques grâce au commutateur de sensibilités. Chaque palier que l'on observe sur ces deux courbes représente un cycle de balayage magnétique. La courbe inférieure représente la différence des deux intégrales précédentes, c'est à dire la variation dans le temps du facteur d'enrichissement. La mesure expérimentale de $\rho$ conduit, pour les deux dispositifs de mesures, à des résultats reproductibles. Dans le cas des Fig. 1 et 2 on trouve:

$$\rho = 0.085 \pm 0.005^*$$

La plus petite valeur de $\rho$ que l'on peut détecter est de l'ordre de 0.001 avec le "montage différentiel" et de 0.005 avec le "montage intégral". Rappelons que, par définition, ce facteur d'enrichissement est nul s'il n'y a aucune séparation et égal à 1 si la séparation est complète.

*Température*

On a opéré dans les conditions suivantes: colonne de longueur 2 m, de diamètre intérieur 4 mm; granulométrie du tamis comprise entre 0.35 et 0.42 mm; volume d'échantillon 2 cm³ TPN; débit d'hélium: 1 cm³/sec.

On a porté sur la Fig. 3 la variation du logarithme du volume de rétention en fonction de l'inverse de la température absolue. On constate que cette variation est linéaire. La pente de la droite permet de calculer l'énergie d'adsorption $E$.[2] On trouve:

$$E = 6{,}160 \text{ cal.mol}^{-1} = 0.27 \text{ eV}$$

A titre indicatif, DOBYCHIN et ses collaborateurs[3] ont trouvé dans les mêmes conditions:

$$E = 6{,}670 \text{ cal.mol}^{-1} = 0.29 \text{ eV}$$

---

* Nous n'avons pas tenu compte dans l'interprétation des résultats de la variation de rapport isotopique de l'oxygène 17. Les molécules de masse (28) et (29) de l'oxyde de carbone sont en effet constituées à partir des isotopes $^{12}C$ et $^{13}C$ du carbone et des isotopes $^{16}O$ et $^{17}O$ de l'oxygène. Le rapport $n(29)/n(28)$ des molécules de masse (29) et (28) s'écrit:

$$n(29)/n(28) = n(^{13}C^{16}O + {}^{12}C^{17}O)/n(^{12}C^{16}O) = n(^{13}C^{16}O)/n(^{12}C^{16}O) + \\ + n(^{12}C^{17}O)/n(^{12}C^{16}O)$$

Pour passer des rapports expérimentaux $n(29)/n(28)$ au rapport $n(^{13}C^{16}O)/n(^{12}C^{16}O)$ il suffit donc de retrancher au rapport expérimental le rapport $n(^{12}C^{17}O)/n(^{12}C^{16}O)$. Pour du CO non enrichi, celui-ci est égal à 0.00037 si bien qu'on peut négliger l'influence d'une éventuelle variation de teneur en oxygène 17.

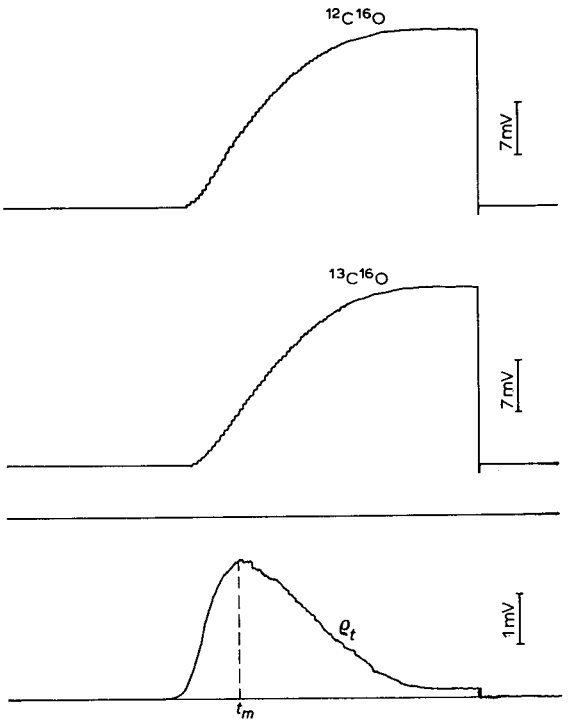


Fig. 2. Reproduction des enregistrements des intégrales des pics correspondant aux isotopes $^{12}C^{16}O$ et $^{13}C^{16}O$ et du facteur d'enrichissement $\rho$.

La Fig. 4 donne la variation des facteurs d'enrichissement et de résolution en fonction de la température. Quand la température décroit de 100° à —30°, le facteur d'enrichissement varie fortement: il passe de 1 % à 9 % alors que le facteur de résolution $R$ passe de 1 à 4. D'autre part, il semble que le facteur de résolution passe par un maximum, faiblement marqué.

*Longueur de la colonne*

On a travaillé à 24° avec du tamis moléculaire 5A broyé entre 0.42 mm et 0.50 mm. Le débit de gaz vecteur a été maintenu constant et égal à 2 cm³/sec.

La Fig. 5 montre que les facteurs d'enrichissement et de résolution varient dans le même sens. On constate un accroissement rapide, mais néanmoins non linéaire de ces deux grandeurs avec la longueur de la colonne. Cette variation du facteur de résolution est connue en chromatographie conventionnelle[2].

*Section de la colonne*

On mesure le volume de rétention, les facteurs d'enrichissement et de résolution donnés par des colonnes de longueur identique et de section croissante.

Les conditions expérimentales sont les suivantes: échantillon d'oxyde de carbone de 4 cm³ TPN; température de la colonne 25°; débit de gaz vecteur 3 cm³/sec.

Les colonnes sont en cuivre de longueur de 3 mètres et de section comprise entre

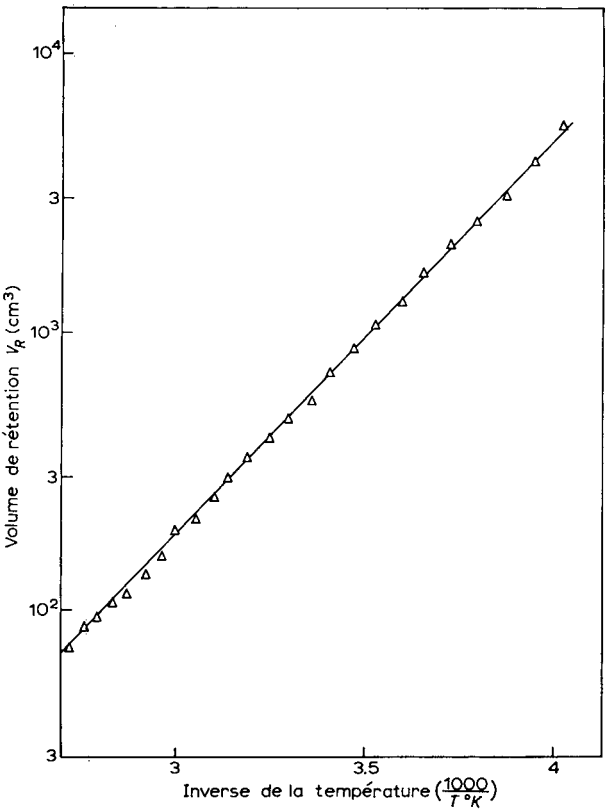


Fig. 3. Variation du volume de rétention en fonction de la température.

3 et 113 mm². Le poids de la phase stationnaire (diamètre des grains compris entre 0.225 et 0.35 mm) nécessaire à leur remplissage est compris entre 6.2 et 224.5 g. On a porté sur le Tableau I les valeurs du volume de rétention, des facteurs de résolution et d'enrichissement pour différentes valeurs de la section des colonnes.

On constate que le volume de rétention augmente avec la section de la colonne. Par contre, le volume de rétention ramené à l'unité de section, reste sensiblement constant. On vérifie ainsi la formule de JAMES ET MARTIN[4] qui relie le volume de rétention à la section de la colonne:

$$V_R = \frac{aL}{V_e/V_v} = aL\left[1 + C\left(\frac{V_{PS}}{V_{PG}}\right)\right]$$

Le Tableau I donne également les valeurs de $\rho$ et $R$ pour différentes valeurs de la section de la colonne. On constate dans les deux cas, l'existence d'un maximum; celui-ci semble difficile à interpréter car à la fois le débit de gaz vecteur par unité de section droite de la colonne, et le volume relatif de l'échantillon par rapport au volume d'adsorbant ont été modifiés.

Pour lever cette ambiguïté, on a étudié les variations des facteurs de résolution

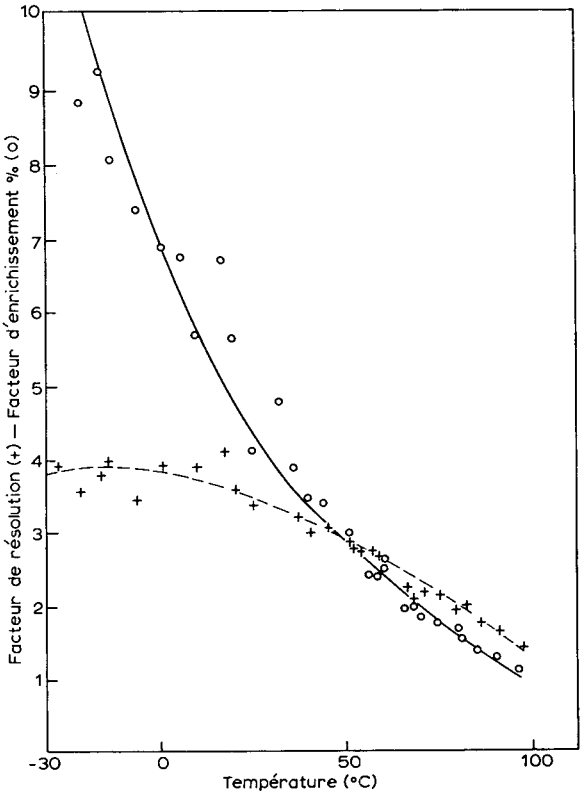


Fig. 4. Variation du facteur de résolution et du facteur d'enrichissement en fonction de la température.

TABLEAU I

VOLUME DE RÉTENTION, FACTEUR DE RÉSOLUTION ET D'ENRICHISSEMENT POUR DES COLONNES DE DIAMÈTRE CROISSANT

| *Section* ($mm^2$) | *Poids de tamis* (*g*) | *Volume de rétention* ($cm^3$) | *Volume de rétention par unité de section* | *Facteur de résolution R* | *Facteur d'enrichissement* $\rho$ (%) |
|---|---|---|---|---|---|
| 3.14 | 6.2 | 454 | 114 | 1.7 | 3.4 |
| 12.5 | 25 | 1543 | 128 | 2.7 | 5.0 |
| 28.3 | 56 | 2696 | 95 | 3.0 | 4.7 |
| 50 | 100 | 5288 | 106 | 3.9 | 4.5 |
| 78.5 | 156 | 7591 | 97 | 4.0 | 2.1 |
| 113 | 224.5 | 10170 | 90 | 2.7 | 1.1 |

et d'enrichissement en fonction de la section droite de la colonne en utilisant un volume d'échantillon et un débit de gaz vecteur proportionnels au volume de phase stationnaire[5]. Les résultats sont portés sur la Fig. 6. On constate que dans ces conditions, le facteur de résolution varie peu. Le facteur d'enrichissement décroît légèrement

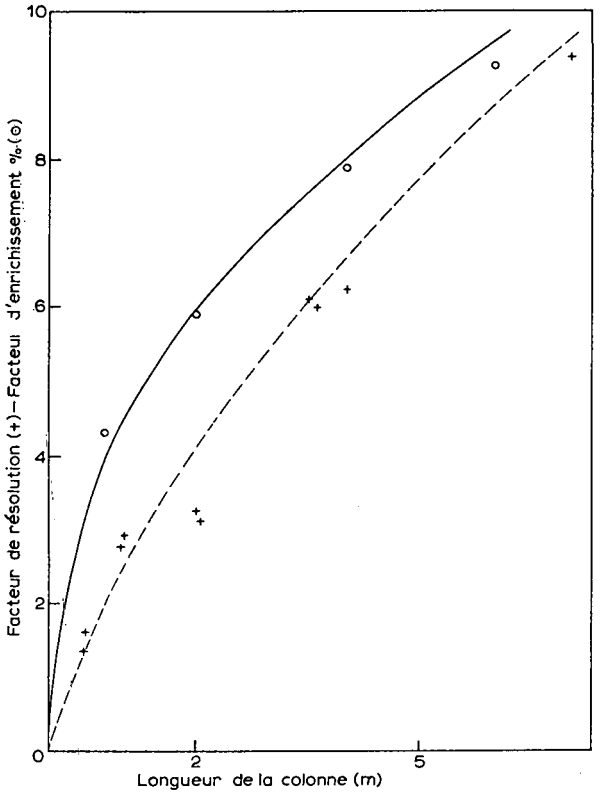


Fig. 5. Variation du facteur de résolution et du facteur d'enrichissement en fonction de la longueur de la colonne.

quand le diamètre des colonnes augmente. A titre indicatif, on passe de $R = 3.2$ et $\rho = 4\%$ avec une quantité de CO de 0.8 cm³ (température et pression normales) sur une colonne de 2 mm de diamètre, à $R = 2.5$ et $\rho = 3\%$ avec 33 cm³ de CO sur une colonne de 12 mm de diamètre. Ainsi, dans le domaine étudié, on peut de cette façon augmenter de 40 fois le volume traité de CO et ne diminuer que d'environ 25 % les facteurs de résolution et d'enrichissement.

*Volume d'échantillon*

Les conditions sont les suivantes:

Colonne: longueur, 2 m; diamètre intérieur, 4 mm; diamètre des grains de tamis, compris entre 0.42 et 0.50 mm; température de la colonne, 0°.

Gaz vecteur: nature, hélium; débit, 1 cm³/sec.

On a utilisé des volumes d'échantillon compris entre 1 et 20 cm³. La Fig. 7 reproduit les pics de concentration obtenus sur les chromatogrammes. Alors que le début des pics est d'autant plus rapproché de l'introduction que le volume de l'échantillon est grand, les courbes d'élution comprises entre le sommet et la fin des pics sont confondues. Ce résultat s'explique de façon simple si l'on considère la courbure des isothermes d'adsorption[6].

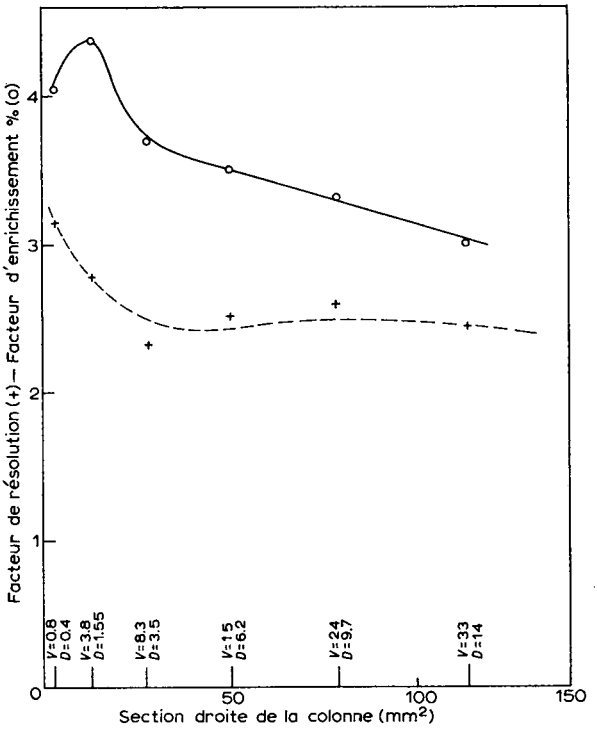


Fig. 6. Variation du facteur de résolution et du facteur d'enrichissement en fonction de la section de la colonne (volume d'échantillon ($V$) et débit ($D$) proportionnels au volume de la colonne).

Les variations des facteurs de résolution et d'enrichissement en fonction du volume de CO introduit sont représentées sur la Fig. 8. On constate que ces deux grandeurs varient de façon approximativement parallèle et décroissent quand le volume d'échantillon augmente.

*Débit de gaz vecteur*

L'augmentation du débit de gaz vecteur diminue le temps de rétention, mais n'affecte pas le volume de rétention.

La Fig. 9 donne la variation de $\rho$ et de $R$ en fonction du débit de gaz vecteur. Les conditions expérimentales sont les suivantes: Colonne: longueur 2 m, diamètre intérieur 4 mm; phase stationnaire: tamis moléculaire 5A broyé entre 0.42 et 0.50 mm; température de la colonne: 0°; volume d'échantillon 2 cm³.

Le gaz vecteur est de l'hélium; on fait varier son débit (mesuré à la pression et à la température ambiantes) de 0.3 cm³/sec à 7 cm³/sec.

On constate que le facteur de résolution passe par un maximum peu marqué.

Quant au facteur d'enrichissement, il diminue légèrement quand on augmente le débit. Il passe de $\rho = 5.5$ % pour $D = 0.5$ cm³/sec à $\rho = 4$ % pour $D = 7$ cm³/sec. On peut donc diminuer d'un ordre de 10 le temps d'expérimentation et limiter la perte relative sur l'enrichissement à 25 %.

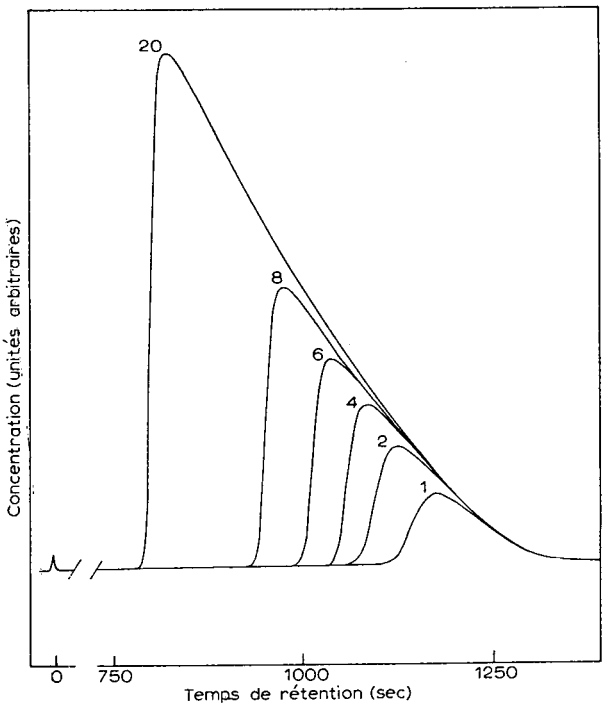


Fig. 7. Forme et position des pics en fonction du volume d'échantillon.

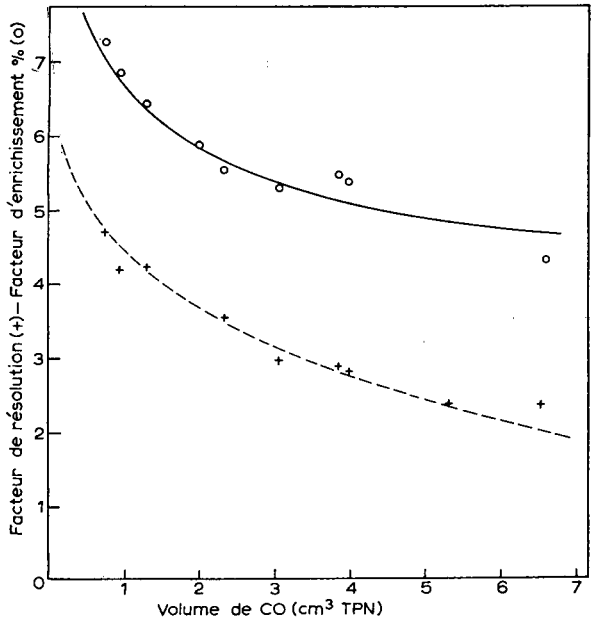


Fig. 8. Variation du facteur de résolution et du facteur d'enrichissement en fonction du volume d'échantillon.

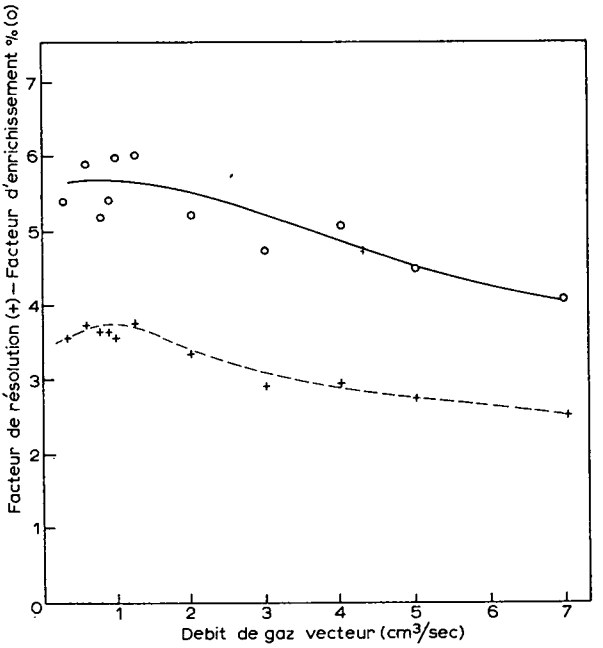


Fig. 9. Variation du facteur de résolution et du facteur d'enrichissement en fonction du débit de gaz vecteur.

*Nature du gaz vecteur*

On opère à zéro degré centigrade sur la colonne précédente. Le débit du gaz vecteur (hélium, argon et néon) est maintenu à 3 cm³/sec pour chaque gaz. On a porté sur le Tableau II les valeurs du volume de rétention, des facteurs de résolution et d'enrichissement.

TABLEAU II

VOLUME DE RÉTENTION, FACTEURS DE RÉSOLUTION ET D'ENRICHISSEMENT POUR DIFFERENTS GAZ VECTEUR

| *Nature du gaz vecteur* | *Volume de rétention* | *Facteur de résolution* | *Facteur d'enrichissement* (%) |
|---|---|---|---|
| Hélium | 2200 cm³ | 2.35 | 5 |
| Néon | 1800 cm³ | 1.87 | 4.8 |
| Argon | 1900 cm³ | 2.09 | 4.5 |

On constate que ces grandeurs sont, dans les conditions expérimentales où l'on s'est situé, peu affectées par la nature du gaz vecteur.

*Échantillons enrichis en carbone-13*

On a mesuré le facteur d'enrichissement d'échantillons d'oxyde de carbone

ayant un rapport isotopique supérieur au rapport naturel (Fournisseurs: Merck, Sharp et Dohme, Montréal).

Les conditions sont les suivantes: Longueur colonne: 2 m; diamètre intérieur 4 mm; diamètre des grains de tamis: compris entre 0.50 et 0.59 mm; débit de gaz vecteur (hélium): 2 $cm^3$/sec; volume d'échantillon 2 $cm^3$.

Les résultats sont portés sur le Tableau III. On constate que le facteur d'enrichissement ne varie pas avec la proportion de carbone-13 contenue dans le mélange isotopique initial.

TABLEAU III

VOLUME DE RÉTENTION, FACTEURS DE RÉSOLUTION ET D'ENRICHISSEMENT D'ÉCHANTILLONS ENRICHIS EN CARBONE-13

| | $R_0 = 1.1\%$ | $R_0 = 20.8\%$ | $R_0 = 93.6\%$ |
|---|---|---|---|
| Volume de rétention ($cm^3$) | 3083 | 3243 | 3260 |
| Facteur de résolution | 2.11 | 2.23 | 2.29 |
| Facteur d'enrichissement (%) | 4.58 | 4.40 | 4.48 |

Enfin, en sectionnant le pic chromatographique et en recueillant les deux fractions, on trouve:

$$R^- = 86\,\%$$
$$R^+ = 99\,\%$$

avec:

$$R_0 = 93.5\,\%$$

Les formules (5) établies dans la première partie de ce travail[1] conduisent à:

$$R^- = 85.3\,\%$$
$$R^+ = 98.2\,\%,$$

en bon accord avec le résultat précédent.

*Enrichissement maximal*

En choisissant convenablement la valeur des différents paramètres on a trouvé:

$$\rho = 13\,\%$$
$$R^+ = 0.77\,R_0$$
$$R^- = 1.30\,R_0 \text{ avec } R_0 = 0.011$$

Les conditions étaient les suivantes:

Colonne. longueur, 6 m; diamètre intérieur, 4 mm. Gaz vecteur: débit, 1 $cm^3$/sec. Échantillon: volume, 2.2 $cm^3$. Durée de l'expérience: 2 h 30 min. Température: —25°.

Pour atteindre un enrichissement isotopique plus grand on a utilisé le chromatographe spécial permettant de recycler les échantillons sur deux colonnes de chromato-

graphie[1]. On a opéré à 25° avec des colonnes de 3 mètres, de section 4 mm, un débit de gaz vecteur de 1 $cm^3$/sec et un volume d'échantillon de 2.2 $cm^3$. Dans ces conditions, le temps de passage dans chaque colonne est de 20 minutes. On obtient ainsi un enrichissement de 17.5 % en moins de deux heures, par passages successifs sur cinq colonnes. Toutefois, le nombre de passages est limité ici à 5 par le pouvoir de résolution: au sixième passage l'échantillon occupe la totalité d'une colonne élémentaire et l'on ne peut plus manoeuvrer la vanne de recyclage.

Un artifice permet d'augmenter le pouvoir de résolution. Au cours de chaque cycle une des colonnes est refroidie à zéro degré jusqu'à ce que l'échantillon ait pénétré en totalité dans la colonne. Dans ces conditions, la largeur du pic à l'entrée de cette colonne est inférieure à la largeur qu'avait le pic à la sortie de la colonne précédente, plus chaude. Dès que l'échantillon a pénétré en totalité dans la colonne froide, on ramène la température à 25°. On peut ainsi obtenir que l'échantillon traverse un nombre important de fois les deux colonnes de chromatographie.

En utilisant 2 colonnes de 3 mètres de longueur, un volume d'échantillon de 0.5 $cm^3$ et un débit de 1 $cm^3$/sec, on a obtenu un enrichissement maximal de 31 %. Il a nécessité 16 passages de l'échantillon sur un élément de colonne, soit une longueur totale traversée de 48 m. Le temps de rétention total était de 7 h 30 min.

## ENRICHISSEMENT DES ISOTOPES 20 ET 22 DU NÉON

On a étudié l'enrichissement isotopique du néon dans différentes conditions expérimentales et sur plusieurs phases stationnaires solides. Rappelons tout d'abord que le néon naturel comporte trois isotopes $^{20}Ne$, $^{21}Ne$ et $^{22}Ne$ dans les proportions: 90.5, 0.28 et 9.21. On s'est intéressé dans ce qui suit, aux seuls isotopes de masse 20 et 22. Dans tous les cas, on trouve que l'isotope 20 a un temps de rétention inférieur à l'isotope 22.

TABLEAU IV

FACTEURS DE RÉSOLUTION ET D'ENRICHISSEMENT POUR DIFFERENTES CONDITIONS EXPÉRIMENTALES

| *Température* (°K) | *Volume d'échantillon* ($cm^3$) | *Débit de gaz vecteur* ($cm^3$/sec) | *Facteur de résolution R* | *Facteur d'enrichissement* ρ (%) |
|---|---|---|---|---|
| 103 | 6 | 0.3 | 2.7 | 2.3 |
| 77 | 6 | 0.5 | 5.2 | 6 |
| 77 | 6 | 2 | 3.7 | 4.4 |
| 77 | 6 | 4 | 3.3 | 3.7 |
| 77 | 2 | 2 | 5.8 | 6.3 |
| 77 | 4 | 2 | 4.9 | 5.4 |
| 77 | 8 | 2 | 3.5 | 4.1 |

Le Tableau IV rassemble les valeurs du facteur de résolution et du facteur d'enrichissement isotopique dans différentes conditions de température, de volume et de débit de gaz vecteur. La phase stationnaire utilisée est le tamis moléculaire 5A; le diamètre de ses grains compris entre 0.42 et 0.50 mm. On constate que les valeurs obtenues sont du même ordre de grandeur que dans le cas des isotopes du carbone.

D'autre part, le Tableau V reproduit les résultats obtenus sur du tamis molécu-

laire, du charbon actif et du silica gel. On travaille à la température de l'azote liquide sur des colonnes de 6 mètres de longueur, (granulométrie 0.35–0.42 mm) avec un débit de gaz vecteur hélium de 1 $cm^3$/sec et un volume d'échantillon de 0.8 $cm^3$.

On constate que c'est le tamis moléculaire qui conduit aux meilleurs résultats. Par ailleurs, les résultats trouvés avec le charbon actif sont très voisins de ceux obtenus par GLUECKAUF[7,8] par chromatographie frontale.

TABLEAU V

TEMPS DE RÉTENTION, FACTEURS DE RÉSOLUTION ET D'ENRICHISSEMENT DU NEON SUR DIFFÉRENTES PHASES STATIONNAIRES SOLIDES

| *Phase stationnaire* | *Temps de rétention (sec)* | *Facteur d'enrichissement ρ (%)* | *Facteur de résolution R* |
|---|---|---|---|
| Tamis moléculaire 5 A | 2220 | 8.6 | 8.3 |
| Charbon actif | 5520 | 5.3 | 7.0 |
| Silica gel | 1560 | 2.3 | 2.6 |

COMPARAISON AVEC LE MODÈLE

Rappelons les formules établies à partir du modèle simplifié décrit dans la première partie:

$$\rho = R\frac{\Delta C}{C} \tag{1}$$

$$\frac{\Delta C}{C} = -{}^3/_2 \cdot \frac{M_2 - M_1}{M_1} - \frac{1}{kT}(E_2 - E_1) \tag{2}$$

Dans le cas de CO comme dans celui du néon, les isotopes lourds sortent toujours les derniers, ce qui montre que, dans le domaine de température étudié, le terme $-1/kT(E_2 - E_1)$ est positif et supérieur au terme faisant intervenir les masses, explicitement:

$${}^3/_2 \cdot \frac{M_2 - M_1}{M_1} .$$

Lorsqu'on examine les résultats expérimentaux, on constate que c'est la température qui a la plus grande influence sur le rapport $\rho/R$. Ainsi, les Tableaux IV et V pour le néon et les Figs. 5, 6, 8, et 9 pour l'oxyde de carbone montrent que $\rho$ et $R$ varient de façon sensiblement proportionnelle lorsqu'on fait varier la longueur de la colonne, sa section, le volume et le rapport isotopique initial de l'échantillon, le débit de gaz vecteur et sa nature, alors que la Fig. 4 montre que le rapport $\rho/R$ décroît très rapidement quand la température croît.

Pour le néon, on peut calculer à partir des valeurs de $\rho$ et $R$ des tableaux IV et V, les valeurs de $\Delta C/C$. On trouve:

$$\frac{\Delta C}{C}\ (77°K - \text{Tamis moléculaire}) = 1.17 \cdot 10^{-2}$$

$$\frac{\Delta C}{C}\ (103°K - \text{Tamis moléculaire}) = 0.84 \cdot 10^{-2}$$

$$\frac{\Delta C}{C}\ (77°K - \text{Charbon actif}) = 0.75 \cdot 10^{-2}$$

$$\frac{\Delta C}{C}\ (77°K - \text{Silica gel}) = 0.87 \cdot 10^{-2}$$

Pour l'oxyde de carbone, la Fig. 4 permet de tracer de façon continue la variation de $\Delta C/C$ en fonction de la température. Cette variation est représentée sur la Fig. 10.

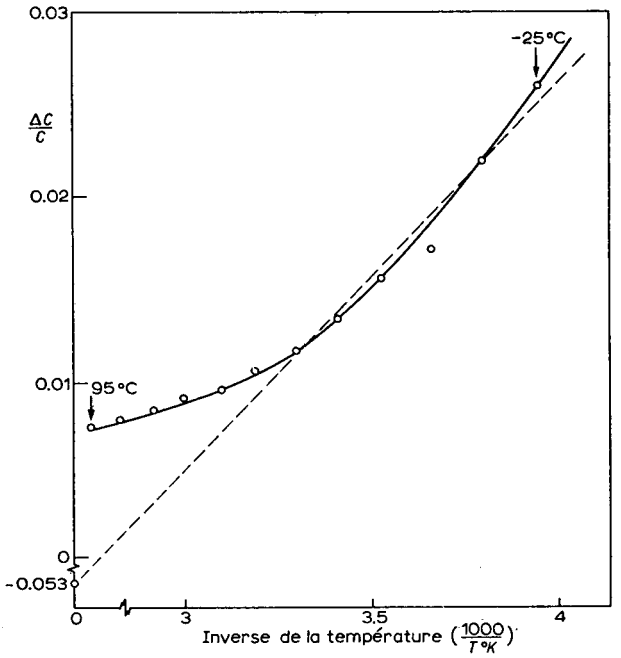


Fig. 10. Variation de $\Delta C/C$ en fonction de la température.

Notons les deux points extrêmes de la courbe:

$$\frac{\Delta C}{C} = 0.0280 \text{ à } -25°C$$

et

$$\frac{\Delta C}{C} = 0.0072 \text{ à } 95°C$$

On constate qu'en accord avec le modèle, $\Delta C/C$ croît avec l'inverse de la température, mais ne suit pas exactement une loi linéaire. On a néanmoins tracé une droite

passant au voisinage des points expérimentaux ainsi que par le point représentatif du terme constant de la formule (2):

$$-{}^3/_2 \cdot \frac{M_2 - M_1}{M_1} = -0.053$$

Cette droite figurée en traits discontinus est représentée par l'équation:

$$\frac{\Delta C}{C} = -0.053 + \frac{19.8}{T} \qquad (3)$$

En identifiant les relations (2) et (3), on déduit:

$$E_2 - E_1 = 39 \text{ cal} \cdot \text{mol}^{-1} = 1.7 \cdot 10^{-3} \text{ eV}$$

Par ailleurs, on avait trouvé à partir de la Fig. 3:

$$E_1 = 6{,}170 \text{ cal} \cdot \text{mol}^{-1} = 270 \cdot 10^{-3} \text{ eV}$$

d'où l'on déduit:

$$\frac{E_2 - E_1}{E_1} = 6 \cdot 10^{-3}$$

On peut rapprocher ces valeurs des valeurs moyennes trouvées par BASMADJIAN[9] pour les isotopes de l'hydrogène sur tamis moléculaire 5A, à savoir:

$$E_{H_2} = 74 \cdot 10^{-3} \text{ eV}$$

et

$$E_{D_2} - E_{H_2} = 6.7 \cdot 10^{-3} \text{ eV}.$$

d'où

$$\frac{E_{D_2} - E_{H_2}}{E_{H_2}} = 9.0 \cdot 10^{-2}$$

Ainsi donc, l'écart relatif des énergies d'adsorption des isotopes du carbone est 15 fois inférieur à celui des isotopes de l'hydrogène.

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RÉSUMÉ

Dans un précédent article, on décrivait l'appareillage utilisé pour suivre la répartition des isotopes dans les pics chromatographiques, dans le cas où le dispositif

chromatographique ne sépare pas complètement les isotopes. On définissait une grandeur nouvelle, le facteur d'enrichissement $\rho$, qui caractérise une expérience de chromatographie isotopique. Selon le modèle proposé, cette grandeur est le produit du pouvoir de résolution qui caractérise l'efficacité analytique de la colonne et de l'écart relatif des constantes d'adsorption des deux isotopes.

Dans le présent travail, on donne les résultats obtenus dans l'enrichissement des isotopes 12 et 13 du carbone et 20 et 22 du néon par chromatographie en phase gazeuse sur phases stationnaires solides. La température et la longueur de la colonne influent fortement sur l'enrichissement isotopique alors que le volume d'échantillon, le débit de gaz vecteur et la section de la colonne entraînent des variations moins importantes. De plus, l'enrichissement est indépendant de la valeur du rapport isotopique initial de l'échantillon. On montre enfin que le modèle proposé rend bien compte des résultats.

SUMMARY

In a preceding article, the apparatus used for following the separation of isotopes in a chromatographic peak was described, for the case where the chromatograph does not completely separate the isotopes. A new magnitude, the enrichment factor $\rho$, is defined, which characterises an isotopic chromatographic experiment. According to the model proposed, this factor is the product of the resolving power, which characterises the analytical efficacy of the column and the relative difference of the adsorption constants of the two isotopes.

In the present paper, the results obtained by gas chromatography on solid stationary phases, for the enrichment of carbon-12 and 13 isotopes and neon-20 and 22 are given. The temperature and length of the column have a considerable influence on the isotopic enrichment, while the sample volume, the rate of flow of the carrier gas and the cross section of the column are less important. In addition, the enrichment is independent of the initial isotopic concentrations in the sample. Finally, it is shown that the proposed model agrees well with the results.

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# STATISTICAL MOMENTS THEORY OF GAS–SOLID CHROMATOGRAPHY

## DIFFUSION CONTROLLED KINETICS

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A new theory of non-equilibrium linear gas–solid chromatography (GSC) following the work of GIDDINGS[1] and MCQUARRIE[2] has been recently published[3–5]. This theory is based on the "exact" solution of a system of partial differential equations describing the mass-balance in the gas chromatographic column. The solution is first obtained in Laplace coordinates, from which statistical moments can be evaluated. By means of a suitable function, these statistical moments can be arranged in a series, which presents the solution of the original system of differential equations in normal coordinates. The expressions for statistical moments up to the fifth order have been calculated[3]. In this paper only the first four moments defined by the following equations will be used.

$$\mu_1 = \left(\frac{L}{u} + \frac{2D_p}{u^2}\right)[1 + \phi K_c(1 + K_n)] \tag{1}$$

$$\mu_2 = \left(\frac{2D_pL}{u^3} + \frac{8D_p^2}{u^4}\right)[1 + \phi K_c \quad (1 + K_n)]^2 +$$

$$+ \left(\frac{2L}{u} + \frac{4D_p}{u^2}\right)\phi K_c\left[\frac{R^2}{D_r}\frac{(1 + K_n)^2}{v(v+2)} + \frac{\phi(1 + K_n)^2}{H_c} + \frac{K_n}{H_n}\right] \tag{2}$$

$$\mu_3 = \left(\frac{12\,D_p^2L}{u^5} + \frac{64\,D_p^3}{u^6}\right)[1 + \phi K_c(1 + K_n)]^3 +$$

$$+ \left(\frac{12\,D_pL}{u^3} + \frac{48\,D_p}{u^4}\right)\phi K_c[1 + \phi K_c(1 + K_n)]\cdot$$

$$\left[\frac{R^2}{D_r}\frac{(\ + K_n)^2}{v(v+2)} + \frac{\phi(1 + K_n)^2}{H_c} + \frac{K_n}{H_n}\right] + \left(\frac{6L}{u} + \frac{12\,D_p}{u^2}\right)\phi K_c\cdot$$

$$\left[\left(\frac{R^2}{D_r}\right)^2\frac{2(1 + K_n)^3}{v^2(v+2)(v+4)} + \frac{R^2}{D_r}\frac{2(1 + K_n)}{v(v+2)}\left(\frac{\phi(1 + K_n)^2}{H_c} + \frac{K_n}{H_n}\right) +\right.$$

$$\left. + \frac{\phi^2(1 + K_n)^3}{H_c^2} + \frac{2\phi K_n(1 + K_n)}{H_cH_n} + \frac{K_n}{H_n^2}\right] \tag{3}$$

$$\mu_4 = \left(\frac{12\,D_p^2L^2}{u^6} + \frac{216\,D_p^3L}{u^7} + \frac{960\,D_p^4}{u^8}\right)[1 + \phi K_c(1 + K_n)]^4 +$$

$$+ \left(\frac{24\,D_pL^2}{u^4} + \frac{288\,D_p^2L}{u^5} + \frac{960\,D_p^3}{u^6}\right)[1 + \phi K_c(1 + K_n)]^2\left[\frac{R^2}{D_r}\frac{(1 + K_n)^2}{v(v + 2)} +\right.$$

$$\left. + \frac{\phi(1 + K_n)^2}{H_c} + \frac{K_n}{H_n}\right]^2 + \left(\frac{12\,L^2}{u^2} + \frac{72\,D_pL}{u^3} + \frac{144\,D_p^2}{u^4}\right)\phi^2K_c^2\left[\frac{R^2}{D_r}\frac{(1 + K_n)^2}{v(v + 2)} +\right.$$

$$\left. + \frac{\phi(1 + K_n)^2}{H_c} + \frac{K_n}{H_n}\right] + \left(\frac{48\,D_pL}{u^3} + \frac{196\,D_p^2}{u^4}\right)\phi K_c[1 + \phi K_c(1 + K_n)]\cdot$$

$$\left[\left(\frac{R^2}{D_r}\right)^2\frac{2(1 + K_n)^3}{v^2(v + 2)(v + 4)} + \frac{R^2}{D_r}\frac{2(1 + K_n)}{v(v + 2)}\left(\frac{\phi(1 + K_n)^2}{H_c} + \frac{K_n}{H_n}\right) +\right.$$

$$\left. + \frac{\phi^2(1 + K_n)^3}{H_c^2} + \frac{2\phi K_n(1 + K_n)}{H_cH_n} + \frac{K_n}{H_n^2}\right] + \left(\frac{24\,L}{u} + \frac{48\,D_p}{u^2}\right)\cdot$$

$$\phi K_c\left[\left(\frac{R^2}{D_r}\right)^3\frac{(5v + 12)(1 + K_n)^4}{v^3(v + 2)^2(v + 4)(v + 6)} + \left(\frac{R^2}{D_r}\right)^2\frac{(1 + K_n)^2}{v^2(v + 2)^2(v + 4)}\cdot\right.$$

$$\left.\cdot\left(\frac{(5v + 12)\phi(1 + K_n)^2}{H_c} + \frac{(6v + 12)K_n}{H_n}\right) + \frac{R^2}{D_r}\frac{1}{v(v + 2)}\left(\frac{3\phi^2(1 + K_n)^4}{H_c^2} +\right.\right.$$

$$\left.\left. + \frac{6\phi K_n(1 + K_n)^2}{H_cH_n} + \frac{(2 + 3K_n)K_n}{H_n^2}\right) + \frac{\phi^3(1 + K_n)^4}{H_c^3} + \frac{3\phi^2K_n(1 + K_n)^2}{H_c^2H_n} +\right.$$

$$\left. + \frac{\phi K_n(2 + 3K_n)}{H_cH_n^2} + \frac{K_n}{H_n^3}\right] \qquad (4)$$

where $v$ is the particle shape factor; $v = 3$ for spheres.

The GC column of length $L$, through which flows the carrier gas with velocity $u$, is homogeneously filled with a porous sorbent of particle radius $R$. The column has the external porosity $\varepsilon_e$ (interparticle void volume in unit volume), and the internal porosity $\varepsilon_i$ (intraparticle void volume in unit volume), the porosity function $\phi$ being defined by $\varepsilon_i/\varepsilon_e$. The molecules of the chromatographed compound can be subject to the following transport processes during passage through the column:

(i) Longitudinal diffusion in the carrier gas characterized by the coefficient $D_p$ in which case $D_p = D_g + Au$, $D_g$ being the gaseous phase diffusion coefficient and $A$ a constant comprising effects of eddy diffusion.

(ii) Mass transfer across the interface around the external grain surface characterized by the rate constant $H_c$.

(iii) Radial diffusion into the pores with the coefficient $D_r$.

(iv) Mass transfer from the internal volume of the pores to the internal adsorbent surface with the rate constant $H_n$.

When the concentration of the compound to be separated is different in the interparticle space from that in the pore volume (volumetric adsorption) then the equilibrium is given by the constant $K_c = c_i/c_e$, where $c_i$ is the equilibrium adsorbate concentration in the pore volume, and $c_e$ is the concentration in the interparticle

volume. The adsorption equilibrium between the internal surface and the concentration in the pore volume is characterized by the constant $K_n = n/\varepsilon_i c_i$; $n$ being the amount adsorbed on the surface of the pore volume unit. The equations (1)–(4) contain well defined physical constants and describe a rather complex model of GSC. In reality, however, it is improbable, that all the transport phenomena considered take place simultaneously in the GS column. In many cases the adsorption kinetics will be controlled by intrinsic (radial) diffusion. Thus the constants $H_c$ and $H_n$ are close to infinity and the terms containing these constants may be neglected. If we further consider only the surface adsorption characterized by the equilibrium constant $K_n$, there is no adsorption in the pore volume and the constant $K_c = 1$. In GSC we usually have the velocity $u$ so large that the term $D_p/u < 1$. Under this condition it can be assumed, that in the individual products of the polynomes only the terms with the lowest power of $D$ and $u$ are significant and the terms with higher powers can be neglected. If we further normalize the moments by dividing the second moment about the mean by the square of the first moment $\mu_1$, the third moment by $\mu_1^3$, and the fourth moment by $\mu_1^4$, we may obtain from (1)–(4):

$$H = \frac{\mu_2}{\mu_1^2} \cdot L = 2A + \frac{2D_g}{u} + \frac{2}{15} \frac{\phi (1 + K_n)^2}{(1 + \phi + \phi K_n)^2} \frac{R^2}{D_r} \cdot u \tag{5}$$

$$Z = \frac{\mu_3}{\mu_1^3} \cdot L^2 = 12 \frac{D_g^2}{u^2} + 24 \frac{AD_g}{u} + 12A^2 + \frac{4}{5} \frac{D_g R^2}{\phi D_r} + \frac{4ARu}{5\phi D_r} + \frac{4}{105} \frac{R^4}{D_r^2} \frac{u^2}{\phi^2} \tag{6}$$

assuming $K_n \gg 1$.

$$F = \frac{\mu_4}{\mu_1^4} \cdot L^3 = \frac{12 D_g^2 L}{u^2} + 12 A^2 L + \frac{24 AD_g L}{u} + \frac{8 D_g R^2 L}{5 D_r \phi^3} +$$

$$+ \frac{8 ALR^2}{5 D_r \phi^3} \cdot u + \frac{32}{105} D_g \left(\frac{R^2}{D_r}\right)^2 \frac{1}{\phi^2} \cdot u + \frac{32}{105} A \left(\frac{R^2}{D_r}\right)^2 \frac{1}{\phi^2} \cdot u^2 +$$

$$+ \frac{4}{75} L \left(\frac{R^2}{D_r}\right)^2 \frac{1}{\phi^2} \cdot u^2 + \frac{8}{525} \left(\frac{R^2}{D_r}\right)^3 \frac{1}{\phi^3} \cdot u^3 \tag{7}$$

assuming $K_n \gg 1$,
where $D_g + Au$ has been inserted for $D_p$. It is clear, that equations (5)–(7) are less complex than equations (1)–(4). In the simpler form they can be used for the experimental verification of the theory outlined.

## EXPERIMENTAL

The individual experiments were carried out with columns packed with activated carbon, pumice and glass beads. The physical properties of these columns are summarized in Table I.

For individual packings these materials were screened through sieves into individual fractions representing a certain particle size. The internal and external porosities of the individual packings were determined on basis of measurements of helium and mercury densities. The elution curves of $CO_2$ were recorded (Perkin Elmer–

TABLE I

COLUMN PARAMETERS

| *No. of column* | *Material* | *Fraction particle radius (mm)* | *Sample weight (g)* | *Length of column (cm)* | *Cross section of empty column ($cm^2$)* | *Inter-stitial porosity* | *Total porosity* |
|---|---|---|---|---|---|---|---|
| 1 | Activated carbon | 0.15–0.21 | 21.1 | 78.5 | 0.73 | 0.406 | 0.843 |
| 2 | Activated carbon | 0.30–0.375 | 20.1 | 79.3 | 0.70 | 0.421 | 0.858 |
| 3 | Activated carbon | 0.50–0.60 | 20.1 | 79.0 | 0.70 | 0.422 | 0.851 |
| 4 | Activated carbon | 0.75–1.0 | 19.6 | 79.8 | 0.68 | 0.422 | 0.847 |
| 5 | Pumice | 0.15–0.21 | 30.3 | 81.2 | 0.67 | 0.341 | 0.761 |
| 6 | Pumice | 0.30–0.375 | 29.10 | 78.8 | 0.67 | 0.361 | 0.781 |
| 7 | Pumice | 0.50–0.60 | 27.2 | 78.8 | 0.69 | 0.420 | 0.840 |
| 8 | Pumice | 0.75–1.0 | 15.15 | 46.8 | 0.70 | 0.322 | 0.742 |
| 9 | Glass | 0.15–0.21 | 90.20 | 79.0 | 0.67 | 0.364 | 0.364 |
| 10 | Glass | 0.75–1.0 | 94.8 | 79.6 | 0.72 | 0.386 | 0.386 |

Model E) on these columns. The system active carbon–$CO_2$ represented the case in which the adsorbate is strongly adsorbed on an adsorbent with high internal porosity; pumice–$CO_2$ represented the case where porous solid is interacting with the non-adsorbing gas. Glass beads were used as materials without internal porosity and without adsorption properties. The carrier gas used in all experiments was hydrogen and its velocity was varied in the range from 3 to 25 cm/sec. The chromatographic curves obtained from all combinations of velocities of the carrier gas and the particle size of individual solids were repeated several times in order to restrict possible experimental errors. The curves were measured and treated by means of a computer. The calculation gave the statistical moments $\mu_1$–$\mu_4$ and some of their functions. From these moments the functions: $(\mu_2/\mu_1^2)L$; $(\mu_3/\mu_1^3)L^2$; $(\mu_4/\mu_1^4)L^3$, in dependence on the velocity $u$ for individual materials with a given particle size have been plotted. Resulting curves are shown in Figs. 1, 2, 3, 4 and 5.

## DISCUSSION

### *First statistical moment*

The meaning of the first statistical moment has already been discussed elsewhere[6]. It should be emphasized, that the first moment gives us the location of the centroid of the area under the chromatographic elution curve (which in general differs from $t_{max}$). The position of this centroid depends on the adsorption equilibrium constant, on the porosity of the solids, on the porosity of the bed, and on the longitudinal diffusion constant $D_p$. The first moment does not depend on any kinetic constant and thus not on the rate at which the equilibrium is established in the column. If we insert in eqn. (1):

$$D_p = D_g + Au$$

and neglect the term $D_g/u^2$ we obtain:

$$\mu_1 = \frac{L + 2A}{u}(1 + \phi + \phi K_n) \tag{8}$$

From this it is obvious that the position of the centroid of the area under the chromatographic elution curve (which is better defined as $t_{max}$) may be influenced by the constant of eddy diffusion $A$ and by the porosity $\phi$ of the column and of the solid. This expression may be of considerable significance for interpreting the chromatographic data, in particular those obtained on materials with a high internal porosity.

*Second statistical moment*

In Figs. 1–3 the function $(\mu_2/\mu_1^2)L$ (denoted as $H$) is plotted in dependence on the velocity $u$. The function $H$ is in some respects analogous[8] to the expression for the HETP, which for GLC was introduced by VAN DEEMTER *et al.*[7]. The relationships presented in Fig. 1 show a number of interesting facts:

(i) The increase of the quantity $H$ with increasing $u$ is noticeable over the entire range of the velocities employed only for the largest particle sizes, the dimensions of which considerably exceed those usually employed in gas chromatography.

(ii) The quantity $H$ does not depend, in the employed range of velocities of the carrier gas, upon the material used or, in a certain velocity range, on the velocity itself. For instance, under the condition $u = 10$–$25$ cm/sec, $R < 0.37$ mm, the quantity $H$ has a constant value amounting very approximately to 0.2 cm. From these experiments we can draw a somewhat surprising conclusion regarding the constant $A$ characterizing the eddy diffusion in gas chromatography. (The $2A$ from our equations is equal to $A$ from the VAN DEEMTER equation[7].) It appears, that the constant $A$ is neither dependent on the character of the surface of the grain nor on its porosity, and that in the range of chosen experimental conditions it is no function of $R$. Since the constant $A$ is of considerable importance not only in the case of the second moment but also for higher moments, we have paid great attention to this question. The experiments were analysed in various possible ways for elucidating the dependence of $A$ on $R$, however, all these analyses only confirm the foregoing statement.

The determination of the term $D_g/u$ whose significance in examining the theory is not very great, has not been studied in detail. For the magnitude of $D_g$ of carbon dioxide in hydrogen we have taken the value of a preceding paper[6] *i.e.* $D_g = 0.55$ cm$^2$/sec. On the other hand considerable importance was attached to the determination of the coefficient of radial diffusion $D_r$. For this coefficient in the system $CO_2$–active charcoal, we obtained the value $2.1 \times 10^{-2}$ cm$^2$/sec, and in the system $CO_2$–pumice for $[(\varepsilon_e + \varepsilon_i)^2/\varepsilon_e\varepsilon_i]D_r = 8 \cdot 10^{-2}$cm$^2$/sec, which follows from (5) if $K_n = 0$. This is in good agreement with the theory, which shows, that the influence of porosity for a non-adsorbing gas is such that $H$ decreases to a quarter of the $H$ value of a strongly adsorbing gas. Concerning the nature of the radial diffusion coefficient $D_r$ we have shown elsewhere[9], that $D_r$ is an effective diffusion coefficient, which is independent of the adsorption constant $K_n$.

*Third statistical moment*

On inspecting the expression for the third statistical moment, given by the relation (3) we find, that it is again a function of all constants characterizing the properties of the bed and the packing, the velocity of the carrier medium, the kinetic properties and the equilibrium constants of adsorption. The expression consists only of positive terms, which means (if we place zero into the mean), that the po-

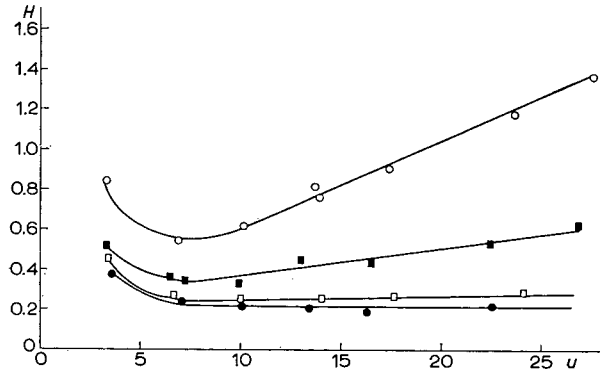


Fig. 1. Dependence of the term $H$ on the velocity of the carrier gas $u$. Column charged with activated carbon particles of different radius. (●) 0.15–0.21 mm; (□) 0.30–0.375 mm; (■) 0.50–0.60 mm; (○) 0.75–1.0 mm.

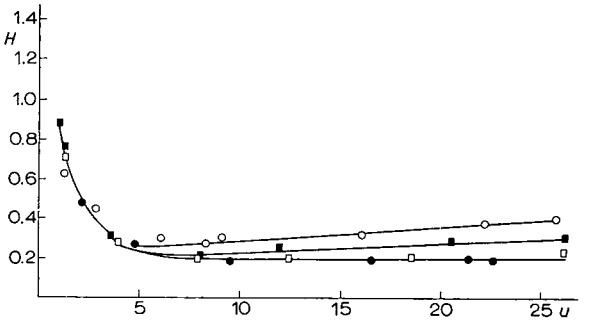


Fig. 2. Dependence of the term $H$ on the velocity of the carrier gas $u$. Column charged with pumice particles of different radius. (●) 0.15–0.21 mm; (□) 0.30–0.375 mm; (■) 0.50–0.60 mm; (○) 0.75–1.0 mm.

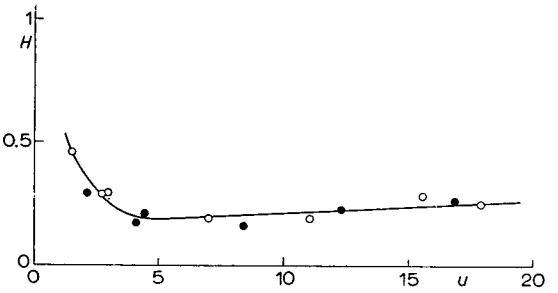


Fig. 3. Dependence of the term $H$ on the velocity of the carrier gas $u$. Column charged with glass beads of different radius. (●) 0.15–0.21 mm; (○) 0.75–1.0 mm.

sitive deviations predominate over the negative ones. Moreover since the third statistical moment never equals zero, this means that the chromatographic curve in GSC as described by the model under consideration must always be asymmetrical. Multiplying the term $\mu_3/\mu_1^3$, which can be denoted as specific asymmetry by the term $L^2$, we obtain the constant $Z$, which is given by the sum of six terms containing characteristic constants. On examination of these terms we find, that also in the case of a linear adsorption isotherm the asymmetry increases with rising particle radius $R$, with increasing constant $D_g$ and $A$ and with decreasing constant $D_r$. As far as the dependence of the constant $Z$ on the velocity $u$ is concerned, we see that the expression will have a minimum. Since two terms are independent of $u$, the value of the minimum is likely to be near the values of these terms. The curve of the dependence of $Z$ on $u$ will have a shape similar to that of the dependence $H$ on $u$. As far as the magnitude and significance of the individual terms is concerned, we must first of all point out the significance of the constant $A$ characterizing the eddy diffusion for the quantity $Z$. A clear survey of the magnitude of the individual terms in the expression for $Z$ is provided by Table II. From this table it is obvious that the terms containing $A$ largely participate in the overall expression. The large terms with $R$ arise because in this case the experiments were undertaken with relatively large particles.

TABLE II

$Z$ TERMS CALCULATED FROM THE THEORETICAL RELATIONSHIPS

$R = 8.75 \cdot 10^{-2}$ cm; $D_r = 2.1 \cdot 10^{-2}$ cm²/sec; $D_g = 5.5 \cdot 10^{-1}$ cm²/sec; $A = 1 \cdot 10^{-1}$ cm; $L = 80.0$ cm; $\phi = 1$

| $u$ | $\frac{12 D_g}{u^2}$ | $\frac{24 A D_g}{u}$ | $12 A^2$ | $\frac{4}{5} D_g \frac{R^2}{D_r}$ | $\frac{4}{5} A \frac{R^2}{D_r} u$ | $\frac{4}{105} \frac{R^4}{D_r^2} u^2$ | $\frac{\mu_3}{\mu_1^3} \cdot L^2 = Z$ |
|---|---|---|---|---|---|---|---|
| 5 | 0.26 | 0.26 | 0.12 | 0.16 | 0.15 | 0.13 | 1.08 |
| 10 | 0.07 | 0.13 | 0.12 | 0.16 | 0.29 | 0.50 | 1.27 |
| 15 | 0.03 | 0.09 | 0.12 | 0.16 | 0.44 | 1.14 | 1.97 |
| 20 | 0.02 | 0.07 | 0.12 | 0.16 | 0.58 | 2.02 | 2.96 |
| 25 | 0.01 | 0.05 | 0.12 | 0.16 | 0.73 | 3.16 | 4.24 |

*Experimental examination of the theory*

Up to now, we have dealt with considerations following from theoretical conclusions and possible simplifications of the individual relations. It is extraordinarily important and interesting, however, to compare the validity and applicability of the theoretical statements with experimental results. Of principal importance for examining the theory were the experiments with $CO_2$ on carbon. The dependence of $H$ on $u$ was utilized for determining the constants $A$ and $D_r$ ($A = 0.1$ cm, $D_r = 2.1 \cdot 10^{-2}$ cm²/sec). In accordance with the foregoing explanation the constant $A$ was regarded as independent of $R$. As magnitude of the particle radius $R$ we introduced the mean value of the respective range of closely limited fractions obtained by screening. The linear velocity of the carrier gas was calculated on the basis of the knowledge of the volumetric flow rate, of the column cross section and the external porosity $\varepsilon_e$. Utilizing experimental data we calculated the dependence of $Z$ upon $u$ according to formula (6). The calculated dependence marked by dashed lines is

TABLE III

$F$ TERMS CALCULATED FROM THE THEORETICAL RELATIONSHIPS

| $u$ | $\frac{12 D_g^2 L}{u^2}$ | $12 A^2 L$ | $\frac{24 A D_g L}{u}$ | $\frac{8 D_g L}{5}\frac{R^2}{D_r}$ | $\frac{8 A L}{5}\frac{R^2}{D_r}u$ | $\frac{32}{105} D_g \frac{R^4}{D_r^2} u$ | $\frac{32}{105} A \frac{R^4}{D_r^2} u^2$ | $\frac{4}{75} L \frac{R^4}{D_r^2} u^2$ | $\frac{8}{505}\frac{R^6}{D_r^3} u^3$ | $\frac{\mu_4}{\mu_1^4} L^3 = F$ |
|---|---|---|---|---|---|---|---|---|---|---|
| 5 | 11.61 | 9.60 | 21.12 | 25.67 | 23.33 | 0.11 | 0.10 | 14.17 | 0.07 | 105.79 |
| 10 | 2.90 | 9.60 | 10.56 | 25.67 | 46.67 | 0.22 | 0.40 | 56.70 | 0.57 | 153.30 |
| 15 | 1.29 | 9.60 | 7.04 | 25.67 | 70.00 | 0.33 | 0.91 | 127.57 | 1.94 | 244.36 |
| 20 | 0.72 | 9.60 | 5.28 | 25.67 | 93.34 | 0.44 | 1.62 | 226.80 | 4.60 | 368.08 |
| 25 | 0.46 | 9.60 | 4.22 | 25.67 | 116.67 | 0.55 | 2.53 | 354.37 | 8.99 | 523.08 |

compared with experimental measurements in Fig. 4. A comparison of the results of the calculations and the experimental data shows an agreement, which can be regarded as very good, because the divergence between theory and experiment is smaller than the assumed range of experimental and calculation errors.

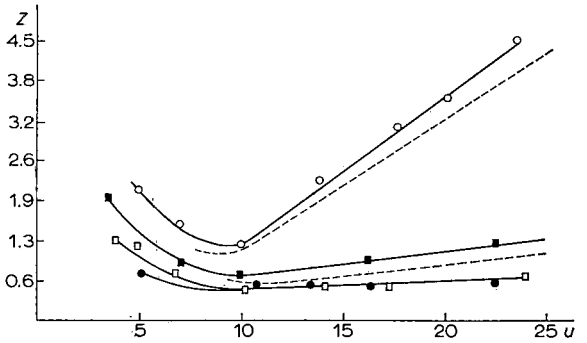


Fig. 4. Dependence of the term $Z$ on the velocity of the carrier gas $u$. Carbon columns as in Fig. 1. Dashed curves represent the values of the term $Z$, derived from the theoretical relationships.

*Fourth statistical moment*

The fourth statistical moment for the Gaussian curve has the value of 3 if we divide this moment by $\mu_2^2$. The deviation from this value shows the excess or flattening of the distribution curve under investigation. A sufficiently simple expression for the ratio $\mu_4/\mu_2^2$ is difficult to derive from the relations for the individual moments. We can, however, easily express the ratio $(\mu_4/\mu_1^4)L^3$, which we denote by $F$. An examination of the relations mentioned shows clearly that this quantity—contrary to the foregoing characteristics of this type—depends in a more complex way on the column length $L$. Its dependence on the velocity $u$ is again similar to the preceding relationships *i.e.* it passes through a minimum. The significance of the individual terms can be estimated by means of Table III.

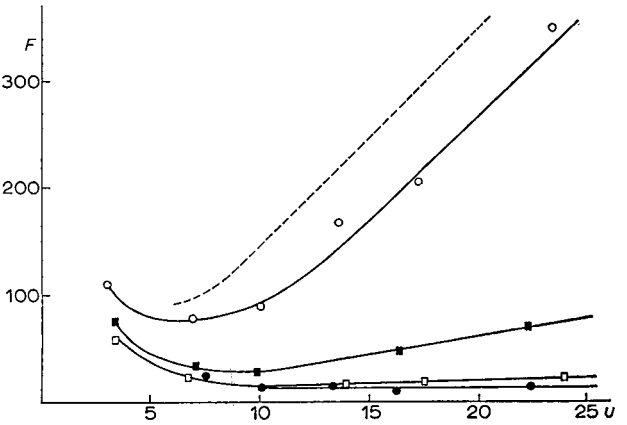


Fig. 5. Dependence of the term $F$ on the velocity of the carrier gas $u$. Carbon column as in Fig. 1. The dashed curve represents the values of the term $F$, derived from the theoretical relationships.

As in the case of the third moment, it was also very interesting to compare the experimental results with the theoretical predictions for the fourth moment. For this we utilized experimental data concerning the passage of $CO_2$ through the column packed with activated carbon, as described earlier, for plotting the expression $(\mu_4/\mu_1^4)L^3$ (this ratio is denoted by $F$) against the velocity $u$ (Fig. 5). By means of the constants already mentioned we then calculated the values for the expression according to eqn. (7). The calculated results are denoted in Fig. 5 by a dashed line.

It is obvious that in this case too, the agreement between experiment and theory is very good. It may be assumed, therefore, that the chosen model and its mathematical description indicate the behaviour of at least certain GSC systems on porous materials and that the simplifications employed are permissible. The theoretical model and its interpretation may be of importance not only for presenting gas chromatographic data, but also for obtaining important physico-chemical constants. On the other hand it must be pointed out that experimental verification and utilization of the theory have overcome only the first steps and that the conclusions so far drawn must be verified also for other adsorbate–adsorbent systems.

ACKNOWLEDGEMENTS

We are indebted to Dr. M. PICEK, PhMr. V. BENEŠOVÁ and L. VIKTOROVÁ for their help in the numerical calculations. The computer programme for calculation of the statistical moments was proposed by Dr. E. KUČERA.

SUMMARY

By means of the system $CO_2$–activated carbon the applicability of the statistical moments theory to gas–solid chromatography was verified under the assumption that the adsorption rate is controlled by interparticle diffusion. The experimental results are in very good agreement with theoretical predictions.

The theory shows that all the gas chromatographic characteristics may be influenced by the column and solid porosity and by the eddy diffusion constant. This fact has to be respected in presenting the gas chromatography data.

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# SIMULATED DISTILLATION BY GAS CHROMATOGRAPHY: A COMPUTER PROGRAM

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## INTRODUCTION

A distillation curve is an important physical characteristic of a hydrocarbon mixture. Physical distillation test methods are generally employed to obtain this property.

A number of investigators have also reported the use of gas chromatography to obtain distillation curves[1–6]. Varying accuracies are reported, depending on the system used and the type of distillation simulated. More recently, an improved and automated system to yield boiling point distribution curves equivalent to that obtained from a 100 theoretical plate distillation column, was reported[2,4]. Whereas instrumentation in the gas chromatographic system proper has already reached a high level of sophistication, simulated distillation by gas chromatography still suffers from the necessity of having to make a large number of reiterative calculations before the raw data can be transformed into conventional true boiling point (TBP) curves. To process the data from a single sample, several hours of manual computations are necessary. A computer program is, therefore, an essential first step to reduce the amount of work connected to handling the raw data, especially if large numbers of samples have to be analyzed routinely.

## GAS CHROMATOGRAPH, COMPUTER AND PLOTTER

The gas chromatographic system used in these investigations is the Varian Aerograph Simulated Distillation Apparatus Model 900. The principle and operating procedures have already been described elsewhere[2,4,7].

The output of the system is in the form of a tape with two columns of data. The numbers in the first column are consecutive from zero up and are called the print numbers. The zero term corresponds to the time of injection. Printout time is once every ten seconds. The four digits in the second column are proportional to the total area on the chromatogram up to that point. Data from this tape have to be key-punched manually to obtain the data cards for the computer program.

The computer used to process the data is an IBM 7094. A Calcomp 570 plotting system, to which a Model 563 Calcomp digital and incremental plotter is attached, is used to present the output of the computer in the form of completely annotated graphs, complete with scale markings, legends, and curve identification symbols.

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COMPUTER PROGRAM

The program is written in FORTRAN. It consists of a main program, a calibration subprogram, a plotter subprogram, each having approximately 120 FORTRAN statements, and several smaller function subprograms. The plotter subprogram uses existing subroutines to make the Calcomp tape, and the calibration subprogram uses several curve-fitting routines, as different correlations between print number and boiling point are tested. The separate subroutines will be discussed under the appropriate headings.

Fig. 1 shows the simplified flow diagram of the program. From the data furnished, the IBM 7094 prepares a magnetic tape which monitors the output of the plotter. This magnetic tape contains all the necessary instructions on how to plot the

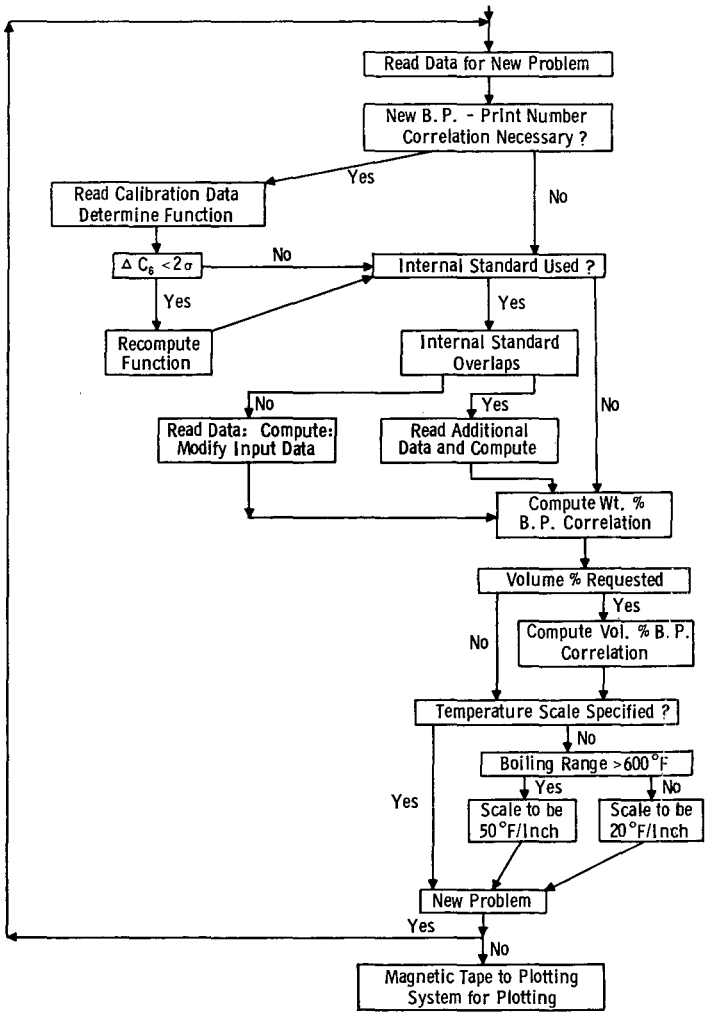


Fig. 1. Simulated distillation computer flow diagram.

calibration curve, the choice of scales, and whether the derived volume percent boiling curve has to be drawn.

## PRINT NUMBER–BOILING POINT CORRELATION

The relationship between print number and boiling point is obtained by processing a synthetic mixture of *n*-hydrocarbons under defined conditions through the chromatograph and plotting the print number, $R$, of each hydrocarbon against its boiling point, $\beta$. Boiling point data were obtained from API Project 44[8]. By connecting these points, a curve is obtained which, on a linear scale for both axes, becomes a straight line for $C_7$ and higher hydrocarbons.

A least square fit is first carried out on these higher boiling components. If $C_6$ is within two standard deviations of this computed line, this line is redetermined with $C_6$ included in the fit. The standard deviation of the correlation is usually a fraction of a print number. The remaining points, $C_3$, $C_4$, $C_5$, and, if necessary, $C_6$, are correlated by the function:

$$\beta = a_1R + a_2 + a_3/(R + a_4) \tag{1}$$

where the $a_i$'s are determined so that the function has the same value and slope as the linear function at $C_6$ (or $C_7$) and the best approximation is obtained (least squares) for the observed values. A nonlinear regression routine from a program library is used for this. The differences between observed and computed values in this curved region are slightly larger than in the linear range. Several more complicated functions gave better fits but were too difficult to work with in a situation where fits were to be made routinely by machine.

## INTERNAL STANDARDS AND HIGH BOILING PRODUCTS

For practical reasons, the time corresponding to a boiling point of 1000°F of the product is equated to an arbitrary limit. Most high resolution TBP's do not exceed this point except for the very few instances where the end point would be just slightly above 1000°F. ASTM D 1160 distillations have a wider range and can go deeper into heavier stocks; for a high resolution TBP, however, the limit of 1000°F is more than adequate. In addition, the print number–boiling point correlation will begin to deviate from linearity some time above 1000°F because the retention time of dotetracontane (boiling point ~ 1000°F) is near the end of the temperature programmed section of the heating curve. In the final isothermal portion of the run, the retention time–boiling point relation is, of course, not linear anymore.

For higher boiling products not completely eluted at that time, an internal standard is used to compute the fraction of sample eluted at the 1000°F point. The internal standard used can be either a simple hydrocarbon or a mixture of hydrocarbons. The mathematical treatment of this case has already been given by WORMAN AND GREEN[9].

We have to distinguish between two cases; *viz.*, the peak(s) from the internal standard either overlap(s) or it (they) do(es) not overlap the chromatogram of the sample proper.

Overlap will be the most general situation. Two runs are now carried out; one of the sample proper and another of the sample to which the internal standard has been added.

No overlap takes place in those cases where the internal standard can be placed in an area of the chromatogram where no components of the sample are present. The procedure is then simplified because only one run has to be carried out. In the usual case the internal standard will elute before the sample proper and a minimum initial boiling point of the sample is necessary because the internal standard used should not be too volatile. For products with an initial boiling point of around 350°F or higher[9], 5–10 % *n*-octane is weighed in as internal standard.

## WEIGHT PERCENT–VOLUME PERCENT CORRELATIONS

Data obtained by physical distillation are generally of the volume percent boiling point type. ASTM D 86, ASTM D 1160, ASTM D 1078, and other distillation test methods all yield relationships between boiling point and volume percent distillate. Even with high resolution TBP's obtained on efficient distillation columns, it is common practice to correlate the volume distillate to the boiling point. To obtain weight percent data, fractions would have to be taken during the distillation and weighed. This is more complicated than direct volume readings.

There is a description of an instrument to yield weight percent boiling curves by physical distillation[10] but it is little known and not extensively used.

Data obtained from simulated distillations are based on weight percents. To convert to volume percent, the density–boiling curve should be known. This relationship is obviously dependent on the composition of the sample; and short of analyzing small fractions for its density, the exact correlation cannot be determined.

For any product boiling in a certain range, densities will vary with structure. In the case of hydrocarbons, a survey shows that on the average the *n*-hydrocarbons have densities which lie between the extreme densities observed for other hydrocarbons boiling in that particular region. The general relation which is used in this program is derived from the density boiling point correlation of the *n*-hydrocarbons. Prime data have been obtained from API Research Project 44[8]. The following equation has been used

$$d = T^{C_2 \, 10^{\{C_1 + C_3 (\log T)^2\}}} \tag{2}$$

where $d$ is the density and $T$ is the atmospheric boiling point in °R. Equation 2 is obtained by plotting the density–boiling point data on a log–log graph and fitting a quadratic curve through these points. The three constants, $C_1$, $C_2$, and $C_3$, have been computed by the method of least squares to yield

$$C_1 = -4.46344$$
$$C_2 = \phantom{-}2.66225$$
$$C_3 = -0.40425$$

The largest deviations occur at the very low values of $T$. For *n*-propane, the observed deviation is slightly more than 1 %. A very much improved correlation is observed for the higher boiling hydrocarbons. Considering the possible error by using

the *n*-hydrocarbon density instead of the true density, the error introduced by this correlation is negligibly small.

VOLUME PERCENT BOILING CURVE

To compute the volume percent curve from the weight percent curve, the weight fraction between each two consecutive print numbers is divided by a density obtained from the density–boiling point correlation for a temperature corresponding to the average of the two print numbers. To obtain the volume fraction at any given temperature, the sum of the quotients of the above computation up to the relative print number is divided by the total sum of these quotients for all the print numbers.

OUTPUT

The final output from the program consists of the plots and the printed output. The printed output from the IBM 7094 is seldom used because information is more easily gained from the plots.

TIME

In a recent test run, three boiling point–retention time calibration curves and five simulated distillations were processed to produce both volume and weight percent curves. The total IBM 7094 computing time was less than one minute; the Calcomp plotting system needed 27 minutes to draw the 13 graphs.

PLOTS

Fig. 2 shows the weight percent TBP curve of a heavy gasoline. This graph is completely machine drawn by the plotter. The original graph is 10 in. wide and drawn

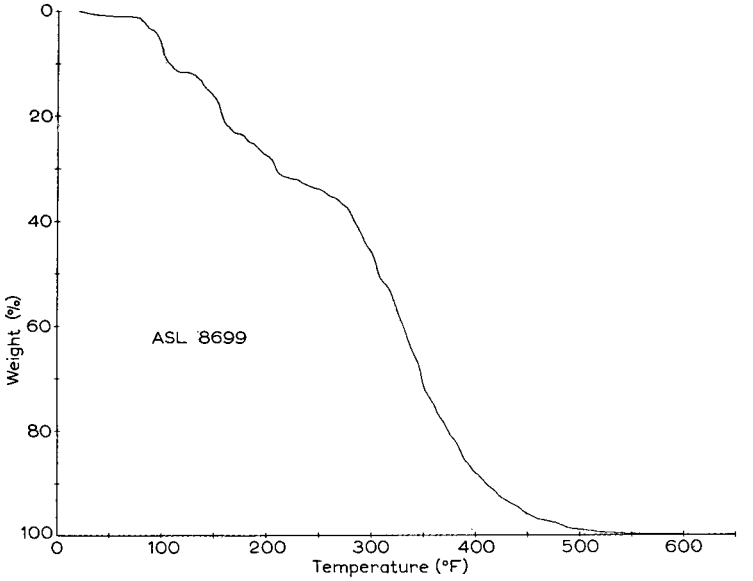


Fig. 2. Weight per cent TBP curve of a heavy gasoline.

on coordinate paper to facilitate interpretation. The crosshatch lines have been screened out in the photographic reduction process.

Details of the FORTRAN code and computer program are available on request.

ACKNOWLEDGMENT

Gas chromatography data for Fig. 2 obtained on a Varian Aerograph Simulated Distillation Chromatograph Model 900, were kindly put at our disposal by Varian Aerograph, Walnut Creek.

SUMMARY

A computer program to process the data from a simulated distillation gas chromatograph is described. Part of the final output is in the form of weight and volume percent true boiling point (TBP) curves which are traced by a Calcomp 570 plotting system from a magnetic tape prepared on an IBM 7094 computer.

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# CALCULATION OF ECL VALUES IN THE GAS–LIQUID CHROMATOGRAPHY OF MULTIPLE-BRANCHED FATTY ACIDS

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The carbon number[1] or equivalent chain length[2,3] (ECL) systems for identifying esters in gas–liquid chromatographic analyses have been used with some success for unique fatty acids[4]. The original concept of the mobility of a given material in a gas–liquid chromatographic separation as due to the contribution of a basic skeleton plus contributions from non-interacting substituents[5–11] has, however, not been used very successfully with fatty acids since in naturally occurring fatty acids there is often interaction between neighbouring substituents or groups. Additionally, the lack of success has been because the position of a given substituent or functional group on or in the chain is of considerable significance in determining the fractional chain length (FCL) contribution due to the particular substituent or group[12–14]. It is generally recognized, however, that FCL values are normally nearly constant in the middle portion of an aliphatic chain. At the end of a fatty acid chain remote from the carboxyl group, FCL values in any chain may be modified in a more or less predictable fashion[15,16] depending on the nature of the group or substituent, but when near the carboxyl group may be subject additionally to the possibility of interaction with the carboxyl group[13,17–19].

In our laboratory, a gas–liquid chromatographic study of the saturated fatty acids of marine lipids gave evidence for the occurrence of three multiple-branched fatty acids in the lipids from all higher species[20]. Subsequent work in another laboratory indicated that these were probably 4,8,12-trimethyltridecanoic, 2,6,10,14-tetramethylpentadecanoic and 3,7,11,15-tetramethylhexadecanoic acids[21]. More recently, further studies in our laboratory have been carried out with both butanediol succinate (BDS) polyester and silicone (SE-30) open-tubular (capillary or Golay) columns[22]. Esters of authentic 2,6,10,14-tetramethylpentadecanoic and 3,7,11,15-tetramethylhexadecanoic acids (courtesy of R. P. HANSEN, D.S.I.R., Wellington, New Zealand) confirmed the identity of these two multiple-branched acids on both substrates. Petroleum has been shown to contain 3,7,11-trimethyldodecanoic acid in association with 2,6,10,14-tetramethylpentadecanoic acid[23] but comparative analyses with the ester of an authentic sample (courtesy of Dr. L. H. SARRETT, Merck, Sharp and Dohme, N.J.) showed that only traces of 3,7,11-trimethyldodecanoic acid were present in the marine lipids examined.

These studies provided accurate retention times on BDS for esters of three known multiple-branched fatty acids and one tentatively identified acid (Table I). The possibility of calculating the retention time of the ester of 4,8,12-trimethyltridecanoic acid was investigated since three model fatty acids were available for

TABLE I

ECL DATA FOR BRANCHED-CHAIN FATTY ACIDS ON BDS OPEN-TUBULAR COLUMNS OF HIGH EFFICIENCY (NO. 1) OR MODERATE EFFICIENCY (NO. 2)

| *Fatty acid* | | *BDS column No. 1; 150°* | | *BDS column No. 2; 170°* | |
|---|---|---|---|---|---|
| | | $r_{18:0}$ | *ECL* | $r_{18:0}$ | *ECL* |
| | 12:0 | 0.088 | 12.00 | — | — |
| | 3,7,11-trimethyldodecanoic | 0.125[a] | 12.87 | — | — |
| *Iso* | 14:0 | — | — | 0.199 | 13.55 |
| | 14:0 | 0.198 | 14.00 | 0.233 | 14.00 |
| | 4,8,12-trimethyltridecanoic (?) | 0.210[b] | 14.14 | ~ 0.250[c] | ~ 14.18 |
| *Iso* | 15:0 | 0.247 | 14.52 | 0.286 | 14.54 |
| *Anteiso* | 15:0 | 0.264 | 14.70 | 0.305 | 14.73 |
| | 15:0 | 0.298 | 15.00 | 0.337 | 15.00 |
| *Iso* | 16:0 | 0.370 | 15.53 | 0.413 | 15.56 |
| *Anteiso* | 16:0 | 0.398 | 15.72 | 0.444 | 15.75 |
| | 2,6,10,14-tetramethylpentadecanoic | 0.413[a] | 15.82 | 0.450 | 15.80 |
| | 16:0 | 0.446 | 16.00 | 0.485 | 16.00 |
| *Iso* | 17:0 | 0.555 | 16.55 | 0.597 | 16.56 |
| *Anteiso* | 17:0 | 0.589 | 16.71 | 0.633 | 16.75 |
| | 17:0 | 0.666 | 17.00 | 0.700 | 17.00 |
| | 3,7,11,15-tetramethylhexadecanoic | 0.673[a] | 17.02 | ~ 0.715[c] | ~ 17.08 |
| *Iso* | 18:0 | 0.825 | 17.52 | 0.852 | 17.56 |
| | 18:0 | 1.000 | 18.00 | 1.00 | 18.00 |

[a] Determined independently of marine lipid fatty acids.
[b] Accurate value for component completely separated from other marine lipid fatty acids.
[c] Estimated value for component incompletely separated from other marine lipid fatty acids.

validating the procedures. The isoprene skeletons of these acids made this appear particularly feasible since there should be no interaction among the methyl groups.

The higher branched-chain fatty acids have been exhaustively studied from various viewpoints and the published material includes a table of separation factors for all of the methyloctadecanoic acids[24]. This data had to be converted into FCL units but no retention data for the appropriate linear fatty acids were provided to complement the tabulated data which were obtained on Reoplex 400 at 216°. To prepare FCL units a log plot line was drawn based on linear fatty acid ester data[25] obtained on Reoplex 400 at 197° ($r_{19:0}$ of 0.431 for methyl hexadecanoate). The FCL units obtained from this line by using the separation factors from the literature[24] in the normal fashion are listed in Table II and plotted against position of methyl substituent in Fig. 1. An apparent discrepancy occurs between the separation factor originally tabulated for 15-methyloctadecanoate and a plot similar to Fig. 1 in the same publication[24]. The FCL value for this acid in Table I and Fig. 1 is therefore an interpolation.

It is particularly evident from Fig. 1 that methyl groups in the 2-5 and 12-17 positions give FCL values differing significantly from the rest. They may be designated by types respectively as carboxyl end ($C_x$) and terminal end ($\omega_y$) numbers, with the less critical positions in between designated $m_x$. The FCL values in Table II were then tested as shown in Table III. It will be noted that in the two shorter chain acids $\omega_5$ FCL values have been employed in preference to $m_8$ and $m_7$ respectively. This usage arises from the obviously greater significance to be assigned to $C_x$ or $\omega_y$ FCL values.

The excellent agreement obtained between the calculated and observed ECL values for these four acids was most unexpected since the FCL values were determined

TABLE II

SEPARATION FACTORS AND FCL VALUES FOR METHYL-BRANCHED OCTADECANOIC ACID METHYL ESTERS WITH TYPE DESIGNATIONS

| *Methyl position* | *Literature*[24] $r_{19:0}$[a] | *FCL*[b] | *Type designation* |
|---|---|---|---|
| 2 | 0.713 | (—) 0.19 | $C_2$ |
| 3 | 0.764 | 0.05 | $C_3$ |
| 4 | 0.833 | 0.36 | $C_4$ |
| 5 | 0.808 | 0.24 | $C_5$ |
| 6 | 0.804 | 0.22 | $m_6$ |
| 7 | 0.806 | 0.23 | $m_7$ |
| 8 | 0.799 | 0.21 | $m_8$ |
| 9 | 0.805 | 0.22 | $m_9$ |
| 10 | 0.806 | 0.23 | $m_{10}$ |
| 11 | 0.811 | 0.26 | $m_{11}$ |
| 12 | 0.819 | 0.29 | $\omega_6$ |
| 13 | 0.812 | 0.27 | $\omega_5$ |
| 14 | 0.844 | 0.41 | $\omega_4$ |
| 15 | 0.672 | (0.43)[c] | $\omega_3$ |
| 16 | 0.898 | 0.62 | $\omega_2$ |
| 17 | 0.870 | 0.51 | $\omega_1$ |

[a] Reoplex 400 at 216°.
[b] Generated from a log plot line with $r_{19:0}$ as 1.00, $r_{16:0}$ as 0.431.
[c] Interpolated value.

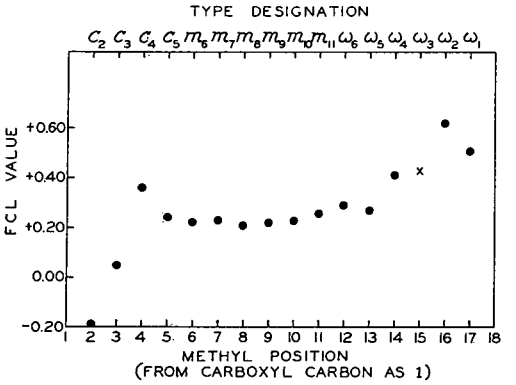


Fig. 1. Plot of FCL value against methyl positions on octadecanoate chain. Type designations at top. Value for $\omega_3$ interpolated.

from a linear log plot based on a line chosen more or less at random from the literature and the arbitrary use of another plot line would generate a quite different family of FCL values. The ECL value for 3,7,11-trimethyldodecanoic acid at 197° on ethylene glycol-adipate (EGA) polyester[26] is 12.80, also close to the calculated value. The probable reason for the good agreement in these examples is that the Reoplex 400 and EGA columns are of comparable polarity to the BDS open-tubular column. Thus octadeca-6,9,12-15-tetraenoate ester falls before eicosenoic ester on the packed columns[25] and also on the BDS open-tubular columns[27]. Retention data obtained on β-cyclodextrin esters as gas–liquid chromatographic substrates were also examined

TABLE III

APPLICATION OF FCL VALUES (TABLE II) TO THE CALCULATION OF ECL VALUES FOR MULTIPLE BRANCHED FATTY ACIDS OBSERVED IN MARINE LIPIDS

Experimental data given at 150° in Table I.

| | | | | | |
|---|---|---|---|---|---|
| *3,7,11,15-Tetramethylhexadecanoate* | | | *2,6,10,14-Tetramethylpentadecanoate* | | |
| Basic chain | | 16.00 | Basic chain | | 15.00 |
| FCL contribution $C_3$ | | 0.05 | FCL contribution $C_2$ | | (—)0.19 |
| FCL contribution $m_7$ | | 0.23 | FCL contribution $m_6$ | | 0.22 |
| FCL contribution $\omega_5$ | | 0.27 | FCL contribution $\omega_5$ | | 0.27 |
| FCL contribution $\omega_1$ | | 0.51 | FCL contribution $\omega_1$ | | 0.51 |
| Calculated | ECL | 17.06 | Calculated | ECL | 15.81 |
| Experimental | ECL | 17.02 | Experimental | ECL | 15.82 |
| *4,8,12-Trimethyltridecanoate* | | | *3,7,11-Trimethyldodecanoate* | | |
| Basic chain | | 13.00 | Basic chain | | 12.00 |
| FCL contribution $C_4$ | | 0.36 | FCL contribution $C_3$ | | 0.05 |
| FCL contribution $\omega_5$ | | 0.27 | FCL contribution $\omega_5$ | | 0.27 |
| FCL contribution $\omega_1$ | | 0.51 | FCL contribution $\omega_1$ | | 0.51 |
| Calculated | ECL | 14.14 | Calculated | ECL | 12.83 |
| Experimental | ECL | 14.14 | Experimental | ECL | 12.87 |

since three multiple-branched acids were included[28]. From the FCL values in Table II the ECL values calculated for the multiple-branched fatty acids were found to be in good agreement (Table IV) with the experimental ECL values obtained with β-cyclodextrin acetate at 220°. This material is, however, of somewhat greater polarity with respect to unsaturated materials. Another experimental log plot line drawn by trial and error (with $r_{19:0}$ of 0.354 for methyl hexadecanoate) generated respective FCL values of 0.04, 0.23, 0.38 and 0.61 for methyl 2-, 3-, 6- and 15-hexadecanoates. As shown in Table IV this set of FCL values gave good agreement with experimental ECL values determined on β-CDX butyrate.

As a rough guide to the choice of log plot lines for generating FCL values from the literature data (Table II) for use in a particular analysis, the reasonably accessible $\omega_1$ (*iso* acid) FCL values may be used to establish a preliminary line. Thus, the typical FCL value of 0.53 found for $\omega_1$ acids at 150° in marine oils (Table I) is very close to that (0.51) inadvertently generated for Table II. The experimental β-CDX acetate FCL value of 0.55 is also fairly close to this value but the experimental β-CDX butyrate value of 0.65 differs considerably from these figures. The trial and error line used to generate the FCL values for β-CDX butyrate (Table IV) was in fact based on the observation that the difference in the $\omega_1$ FCL values for the acetate FCL values was 0.04.

The validity or accuracy of the FCL values given in Table II is not guaranteed beyond the original data. As previously pointed out the 15-methyloctadecanoate figure is an interpolation. Some reservations must also be held regarding the 16-methyloctadecanoate ($\omega_2$) FCL value of 0.62. As shown in Table I the $\omega_1$ and $\omega_2$ FCL values obtained with authentic esters on the open-tubular BDS column at 150° were consistent at respectively about 0.53 and 0.71. These figures are in good agreement with typical values obtained from literature data[25] of about 0.52 for $\omega_1$ and about 0.72 for $\omega_2$ on EGA at 197°. A survey of all available literature data gave respective

TABLE IV

APPLICATION OF FCL VALUES TO THE CALCULATION OF ECL VALUES FOR MULTIPLE-BRANCHED FATTY ACIDS ANALYSED ON β-CDX ACETATE AND BUTYRATE SUBSTRATES[28]

| | β-CDX | |
|---|---|---|
| | *Acetate*[a] | *Butyrate*[b] |
| *3,6,13-Trimethyltetradecanoate* | | |
| Basic chain | 14.00 | 14.00 |
| FCL contribution $C_3$ | 0.05 | 0.23 |
| FCL contribution $m_6$ | 0.21 | 0.38 |
| FCL contribution $\omega_1$ | 0.51 | 0.61 |
| Calculated ECL | 14.77 | 15.22 |
| Experimental ECL | 14.75 | 15.20 |
| *3,6-Dimethylpentadecanoate* | | |
| Basic chain | 15.00 | 15.00 |
| FCL contribution $C_3$ | 0.05 | 0.23 |
| FCL contribution $m_6$ | 0.21 | 0.38 |
| Calculated ECL | 15.26 | 15.61 |
| Experimental ECL | 15.15 | 15.60 |
| *2,14-Dimethylpentadecanoate* | | |
| Basic chain | 15.00 | 15.00 |
| FCL contribution $C_2$ | (—)0.19 | 0.04 |
| FCL contribution $\omega_1$ | 0.51 | 0.61 |
| Calculated ECL | 15.32 | 15.65 |
| Experimental ECL | 15.25 | 15.70 |

[a] FCL values from Table II.
[b] For derivation of FCL values see text.

average FCL values of 0.57 (from 32 figures) and 0.73 (from 19 figures), but no pattern could be detected associating variations with polyester polarity or operating temperature.

The large FCL value of a $C_4$ isomer is substantiated by the calculation for 4,8,12-trimethyltridecanoate and by gas–liquid chromatographic analyses of certain methyl-branched fatty acids on a polypropylene glycol open-tubular column[29,30]. In these reports it is shown that 4-methyloctadecanoate falls between the 12- and 14-methyloctadecanoates, but 4-methylhexadecanoate falls between the 10- and 12-methylhexadecanoates. This means, in effect, that the $C_4$ FCL value in this analysis would be numerically between the FCL values for $\omega_4$ and $\omega_6$. This is in fact clearly indicated in Fig. 1 and Table II and may be a general rule. The importance of viewing isomers with methyl branches near the terminal end of the chain in terms of $\omega_y$ is supported by the similar relationship of the $C_4$ to the $\omega_5$ position in both the methyloctadecanoate and methylhexadecanoate series.

The impression may be gained from the data in Table I and the close similarity of literature ECL values for 3,7,11-trimethyldodecanoic acid on EGA that the ECL values on polyester substrates for multiple-branched fatty acids are subject to very little variation. This is not necessarily correct, as shown by the literature data for the β-CDX esters[28] and other studies[22,31].

Apolar substrates have not been evaluated for additive FCL values since it has been shown that on SE-30 and QF-1 columns[22,32] the effect of relative concentration

makes retention times for small components unreliable. It is not known if this is true of the widely used Apiezon substrates.

## EXPERIMENTAL

The data in Table I were obtained with BDS columns (150 ft. in length × 0.01 in. I.D.) in a Perkin-Elmer 226 gas chromatograph. Injection port temperature was 260°, carrier gas helium at 40 p.s.i.g. A No. 2 inlet splitter was employed. The marine oil sample was obtained by hydrogenating the methyl esters of cod liver oil triglycerides. Methyl esters of several authentic *iso* and *anteiso* fatty acids were found to coincide with appropriate peaks, and homologues were identified by plotting procedures. Authentic multiple-branched fatty acids were also co-chromatographed with pure linear fatty acids. Column No. 1 was new, column No. 2 had lost considerable efficiency with prolonged use[18].

## SUMMARY

The generation from literature data of fractional chain length (FCL) values for single methyl substituents in esters of isomeric monomethyl-branched fatty acids is described. The addition of FCL values to the basic chain length gives good agreement between calculated and experimental equivalent chain length (ECL) values for esters of several multiple-branched fatty acids of known structure and is used to verify the structure tentatively assigned to another multiple-branched fatty acid ester.

## NOTE ADDED IN PROOF

The tabulated retention data[24] for 15-methyloctadecanoate are in error, but the associated figure is correct[33]. The FCL value for an $\omega_3$ acid can therefore be taken as marginally greater than that for the $\omega_4$ acid.

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# THE DETERMINATION OF SUGARS AND AMINO SUGARS IN THE HYDROLYSATES OF MUCOPOLYSACCHARIDES BY GAS–LIQUID CHROMATOGRAPHY

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## INTRODUCTION

Recent progress has been made in the application of gas–liquid chromatography to the separation of carbohydrates and related polyhydroxy compounds. As these materials, themselves, are not amenable to gas–liquid chromatography, efforts have been made to convert them to sufficiently volatile derivatives which can be conveniently prepared on a micro scale. At the present time there would seem to be little doubt that the most satisfactory derivatives both from the point of view of performance and ease of preparation are the O-trimethylsilyl ethers of the carbohydrates. A number of papers have appeared describing separations using these derivatives[1–4] and in the latter three cases quantitative results are cited for both neutral and amino sugars. In our previous communication[5] we described the separation and estimation of the neutral monosaccharides found in hydrolysates of human gastric mucopolysaccharides and saliva, and in this work the technique has been extended to include quantitative data on the two amino sugars D-galactosamine and D-glucosamine normally found in the above biological materials.

### *Materials*

Pyridine (AnalaR grade; British Drug Houses Ltd., Poole, Dorset) was dried overnight with BaO and distilled. The distillate was kept over $CaH_2$ in an amber-coloured bottle.

Trimethylchlorosilane (reagent grade; Hopkin & Williams Ltd., Chadwell Heath, Essex), hexamethyldisilazane (Koch-Light Laboratories Ltd., Colnbrook, Bucks.), and ethyl chloroformate (reagent grade; British Drug Houses Ltd.) were used directly. D-Galactitol, α-D-glucose, α-D-galactose, β-D-mannose, α-L-fucose, D-glucosamine and D-galactosamine (AnalaR or reagent grade; British Drug Houses Ltd.) were chromatographically pure and used as supplied.

"De-Acidite" G (7–9 % crosslinking) 52–100 mesh or 100–200 mesh was treated as follows. The resin was suspended in water, poured into a column and washed with several column volumes of 2 *M* $Na_2CO_3$ until chloride-free. Washing was continued with water until carbonate-free. Dowex 50 W (X 12,200–400 mesh) was regenerated with NaOH followed by HCl and water.

### *Preparation of N-carboethoxy D-glucosamine and N-carboethoxy D-galactosamine*

D-Glucosamine hydrochloride or D-galactosamine hydrochloride (2 g) was

dissolved in water (20 ml) and to the resulting solution was added ethyl chloroformate (1 ml) and sodium bicarbonate (1.7 g). The reaction mixture was allowed to stand at room temperature overnight. The product was deionized firstly by means of an electrolytic desalter and then by columns of "De-acidite" G (7–9 % crosslinking, 52–100 mesh) and Dowex 50W (X 12, 200–400 mesh). The effluent from the columns was finally freeze-dried to afford the N-carboethoxy amino sugars. Purification was carried out by repeated recrystallisations from propan-1-ol for the D-glucosamine derivative (m.p. 175°) and acetone for the D-galactosamine derivative (m.p. 90°).

## METHODS

### *Gas–liquid chromatography*

The separations were obtained on a Pye Series 104 Chromatograph equipped with a hydrogen flame detector, temperature programming unit and a Honeywell-Brown recorder. Column packings were prepared by coating Chromosorb G (AWDMCS, 80–100 mesh) (Johns-Manville Corp., New York), with a solution of Apiezon L and neopentyl glycol adipate (Applied Science Laboratory Inc., State College, Conn., U.S.A.) in benzene to give a final concentration of 2 % (w/w) and 4 % (w/w) respectively, in the final dried packing.

The usual Pye (5 ft. × 3/32 in. I.D.) coiled glass columns were packed with the above material by means of a vacuum applied to the end of the columns, while adding the packing at the other end with simultaneous vibration. Columns were finally conditioned by heating to 245° for 48 h with a nitrogen flow of 100 ml/min and then for a further 24 h at 210° with the same flow rate.

The separations obtained in this report were generally achieved under the following conditions:

Nitrogen carrier gas flow rate 40–45 ml/min; linear temperature programming at 1.5°/min from an initial temperature of 130° to a final temperature of 210°.

Detector conditions: hydrogen flow rate 40–45 ml/min, air flow rate 500 ml/min approx., detector voltage, 45 V.

The amplifier was generally set to an attenuation of $1 \times 10^3$ and the chart speed at 10 in/h.

The stainless steel injection port supplied with the Pye 104 series Chromatograph can only be used to inject liquid samples on to the column. However, widening out the narrow hole in the port to one 5/32 in. diameter rendered it very suitable for the solid injection of small glass tubes (1 cm × 0.4 cm) containing plugs of glass wool. The products of the silylation reaction were injected directly on to these glass wool plugs and the volatile solvents rapidly removed by means of a stream of nitrogen. A few minutes later the glass tube was dropped into the top of the column after shutting off the carrier gas and removing the screw cap and septum from the injection port. Immediately following addition, the septum and cap were replaced and the carrier gas supply turned on. At the end of each run the glass tube was removed from the column by means of a piece of thin wire inserted into the glass wool plug. Peak areas were measured by multiplying the peak height by the width of the peak at half its height.

### *Hydrolysis of the mucopolysaccharides and preparation of the trimethylsilyl derivatives*

The mucopolysaccharide solutions or suspensions were hydrolysed by rendering them 0.3 *N*, 0.5 *N* and 2 *N* with appropriate amounts of conc. HCl. Usually 5–10 ml

of the material was taken for analysis and the acidified product hydrolysed in a 25 ml screw-capped bottle at 100° for 16 h (overnight). After cooling, a suitable amount of the internal standard (D-galactitol) based on the original volume of mucopolysaccharide solution was added to the material in the screw-capped bottle and the bottle washed out with an equal volume of water in the case of the 0.3 *N* and 0.5 *N* hydrolysates, but with five times its volume of water for the 2 *N* hydrolysates. These solutions were now deacidified by passing down small columns (1 cm × 8 cm) of "De-acidite" G (7–9 % crosslinking 52–100 mesh), the first 4 ml of eluent being rejected and the next 5 ml being collected in a test tube. Occasionally as considerable quantities of $CO_2$ were liberated during the passage of the hydrolysate down the columns it was sometimes found advantageous to disturb the resin from time to time with a piece of wire to dislodge gas locks. A column of the above size could cater for about 15 ml of 0.3 *N* HCl hydrolysate, and the pH of the eluent was normally between 6.0 and 7.0. Five millilitres of the eluent from the "De-acidite" G column were now treated with 0.3 ml of a freshly prepared saturated solution of $NaHCO_3$ followed by 0.025 ml (2 drops) of ethyl chloroformate; the tube was immediately corked, shaken vigorously and allowed to stand at room temperature for about 1 h. The product was deionized by passing down a small column (1 cm diam.) containing a layer of "De-acidite" G (7–9 % crosslinking, 100–200 mesh) (2 cm height), over which was carefully laid a layer of Dowex 50W (X 12, 200–400 mesh) $H^+$ form (2 cm height). The first 2.5 ml of eluate was rejected and the next 2 ml collected and pipetted into a flat bottomed phial (20 ml) equipped with a well-fitting polythene stopper. The aqueous solution of sugars and N-carboethoxylated amino sugars so obtained was evaporated to dryness in a vacuum desiccator over NaOH pellets, and finally silylated with the pyridine–silanes reagent (0.4 ml). This amount of reagent was found to give a maximum yield for up to at least 8 mg of carbohydrate material. The reaction mixture so obtained was allowed to stand at room temperature for about 30 min, this being ample time for complete reaction, although samples could be left much longer without any appreciable diminution of peak areas. A 10 μl Hamilton syringe was used to inject a suitable amount (usually 5–20 μl) on to the glass plug for chromatography.

The pyridine–silanes reagent was prepared by taking the dried pyridine (10 ml) and adding to it hexamethyldisilazane (3 ml) and trimethylchlorosilane (2 ml). The container was stoppered immediately and the liquid swirled gently. This reagent gave satisfactory results for up to at least 3 days, standing at room temperature.

The assay for fucose by the thioglycollic acid technique[6] was carried out on samples of eluates from the "De-acidite" G columns. The determination of total hexosamine by the Elson-Morgan technique modified by BOAS[7] was also carried out on these eluates. These samples were also used for the determination of D-glucosamine and D-galactosamine carried out on the Technicon automatic amino acid analyser (Technicon Instrument Co. Ltd., Chertsey, Surrey), with the usual buffer conditions for a 20-hour chromatogram.

## RESULTS

### *Preparation of N-carboethoxy D-glucosamine, N-carboethoxy D-galactosamine and their respective trimethylsilyl ethers for chromatography*

In this series of experiments, D-galactitol was used to follow the N-carbo-

ethoxylation of the two amino sugars in order to determine the optimum reaction conditions for quantitative yields. Standard solutions of the two amino sugars and D-galactitol were prepared by dissolving suitable weighed amounts in water. Aliquots (5 ml) of these standards were taken and reacted with quantities of saturated sodium bicarbonate solution, varying between 0 and 0.4 ml, while the ethyl chloroformate was varied between 0.01 ml–0.04 ml. It was found that except where sodium bicarbonate was absent, the peak areas obtained on subsequent gas–liquid chromatography were not affected within the above limits of the reagents. The reaction time in all cases was one hour at room temperature. In view of the above results, it was decided to use the middle of the range for further experiments, *i.e.* 0.3 ml sodium bicarbonate solution and 0.025 ml ethyl chloroformate. Investigations into the reaction time were now carried out using the above quantities of reagents, and 5 ml of the standard solution. These results are shown in Table I. It will be seen that the N-carboethoxylation was complete for both amino sugars within 15 min, and that the reaction conditions had no deleterious effect on the glucose present, even in the final experiment, which was heated to 80°. In view of these results, one hour at room temperature was generally employed for the reaction.

TABLE I

THE REACTION OF D-GLUCOSAMINE AND D-GALACTOSAMINE WITH ETHYL CHLOROFORMATE IN THE PRESENCE OF SODIUM BICARBONATE

A solution (5 ml) containing D-glucose, D-galactitol, D-glucosamine (1 m$M$/l) and D-galactosamine (0.5 m$M$/l) was treated with saturated $NaHCO_3$ (0.3 ml) and Cl·COOEt (0.025 ml). After the appropriate reaction time, the mixture was treated with ion exchange resin as described in the text, and finally silylated (5 $\mu$l taken for analysis). Amount recovered in respect to D-galactitol.

| *Reaction time (min)* | D-*Glucose* (m$M$/l) | D-*Glucosamine* (m$M$/l) | D-*Galactosamine* (m$M$/l) |
|---|---|---|---|
| 15 | 0.95 | 1.01 | 0.51 |
| 30 | 0.95 | 0.98 | 0.50 |
| 60 | 0.99 | 0.95 | 0.48 |
| 90 | 0.98 | 1.03 | 0.52 |
| 90 + 15 at 80° | 0.96 | 0.99 | 0.50 |
| Mean values | 0.97 | 0.99 | 0.50 |

The reactivity of the N-carboethoxy amino sugars with the pyridine–silanes reagent was compared to a typical hexose D-glucose and also with the internal standard D-galactitol. Known weights of the above compounds were dissolved in water and evaporated in the usual manner in a vacuum desiccator. The product was silylated as usual with 0.4 ml reagent, and 5 $\mu$l analysed by gas–liquid chromatography at varying time intervals. The areas of the various peaks for the anomers of each sugar were added together, and plotted against the silylation times as shown in Fig. 1. It will be seen that all the components react at approximately the same rate and that the reaction is complete within 10 min.

*Anomerisation of N-carboethoxy* D-*glucosamine and N-carboethoxy* D-*galactosamine*

When samples of the recrystallised N-carboethoxy amino sugars were reacted with the pyridine–silanes reagent, only one major peak was obtained for each sugar.

However, when these derivatives were prepared in the usual manner from the amino sugars, evaporated and the residue treated with the pyridine–silanes reagent, a second peak with a longer retention time appeared in addition to the original peak, for both sugars. This was considered to be derived from the other anomer of each respective N-carboethoxy amino sugar. For quantitation the areas of both peaks were added together. No attempt was made to correlate each peak to its respective anomeric form ($\alpha$ or $\beta$).

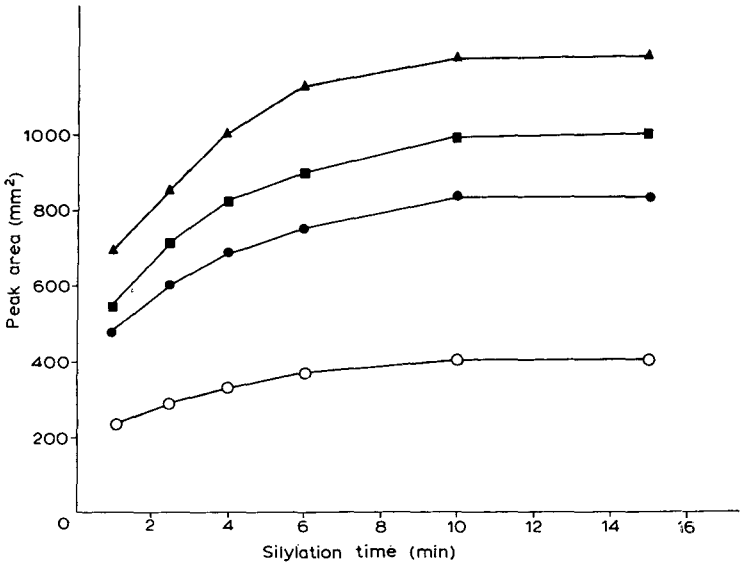


Fig. 1. The reactivity of the N-carboethoxy derivatives of D-glucosamine and D-galactosamine with the pyridine–silanes reagent. A solution (2 ml) containing D-glucose, D-galactitol, N-carboethoxy D-glucosamine (1 m$M$/l) and N-carboethoxy D-galactosamine (0.5 m$M$/l) was evaporated and the residue silylated in the usual manner with reagent (0.4 ml). 5 $\mu$l was taken for analysis. (▲) D-galactitol; (■) D-glucose; (●) N-carboethoxy D-glucosamine; (○) N-carboethoxy D-galactosamine.

*Quantitation of the pure monosaccharides*

Due to the anomerisation of the various sugars in water, subsequent chromatography gave two or more peaks from each monosaccharide[5]. The retention times for a mixture of L-fucose, D-galactose, D-mannose, D-glucose, N-carboethoxy D-glucosamine, N-carboethoxy D-galactosamine and the internal standard D-galactitol obtained by evaporating an aqueous equilibrium solution are shown in Fig. 2. The elution order was as follows: $\gamma$-fucose, $\alpha$-fucose, $\beta$-fucose, $\alpha$-mannose, $\gamma$-galactose, $\alpha$-galactose, D-galactitol, $\alpha$-glucose, $\beta$-mannose and $\beta$-galactose together, $\beta$-glucose, N-carboethoxy galactosamine (1st peak), N-carboethoxy glucosamine (1st peak), N-carboethoxy galactosamine (2nd peak), and finally N-carboethoxy glucosamine (2nd peak). It will be seen that under these conditions the $\beta$-mannose and $\beta$-galactose peaks failed to separate, so that it became necessary to find the relationship of the $\beta$-anomer of D-mannose to its $\alpha$-anomer. This was determined by preparing a solution of all the sugars with the exception of D-galactose and carrying out the analytical technique including rendering the mixture 0.3 $N$ and 0.5 $N$ with respect to hydrochloric acid.

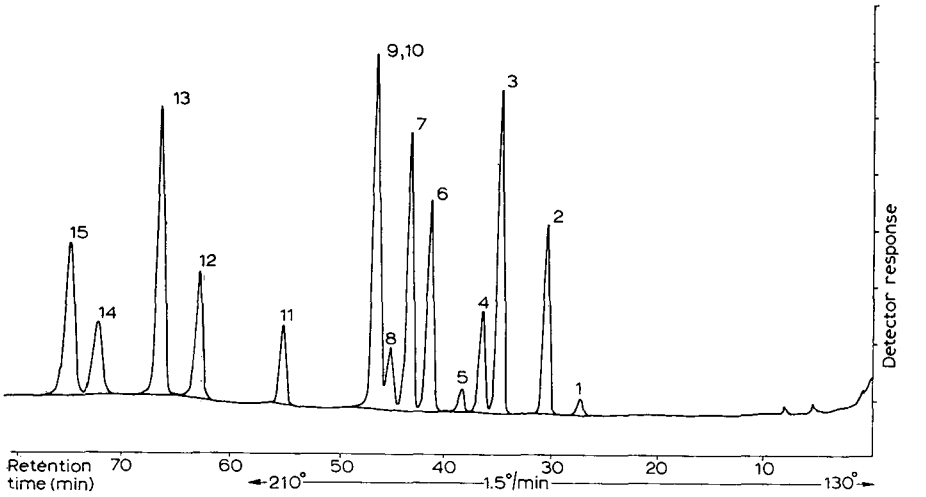


Fig. 2. Gas–liquid chromatography of an aqueous equilibrium mixture of the trimethylsilyl derivatives of D-galactose, D-glucose, D-mannose, L-fucose, N-carboethoxy D-glucosamine and N-carboethoxy D-galactosamine on Apiezon L + neopentyl glycol adipate polyester, with 40 ml $N_2$/min and linear temperature programming at 1.5°/min. The following are the assignments given to the various peaks in order of increasing retention times: 1 = $\gamma$-fucose; 2 = $\alpha$-fucose; 3 = $\beta$-fucose; 4 = $\alpha$-mannose; 5 = $\gamma$-galactose; 6 = $\alpha$-galactose; 7 = galactitol (std.); 8 = $\alpha$-glucose; 9 and 10 = $\beta$-mannose and $\beta$-galactose; 11 = $\beta$-glucose; 12 and 14 = N-carboethoxy galactosamine; 13 and 15 = N-carboethoxy glucosamine.

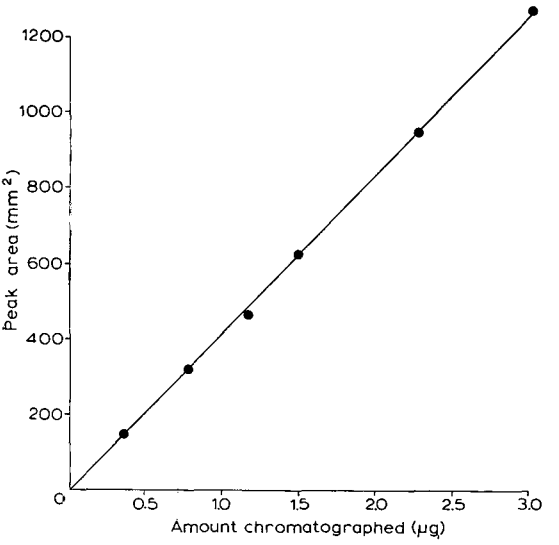


Fig. 3. Proportionality of the gas chromatographic response in terms of peak area to the quantity of N-carboethoxy D-glucosamine silylated for analysis.

Under these conditions it was found that the α-anomer varied between 68–70 % and the β-anomer between 32–30 %. This high level of reproducibility made it possible to calculate the total mannose content merely by measuring the α-mannose peak. The value of the β-mannose peak so calculated was subtracted from the measured β-galactose peak to give the true β-galactose value. The peak areas produced by the trimethylsilyl derivatives of the four sugars, two N-carboethoxy amino sugars and D-galactitol were plotted against the amount chromatographed (0.4–4 μg). In all cases, a linear plot passing through the origin was obtained when the various peaks for each sugar were added together. The plot for N-carboethoxy D-glucosamine is shown in Fig. 3.

By measurement of the slope of the plots obtained, it was possible to calculate the response of the hydrogen flame detector to each sugar derivative and also for the D-galactitol derivative. It was found that the detector response to the three hexose, L-fucose and D-galactitol derivatives was identical for the same weight of each derivative chromatographed. However, although the trimethylsilyl derivatives of the two N-carboethoxy amino sugars each gave an identical response, this response was somewhat less (20 % approx.) than for the other sugars. In this way, factors were determined to correct the observed peak areas for each sugar per mole to that area obtained per mole of D-glucose. These were as follows: D-glucose, D-galactose, D-mannose, 1.00; L-fucose, 1.20; D-galactitol, 0.88; D-glucosamine and D-galactosamine, 1.24.

*Quantitative analysis of pure monosaccharides by the gas chromatographic technique*

A mixture of the four sugars and two amino sugars was prepared in the following concentrations: L-fucose, D-galactose and D-glucosamine, 1 m$M$/l; D-galactosamine, 0.5 m$M$/l; and D-mannose and D-glucose, 0.25 m$M$/l. This solution (5 ml aliquots) was now treated with the required amount of conc. hydrochloric acid and internal standard (0.2 ml of a 10 m$M$/l solution) and subjected to the full analytical technique except that the overnight hydrolysis at 100° was omitted. The results obtained are reproduced in Table II. It will be seen that excellent agreement was obtained for every sugar. The above series of experiments was repeated except that the hydrolysis step at 100° overnight was now included (Table III). It will be seen that the neutral monosaccharides were recovered completely at 0.3 $N$ but at 0.5 $N$ slight destruction occurs, leading to somewhat low recoveries of L-fucose and D-galactose. For the 2$N$ conditions, this loss was considerable. The amino sugars were recovered unchanged under all these conditions.

*Comparison of the gas–liquid chromatography technique with the Technicon Auto-Analyser and the manual Elson-Morgan method*

A series of standard solutions of D-glucosamine and D-galactosamine were made up and their concentration determined by each of the above methods. In the auto-analyser technique, the buffer conditions were those normally employed for the determination of amino acids and nor-leucine was used as an internal standard. The Elson-Morgan technique measured total hexosamine[6]. The gas–liquid chromatography was carried out as previously described with D-galactitol as the internal standard. The results are shown in Table IV. It will be seen that good agreement was obtained between the three techniques.

TABLE II

QUANTITATIVE ANALYSIS OF A MIXTURE OF PURE MONOSACCHARIDES BY THE GAS CHROMATOGRAPHIC TECHNIQUE

A standard solution of the six sugars was prepared, acidified with the appropriate quantity of HCl and subjected to the usual analytical procedure as described in the text. All values are expressed in m$M$/l.

| *Hydrolysis normality* | D-*Galactose* (*1.00 mM/l*) | L-*Fucose* (*1.00 mM/l*) | D-*Mannose* (*0.250 mM/l*) | D-*Glucose* (*0.250 mM/l*) | D-*Glucosamine* (*1.00 mM/l*) | D-*Galactosamine* (*0.500 mM/l*) |
|---|---|---|---|---|---|---|
| 0.3 | 0.97 | 1.02 | 0.240 | 0.240 | 1.01 | 0.500 |
| 0.3 | 1.01 | 0.99 | 0.240 | 0.234 | 1.00 | 0.500 |
| 0.3 | 0.99 | 1.04 | 0.247 | 0.255 | 1.01 | 0.514 |
| 0.3 | 0.95 | 1.01 | 0.245 | 0.239 | 1.02 | 0.494 |
| 0.5 | 0.98 | 1.02 | 0.252 | 0.240 | 1.03 | 0.530 |
| 0.5 | 1.06 | 1.00 | 0.250 | 0.248 | 1.02 | 0.495 |
| 0.5 | 0.97 | 1.00 | 0.255 | 0.243 | 0.99 | 0.491 |
| Mean values | 0.99 | 1.01 | 0.247 | 0.245 | 1.01 | 0.504 |
| 2 | 1.02 | 1.04 | 0.240 | 0.253 | 1.00 | 0.500 |
| 2 | 1.01 | 1.04 | 0.242 | 0.236 | 1.04 | 0.500 |
| 2 | 0.98 | 1.00 | 0.245 | 0.248 | 1.00 | 0.491 |
| Mean values | 1.00 | 1.03 | 0.243 | 0.246 | 1.01 | 0.494 |

TABLE III

THE EFFECT OF INCREASING ACID STRENGTH ON THE RECOVERIES OF PURE MONOSACCHARIDES

A standard solution of the six sugars was prepared, hydrolysed at 100° overnight with the appropriate quantity of HCl and subjected to the usual analytical procedure as described in the text. All values are expressed in m$M$/l.

| *Hydrolysis normality* | D-*Galactose* (*1.00 mM/l*) | L-*Fucose* (*1.00 mM/l*) | D-*Mannose* (*0.250 mM/l*) | D-*Glucose* (*0.250 mM/l*) | D-*Glucosamine* (*1.00 mM/l*) | D-*Galactosamine* (*0.500 mM/l*) |
|---|---|---|---|---|---|---|
| 0.3 | 1.01 | 1.00 | 0.236 | 0.240 | 1.00 | 0.482 |
| 0.3 | 0.98 | 1.00 | 0.240 | 0.260 | 0.98 | 0.471 |
| 0.3 | 0.98 | 0.97 | 0.253 | 0.255 | 1.01 | 0.483 |
| 0.3 | 0.97 | 0.99 | 0.244 | 0.260 | 1.02 | 0.496 |
| Mean values | 0.98 | 0.99 | 0.244 | 0.254 | 1.00 | 0.483 |
| 0.5 | 0.89 | 0.96 | 0.243 | 0.245 | 1.00 | 0.493 |
| 0.5 | 0.96 | 0.95 | 0.236 | 0.258 | 0.98 | 0.480 |
| 0.5 | 0.94 | 0.97 | 0.244 | 0.250 | 1.00 | 0.495 |
| 0.5 | 0.94 | 0.98 | 0.249 | 0.253 | 1.02 | 0.500 |
| Mean values | 0.93 | 0.96 | 0.243 | 0.251 | 1.00 | 0.492 |
| 2 | 0.72 | 0.66 | 0.122 | 0.180 | 1.04 | 0.495 |
| 2 | 0.62 | 0.60 | 0.144 | 0.166 | 1.03 | 0.512 |
| 2 | 0.69 | 0.43 | 0.102 | 0.178 | 1.03 | 0.472 |
| 2 | 0.72 | 0.73 | 0.183 | 0.167 | 1.01 | 0.492 |
| Mean values | 0.64 | 0.63 | 0.138 | 0.173 | 1.03 | 0.493 |

TABLE IV

A COMPARISON OF THE GAS–LIQUID CHROMATOGRAPHIC, TECHNICON AUTO ANALYSER AND ELSON-MORGAN TECHNIQUES FOR THE DETERMINATION OF HEXOSAMINES

$GNH_2$ = D-glucosamine; Gal $NH_2$ = D-galactosamine. The analytical procedures are described in the text.

| *Amount taken (mM/l)* | | *Amount recovered (mM/l)* | | | | |
|---|---|---|---|---|---|---|
| $GNH_2$ | *Gal* $NH_2$ | *Technicon Auto Analyser* | | *Gas–liquid chromatography* | | *Elson-Morgan* |
| | | $GNH_2$ | *Gal* $NH_2$ | $GNH_2$ | *Gal* $NH_2$ | *Total hexosamine* |
| 0.20 | 0.10 | 0.198 | 0.091 | 0.201 | 0.090 | 0.29 |
| 0.30 | 0.15 | 0.281 | 0.146 | 0.320 | 0.158 | 0.50 |
| 0.36 | 0.18 | 0.336 | 0.178 | 0.340 | 0.175 | 0.57 |
| 0.40 | 0.20 | 0.391 | 0.179 | 0.440 | 0.207 | 0.61 |
| 0.60 | 0.30 | 0.567 | 0.290 | 0.620 | 0.316 | 0.93 |

*The release of monosaccharides from gastric secretion during acidic hydrolysis*

In this series of experiments, gastric secretion obtained from patients under fasting conditions was subjected to hydrolysis under varying normalities of HCl at 100° overnight. These results are shown in Table V. It will be seen that the sugars are generally released in the following order: L-fucose first, then the three hexoses at

TABLE V

THE RELEASE OF MONOSACCHARIDES FROM GASTRIC SECRETIONS DURING ACIDIC HYDROLYSIS

All gastric secretions were hydrolysed overnight at 100° with the appropriate quantity of HCl. The analytical procedure was as described in the text. All values are expressed as m*M*/l.

| *Hydrolysis normality* | D-*Galactose* | L-*Fucose* | | D-*Mannose* | D-*Glucose* | D-*Galactosamine* | D-*Glucosamine* | *Total hexosamine Elson-Morgan* |
|---|---|---|---|---|---|---|---|---|
| | | *Gas chromatography* | *Thioglycollic acid* | | | | | |
| *Gastric secretion (1)* | | | | | | | | |
| 0.1 | 0.310 | 0.217 | 0.23 | 0.074 | 0.077 | 0.120 | 0.253 | 0.48 |
| 0.3 | 0.315 | 0.230 | 0.22 | 0.074 | 0.074 | 0.150 | 0.303 | 0.47 |
| 0.5 | 0.300 | 0.217 | 0.22 | 0.074 | 0.090 | 0.133 | 0.288 | 0.47 |
| 1.0 | 0.220 | 0.110 | 0.13 | 0.060 | 0.070 | 0.130 | 0.270 | 0.47 |
| 2.0 | 0.123 | 0.070 | 0.10 | 0.030 | 0.043 | 0.135 | 0.280 | 0.42 |
| 3.0 | — | — | — | — | — | 0.145 | 0.270 | 0.42 |
| *Gastric secretion (2)* | | | | | | | | |
| 0.05 | 0.64 | 0.274 | 0.34 | 0.114 | 0.230 | 0.099 | 0.420 | 0.82 |
| 0.1 | 0.78 | 0.371 | 0.33 | 0.171 | 0.291 | 0.142 | 0.560 | 0.86 |
| 0.3 | 0.75 | 0.355 | 0.35 | 0.150 | 0.290 | 0.155 | 0.631 | 0.85 |
| 0.5 | 0.74 | 0.355 | 0.35 | 0.150 | 0.293 | 0.165 | 0.641 | 0.85 |
| 1 | 0.67 | 0.330 | 0.34 | 0.130 | 0.243 | 0.167 | 0.625 | 0.84 |
| 2 | 0.24 | 0.074 | 0.09 | 0.151 | 0.103 | 0.170 | 0.624 | 0.82 |
| 3 | — | — | — | — | — | 0.180 | 0.647 | 0.80 |
| *Gastric secretion (3)* | | | | | | | | |
| 0.3 | 0.923 | 0.403 | 0.39 | 0.135 | 0.150 | 0.253 | 0.916 | 1.26 |
| 0.5 | 1.10 | 0.421 | 0.39 | 0.160 | 0.253 | 0.276 | 1.03 | 1.48 |
| 2 | — | — | — | — | — | 0.353 | 1.05 | 1.48 |
| 3 | — | — | — | — | — | 0.360 | 1.00 | 1.48 |

approximately the same rate and finally D-glucosamine and D-galactosamine. These and other experiments not shown in Table V, led to the conclusion that in general L-fucose was released completely at 0.3 *N* HCl, and very often the three hexoses were also released. At 0.5 *N* HCl, L-fucose was showing a certain amount of destruction as also were the hexoses in some cases, although in general only slightly. D-glucosamine usually reached a maximum value at this normality of acid, and in certain cases, D-galactosamine also. In many instances, however, it was found necessary to resort to 2 *N* HCl for complete release of D-galactosamine. Under these conditions all the neutral sugars suffered destruction, making their analytical values useless. In view of these results, it was decided to standardise 0.3 *N*, 0.5 *N* and 2 *N* HCl hydrolysis conditions for all material under investigation, to obtain a complete record for every sugar. Where only amino sugars were being investigated, however, it was necessary to carry out the 2 *N* HCl hydrolysis only. The reproducibility of a series of hydrolyses on the gastric secretion obtained from one individual is shown in Table VI. The agreement obtained under any one set of conditions was excellent, although the values of the various sugars vary under different hydrolytic conditions, due to destruction on the one hand and further sugar release on the other.

It should be noted that in several of the tables, values for L-fucose as determined by the thioglycollic acid method, and total hexosamine by the Elson-Morgan technique have been included for comparison with the gas–liquid chromatography figures.

TABLE VI

QUANTITATIVE ANALYSIS OF MONOSACCHARIDES OBTAINED BY THE HYDROLYSIS OF A POOLED GASTRIC SECRETION

The gastric secretion was hydrolysed overnight at 100° with the appropriate quantity of HCl. Aliquots were taken for analysis by the procedures described in the text. All values are expressed in m*M*/l.

| *Hydrolysis normality* | D-*Galactose* | L-*Fucose* | D-*Mannose* | D-*Glucose* | D-*Glucos-amine* | D-*Galactos-amine* |
|---|---|---|---|---|---|---|
| 0.3 | 0.664 | 0.562 | 0.137 | 0.121 | 0.594 | 0.107 |
| 0.3 | 0.645 | 0.553 | 0.134 | 0.121 | 0.564 | 0.117 |
| 0.3 | 0.621 | 0.560 | 0.135 | 0.117 | 0.560 | 0.112 |
| 0.3 | 0.660 | 0.570 | 0.135 | 0.122 | 0.560 | 0.111 |
| Mean values | 0.650 | 0.562 | 0.135 | 0.120 | 0.570 | 0.112 |
| 0.5 | 0.585 | 0.492 | 0.136 | 0.110 | 0.643 | 0.154 |
| 0.5 | 0.615 | 0.455 | 0.130 | 0.103 | 0.664 | 0.160 |
| 0.5 | 0.600 | 0.500 | 0.131 | 0.105 | 0.680 | 0.162 |
| 0.5 | 0.630 | 0.502 | 0.133 | 0.111 | 0.674 | 0.160 |
| Mean values | 0.607 | 0.488 | 0.132 | 0.108 | 0.665 | 0.159 |
| 2 | — | — | — | — | 0.630 | 0.162 |
| 2 | — | — | — | — | 0.690 | 0.167 |
| 2 | — | — | — | — | 0.655 | 0.155 |
| 2 | — | — | — | — | 0.660 | 0.162 |
| 2 | — | — | — | — | 0.640 | 0.165 |
| 2 | — | — | — | — | 0.630 | 0.158 |
| Mean values | — | — | — | — | 0.651 | 0.162 |

Generally good agreement was obtained. A typical chromatogram for a gastric secretion hydrolysed with 0.5 *N* HCl is reproduced in Fig. 4.

Investigations undertaken with saliva showed that its component sugars were released under very similar conditions to the gastric mucopolysaccharides and the same hydrolysis conditions were therefore standardised for experiments on this material.

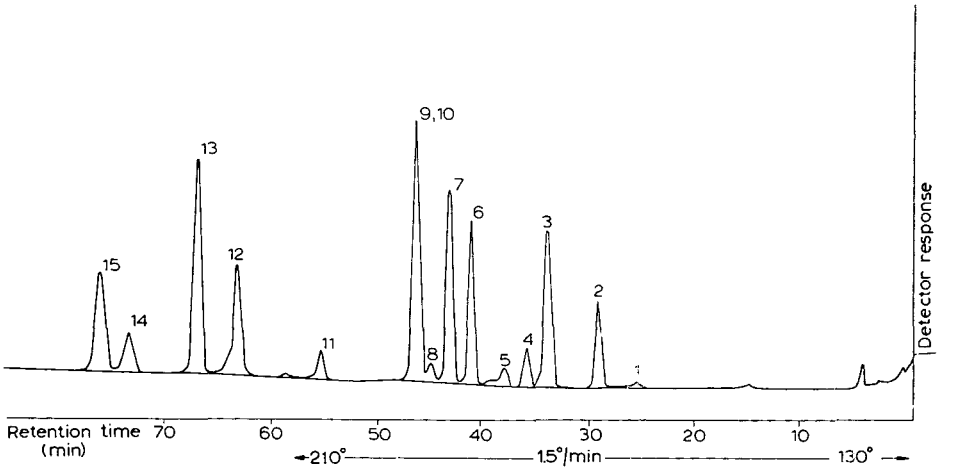


Fig. 4. A typical gas–liquid chromatogram of a gastric secretion hydrolysed with 0.5 *N* HCl and analysed as described in the text. The chromatogram was obtained under the same conditions as given in the legend of Fig. 2, and similar assignments have been given to the peaks.

## DISCUSSION

Previous investigators[2,4] working on the estimation of amino sugars by gas–liquid chromatography, have commonly used the trimethylsilyl ethers of the N-acetyl amino sugars. During preliminary work, the trimethylsilyl derivatives of both the free and N-acetylated amino sugars were examined. It was found that the derivatives of the free amino sugars gave much smaller peaks than would have been expected, and that these peaks had a similar retention time to α-galactose. It was therefore considered that these derivatives did not merit further investigation. The trimethylsilyl ethers of the N-acetyl amino sugars gave only one peak when prepared from each respective crystalline solid N-acetyl amino sugar, but when prepared by acetylation of the amino sugars directly, two major peaks appeared and also sometimes a third minor peak in both cases. Attempts to quantitate the derivatives of the two N-acetyl amino sugars were only partially successful as the reproducibility from sample to sample was poor. This appeared to be due to decomposition or absorption on the column, with consequent reduction in the expected peak areas. BISHOP, COOPER AND MURRAY[8] mention the decomposition of fully acetylated amino sugars on gas chromatography columns, and it may well be that similar reactions occur with these N-acetyl-O-trimethylsilyl derivatives. RICHEY, RICHEY JR. AND SCHRAER[3] carried out quantitative experiments on both N-acetyl glucosamine and N-acetyl galactosamine and also apparently suffered from column absorption effects, necessitating rigorously controlled conditions

for successful quantitative analysis. In view of these preliminary experiments with N-acetylated amino sugars, it was decided to investigate the possibility of using other derivatives for gas–liquid chromatography, namely the trimethylsilyl ethers of the N-carboethoxy amino sugars. The experiments carried out with these derivatives showed that they did not suffer any absorption or apparent decomposition on the columns used, and as expected, only two peaks were obtained from each of the two amino sugars. The detector response for the two derivatives was also identical. These findings indicate the superiority of these derivatives over the N-acetyl-O-trimethylsilyl compounds; in addition, their preparation is easier and does not require the use of an internal standard to follow the N-carboethoxylation step in the procedure. Solid injection was again adopted[5,9] in order to allow the use of large volumes of sample (10 $\mu$l or more). This technique had the further advantage of eliminating column contamination, it only being necessary to replace the glass wool plugs occasionally when they became very dirty. In our previous communication[5] it was noted that contamination of the sample with water vapour resulted in a highly objectionable rising base line which often stretched as far as $\beta$-glucose; it has been found, however, that the hydrogen flame detector does not show this effect, so that complete exclusion of water from the sample and pyridine–silanes reagent is not now of paramount importance. Furthermore, the flame detector has been found preferable as it does not suffer from contamination and response variations to the same extent as the argon detector when used with these silicon-containing derivatives. The flame detector has now been in daily use for over six months and has given no trouble apart from requiring occasional jet cleaning, indicating its suitability for the analysis of derivatives of the type under investigation.

It was found that the separation of the various sugar anomers could be controlled by slight variations in the ratio of the Apiezon L to the neopentyl glycol adipate. It was felt that this was a more convenient technique than searching for one stationary phase of a suitable polarity to bring about the required separations. It might be considered that the estimation of D-mannose using only the $\alpha$-mannose peak is not completely satisfactory and indeed this would probably be the case where the ratio of D-mannose to D-galactose is high; in the case of gastric secretion and saliva, however, where the D-mannose content is generally comparatively low, the estimation of the $\alpha$-mannose peak only gives highly reproducible results for both D-galactose and D-mannose.

It was found that the response per unit mass of the hydrogen flame detector to the various trimethylsilyl derivatives of the four neutral sugars and for D-galactitol was identical, thereby indicating that the trimethylsilylation reaction was quantitative. In the case of the two N-carboethoxy amino sugars, however, the response per unit mass was about 20 % less than might have been expected, presumably due to the different structural group present in these compounds[10], as no evidence for column absorption tendencies was noted.

The results show that for the pure amino sugars, excellent agreement was obtained between the Elson-Morgan technique and that involving the Technicon Auto-Analyser, but obviously that the gas chromatographic technique is much quicker than the Auto-Analyser and superior to the Elson-Morgan technique, which only gives the total hexosamine value. It should be noted that the total hexosamine values for gastric secretion determined by the Elson-Morgan method do not always

agree with the gas chromatography method and, in general, are often significantly higher, particularly where complete acidic hydrolysis has not been achieved. This is considered to be due to two factors, firstly that the colour reaction is still complete even though the amino sugars are not completely released and secondly that the neutral sugars and amino acids present in the hydrolysates have combined to form a chromogen which contributes to the Elson-Morgan reading[11,12].

It is apparent, therefore, that gas chromatography marks a notable advance over the above technique in that it can be conveniently used to follow the release of each amino sugar in the hydrolysates of mucopolysaccharides, and also, of course, for the concomitant release of any neutral sugars present as well, thereby affording information on structural positions of monosaccharides present in the mucopolysaccharides. The experiments carried out with gastric secretions to determine the optimum hydrolytic conditions for the various sugars show the importance of these conditions to obtain complete hydrolysis without significant loss of the more labile neutral sugars. It was found impossible to arrive at one particular acid strength to release all the sugars without some destruction of the more labile ones and furthermore, gastric secretions tended to vary in their resistance to hydrolysis; for this reason, it was necessary to standardise three different hydrolytic conditions to obtain full analytical data for the four neutral and two amino sugars normally present in gastric secretions. It should be mentioned that the gas chromatographic technique for analysing gastric secretions has now been in daily use for some six months and have given virtually no trouble during this time, thereby indicating its suitability for the analysis of these materials on a routine basis.

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SUMMARY

Conditions are described for the simultaneous gas chromatographic determinations of L-fucose, D-mannose, D-galactose, D-glucose, D-glucosamine and D-galactosamine.

The technique involves the carboethoxylation of the amino group present in the amino sugars followed by trimethylsilylation of the hydroxyl groups. The neutral monosaccharides are trimethylsilylated directly in the usual manner and the final reaction mixture analysed by gas–liquid chromatography on a single column with temperature programming.

The release of the component monosaccharides from human gastric mucopolysaccharides and saliva with increasing acidity was followed by gas–liquid chromatographic determinations on the hydrolysates.

Quantitative data on standard mixtures of amino sugars showed good agreement between this technique and others, involving either the Technicon Auto-Analyser, or the manual Elson-Morgan method.

The gas chromatographic technique is considered to be superior to either of the

above methods of analysis, particularly with regard to sensitivity, speed and convenience.

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# GAS–LIQUID CHROMATOGRAPHY OF ALDITOL ACETATES AND ITS APPLICATION TO THE ANALYSIS OF SUGARS IN COMPLEX HYDROLYSATES

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## INTRODUCTION

Three main groups of derivatives have been successfully used for gas–liquid chromatography (GLC) of sugars, *viz.* methyl ethers, trimethylsilyl (TMS) ethers and acetates[1]. Although O-methylated sugars can be prepared practically quantitatively, the technique does not lend itself to routine handling of hydrolysates and will probably be reserved for structural studies of polysaccharides. Both TMS ethers and acetates are easy to prepare but the TMS ethers cannot be prepared quantitatively; SWEELEY *et al.*[2] quote 90 % conversion for glucose; PERRY[3], 85 % conversion for glucosamine. In addition, many of the TMS derivatives cannot be obtained in crystalline form. Thus the acetates are the most suitable derivatives for investigating complex hydrolysates as most can be obtained in crystalline form and characterised by melting point as well as by GLC. The work of GUNNER, JONES AND PERRY[4,5] established the thermal stability of alditol acetates and they also succeeded in separating some of these compounds. Much improved separations have since been reported by SAWARDEKER, SLONEKER AND JEANNES[6]. It is essential to reduce the sugars to the corresponding alcohols to avoid the multiplicity of peaks which can arise with a hydrolysate containing a range of sugars each capable of producing two anomeric and two ring isomers, all of which may be separated during GLC.

The technique described in this paper was developed primarily to deal with soil hydrolysates. Such hydrolysates contain a wide range of sugars as shown by paper chromatography, and in addition a vast range of other organic and inorganic materials. The carbohydrate content of soils may be less than 0.1 % in mineral soils and as high as 20 % in peats. A technique which can be applied successfully to soil hydrolysates can probably be used with most other complex hydrolysates.

## METHODS

Gas–liquid chromatography was carried out using a Perkin Elmer 801 gas chromatograph with dual flame ionisation detector and controlled heating of both injection port and detector. Glass columns 1.84 m long by 0.32 cm internal diameter were used.

*Materials*

*Solid supports.* Embacel (May and Baker) acid washed Chromosorb, 60 to 100 mesh, and hexamethyldisilazane (HMDS) coated Chromosorb W, 60 to 80 mesh, (Johns Mansville) were used.

*Liquid phases.* A range of liquid phases were investigated including those described by GUNNER, JONES AND PERRY[4,5] but only the following were found useful.

LAC IR 296 (Cambridge Laboratories, England)—a polymer of diethylene glycol adipate.

DD 071 (Perkin Elmer)—as for LAC IR 296 but reported to possess improved thermal stability.

ECNSS-M (Applied Science Laboratories, Pa., U.S.A.)—a medium polarity liquid phase based on a chemical combination of a cyanoethyl silicone polymer with ethylene glycol succinate polyester.

The best results in terms of peak shape, resolution and useful life of columns were obtained with 10 % w/w liquid phase on the support. Smaller amounts tended to result in peak tailing and larger amounts caused severe liquid phase bleed. Conditioning the 10 % columns for several days at 220° in a stream of nitrogen was necessary for satisfactory results and resolution gradually improved during the first two or three weeks of continual use. Cooling below about 100° even for a few minutes and then reheating to working temperature resulted in broad peaks which only became sharp after overnight heating at 220°. Retention volumes gradually decreased over a period of several weeks.

Separations of the alditol acetates were possible using carrier gas flow rates from 30 to 100 ml nitrogen per minute at temperatures from 180 to 220°. The best combination of these two parameters was found to be a flow rate between 30 and 50 ml nitrogen per minute and a linear temperature programme from 170 to 220° at 0.8°/min. The temperature programme was commenced immediately after the injection.

*Standard alditol acetates.* Alditol acetates were obtained by reduction of the parent sugars with sodium borohydride in aqueous solution overnight. Excess borohydride was destroyed by addition of acetone and sodium removed from the reaction mixture by passage through $H^+$ Dowex-50. Borate was removed as far as possible by continued addition and removal of methanol, using a rotary evaporator. The alditols were acetylated at room temperature overnight using acetic anhydride and redistilled pyridine (50/50). The alditol acetates were recrystallised from ethanol or methanol until only a single peak was given during GLC. The acetates which could not be obtained in crystalline form were purified by vacuum distillation onto a cold finger and by the column procedure, described below, for the purification of alditol acetates obtained from hydrolysates. A few preparations were obtained by acidic acetylation (2 % v/v concentrated $H_2SO_4$ in acetic anhydride, 60° for 10 min) of the alditols without removal of sodium or borate as described for the treatment of hydrolysates.

*Treatment of soil hydrolysates*

3 g of air dry soil were mixed to a slurry with 4 ml 72 % $H_2SO_4$ (v/v) and allowed to stand 2 h at room temperature. The acid concentration was reduced to 1 *N* and the suspension refluxed for 16 h. The clear supernatant obtained by centrifugation was decolourised by charcoal and the carbohydrate content determined with an-

throne[7]. The clarified solution was passed through a column of $Na^+$ Dowex-50 to remove iron and the eluate of about 300 ml adjusted to pH 7 with sodium bicarbonate before evaporation just to dryness in a rotary evaporator. The sugars were quantitatively extracted with methanol (6 × 50 ml) and the volume reduced to about 20 ml. About 1 mg of solid sodium borohydride was added and the solution allowed to stand overnight. A few drops of acetone were added, the sample evaporated to dryness and the reduced sugars acetylated using 2.5 % (v/v) concentrated $H_2SO_4$ in acetic anhydride at 60° for 10 min. The reaction mixture was cooled in ice, water added cautiously and then in sufficient quantity to render the reaction mixture immiscible with chloroform. The alditol acetates were extracted using chloroform. The bulked chloroform extracts were evaporated to dryness, and the sample taken up in a few drops of chloroform–methanol (50/50) and transferred quantitatively to a small wad of cotton wool. The solvents were *completely* removed in a stream of warm air when the sample on cotton wool was placed on top of a column (2 cm × 0.5 cm diameter) of Silica Gel G poured in benzene. At least 6 ml benzene were passed through the column under pressure. The alditol acetates, which remained on the column during the benzene washing were quantitatively eluted using several ml of chloroform–methanol (50/50). The solvent mixture was evaporated to dryness and the residue taken up in a known volume of chloroform for injection into the gas chromatograph. When possible the volume of solvent injected into the GLC column was kept to 1 $\mu$l or less to avoid liquid phase bleed.

## RESULTS AND DISCUSSION

### *Separation of standard alditol acetates*

Table I shows the separations obtained by gas–liquid chromatography of mixtures of standard alditol acetates on columns packed with 10 % w/w DD 071 and 10 % w/w ECNSS-M on HMDS coated Chromosorb W and the separations obtained by chromatography of the same mixtures of standards on a column packed with 5 % DD 071 plus 5 % ECNSS-M on HMDS coated Chromosorb W. The liquid phases based on diethylene glycol adipate were particularly useful for the separation of tetritol, pentitol and 6-deoxy hexitol acetates. However, this system did not resolve galactitol and glucitol acetates and thus has limited use for the study of complex hydrolysates. Conversely the other liquid phase (ECNSS-M) was useful for separation of hexitol acetates but did not resolve satisfactorily arabitol from ribitol and rhamnitol from fucitol acetate. A useful separation of all the alditol acetates was given by the mixed liquid phase system. The separation of hexitol acetates was favoured by an increase in the proportion of ECNSS-M while the total liquid phase concentration was maintained at 10 % w/w and *vice versa*. Thus the two liquid phases can be used separately to produce the best resolution within the group of sugars in which the experimenter is most interested or a mixture used to give reasonable separations over the complete range of compounds from glycerol to iditol acetate.

The order of appearance of the alditol acetates from the column is of some interest. BISHOP[1] stated that there appeared to be no rationale between the retention volumes of aldose derivatives and corresponding structures[4,5]. The sequence of alditol acetates shown in Table I under ECNSS-M has been obtained by SAWARDEKER, SLONEKER AND JEANNES[6] and also by WILLIAMS AND JONES[8] for acetylated iso-

TABLE I

RELATIVE RETENTION TIMES OF ALDITOL ACETATES AND RELATED COMPOUNDS[a]

| | *Melting*[b] *point* | *Liquid phase* | | |
|---|---|---|---|---|
| | | *10% LAC IR 296 or 10% DD 071* | *10% ECNSS-M* | *5% DD 071 + 5% ECNSS-M* |
| Tri-O-acetyl-D-glycerol | Syrup | 10 | 13 | 11 |
| Tetra-O-acetyl-D-erythritol | 85–86 | 39 | 42 | 40 |
| Tetra-O-acetyl-D-threitol | Syrup | 45 | 50 | 46 |
| Tetra-O-acetyl-2-deoxy-D-ribitol | Syrup | 57 | 60 | 58 |
| Penta-O-acetyl-6-deoxy-L-mannitol (rhamnitol) | Syrup[c] | 68 | 66 | 67 |
| Penta-O-acetyl-6-deoxy-L-galactitol (fucitol) | 126.5–127.5 | 73 | 69 | 72 |
| Tetra-O-acetyl-penta-erythritol | 74–75 | 70 | 72 | 72 |
| Penta-O-acetyl-D-ribitol | Syrup | 84 | 83 | 84 |
| Penta-O-acetyl-L-arabitol | 73–75 | 88 | 87 | 88 |
| Penta-O-acetyl-D-xylitol | 60.5–62 | 100[d] | 100 | 100 |
| Hexa-O-acetyl-D-allitol | 62.5–64.5 | 128 | 120 | 126 |
| 3-O-methyl-penta-O-acetyl-D-glucitol | Syrup[e] | 130 | 124 | 129 |
| Hexa-O-acetyl-D-mannitol | 123.5–124 | 137 | 126 | 134 |
| Hexa-O-acetyl-D-altritol | Syrup | 136 | 127 | 134 |
| Hexa-O-acetyl-D-galactitol | 165–166.5 | 146 | 133 | 142 |
| Hexa-O-acetyl-D-glucitol | 97.5–98.5 | 146 | 141 | 146 |
| Hexa-O-acetyl-D-iditol | 121.5–122.5 | 153 | 155 | 155 |
| Hexa-O-acetyl-myoinositol | 216.5–217.5 | 152 | 157 | 155 |

[a] Using linear temperature programme from 170 to 220° at 0.8° per minute.
[b] Corrected melting points obtained using a Kofler hot stage microscope.
[c] Crystals appeared but were unmanageable.
[d] The retention time for xylitol acetate was from 35 to 55 min depending on the carrier gas flow rate and age of the column.
[e] Contained several percent of glucitol acetate.

deoxyalditols. However, on other liquid phases glucitol and galactitol acetates have a reversed order of appearance[4,5], and Table I shows that this is also true of altritol and mannitol acetates on different liquid phases. GLC of the TMS ethers of the alditols has yielded a quite different sequence of retention times and confirms that at present there is no complete relationship between retention times and structure. With the exception of mannitol and galactitol acetates those alditols with the most groups in *cis* relationship on one side of the conventional zigzag planar chain have the longest retention times, *i.e.* those molecules with the closest approach of the acetyl groups remain on the column longest. It is possible that the zigzag planar carbon chain is not maintained when O-acetyl or more particularly O-trimethylsilyl groups are present on the molecule at temperatures near 200°, particularly when these groups have a *cis* relationship on alternate carbon atoms in the chain.

*Application of the method to complex hydrolysates*

*Removal of salts and reduction.* The clarification of hydrolysates and removal of iron by Dowex-50 were not necessary when dealing with hydrolysates of purified polysaccharides. However, with soil hydrolysates removal of iron was particularly

necessary otherwise considerable losses (75 %) of sugars occurred during rotary evaporation to dryness due to reduction of iron and consequent oxidation of some of the sugars. Similarly the methanol extractions can be omitted when the hydrolysate contains higher ratios of sugars to acid than can be obtained with soil hydrolysates. Large amounts of salt caused problems during acetylation when only microgram quantities of sugar were present. The borohydride reduction can be carried out directly in aqueous solution after neutralisation of the hydrolysate with sodium bicarbonate. The rate of reduction of the sugars at room temperature was followed using the anthrone procedure and was not entirely complete after 2 h, but no anthrone reaction could be detected after 5 or 6 h. Normally the reduction was allowed to proceed overnight.

*Acetylation.* Initially it was intended to carry out acetylation of the reduced sugars using acetic anhydride and pyridine. However, this was not possible presumably due to the fact that borate could not be completely removed from the reaction mixture even after exhaustive addition and evaporation of methanol after treatment with $H^+$ Dowex-50 to remove sodium. The same problem was encountered when 100 $\mu$g quantities of individual sugars were subjected to the reduction and basic acetylation procedure; alditol acetates were not detected in the final reaction mixtures until considerably larger quantities of starting material were used. These difficulties were overcome by the use of an acid catalyst during the acetylation and microgram quantities of sugar were reduced and acetylated by this method in the presence of large excesses of borate. The method is not particularly suitable for the acetylation of glycerol and the tetritols as other minor components appear in the reaction mixture. The amounts of these components is minimal ($< 5$ %) if the acetic anhydride contains only 2.5 % (v/v) concentrated $H_2SO_4$.

*Extraction of acetylated products.* The acid acetylation led to the formation of a number of artefacts which were produced by heating 2.5 % (v/v) concentrated $H_2SO_4$ in acetic anhydride in the absence of other materials. These artefacts ran with the alditol acetates during GLC so that their removal was essential. This was readily accomplished since they ran faster than alditol acetates during thin layer chromatography (TLC) on Silica Gel G nach Stahl using methanol in benzene (4 % v/v). Acetates were detected by the ferric hydroxamate spray of TATE AND BISHOP[9]. The position of the artefacts was determined by removal of successive bands of the thin layer ahead of the alditol acetates and extraction of any materials present by chloroform which was then concentrated and injected into the gas chromatograph. The separation of the alditol acetates from the artefacts was improved by repeated TLC in benzene as the acetates remained stationary in this solvent but the artefacts moved slowly. This information led to the development of the column procedure described. The elution of the artefacts from such columns using benzene was confirmed by concentration and injection of the benzene washings into the gas chromatograph. In addition to the removal of a number of peaks due to artefacts this procedure also removed materials which caused serious tailing after injection of the sample. Such materials ran near the front during TLC in methanol in benzene (4 % v/v) and were detected by iodine staining. Similar tailing after injection into the gas chromatograph often occurs with sugar acetates produced using pyridine and acetic anhydride. The column procedure removed the materials responsible for this tailing.

The chromatograms shown in Fig. 1 represent the monosaccharide composition

of complex carbohydrates from partly decomposed plant remains, a soil polysaccharide, the whole soil before removal of these two fractions and finally a peat.

Myoinositol may be used as an internal standard as it is commercially available in relatively pure form and was well separated from the hexitols under the conditions described. This necessitates two GLC runs on each hydrolysate because myoinositol was present in all the soils investigated. However, if this is done with the two different liquid phases, some predictions can be made concerning the identity of sugars responsible for some of the unlabelled peaks present on the chromatograms.

The chromatogram which shows the peat sugars in Fig. 1 is the result of addition of a known quantity of myoinositol to the hydrolysate of the peat soil. The relative

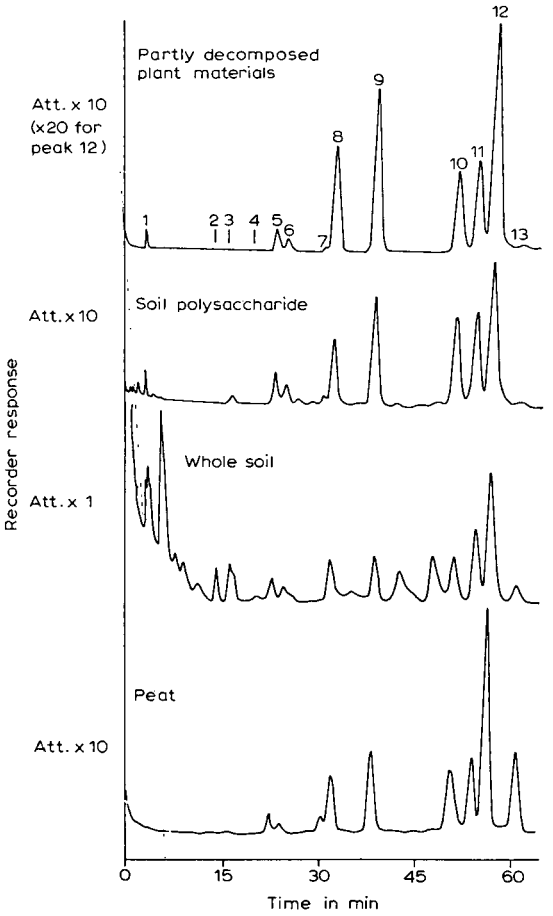


Fig. 1. Gas–liquid chromatography of alditol acetates from complex hydrolysates. Liquid phase: 10% w/w ECNSS-M on Chromosorb W coated with hexamethyldisilazane. Flow rate: 40 ml/min. Temperature: 170° by 0.8°/min to 220°. Attenuation: × 10; × 1 for whole soil sample; × 20 for glucitol acetate peak (peak 12) on the run for partly decomposed plant remains. Designation of peaks: 1 = Glycerol acetate; 2 = erythritol acetate; 3 = threitol acetate; 4 = 2-deoxy-ribitol acetate; 5 = rhamnitol acetate; 6 = fucitol acetate; 7 = ribitol acetate; 8 = arabitol acetate; 9 = xylitol acetate; 10 = mannitol acetate; 11 = galactitol acetate; 12 = glucitol acetate; 13 = myoinositol acetate. Other peaks have not yet been identified.

responses of the alditol acetates compared with myoinositol acetate are being determined and the procedure applied to the study of the fate of plant polysaccharides during decomposition in the soil and as a method for following the fractionation of complex mixtures of polysaccharides. The small amounts of sugar which can be quantitatively analysed render the technique particularly useful for monitoring polysaccharides from chromatographic columns.

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SUMMARY

A procedure is described for the quantitative conversion of sugars in a complex hydrolysate to the corresponding alditol acetates. The conditions which allow the separation of alditol acetates by gas–liquid chromatography are defined and several examples of the application of these techniques to natural complex polysaccharide materials are given.

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# CHROMATOGRAPHIC IDENTIFICATION OF CARBONYL COMPOUNDS*

## IV. GAS CHROMATOGRAPHY OF ALDEHYDE 2,4-DINITROPHENYLHYDRAZONES

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For the purpose of gas chromatographic analysis, the dinitrophenylhydrazones must be converted into volatile compounds. If aldehyde dinitrophenylhydrazones are heated with diketones[4,5], keto aldehydes[4,5] or keto acids[6], the bound aldehydes can be liberated. RALLS[7,8] has developed a method in which the precipitated dinitrophenylhydrazones of aldehydes are heated together with 2-oxoglutaric acid in a glass capillary tube before the sample is subjected to gas chromatography. Some applications and modifications of this method have been reported[9,10]. In the method of KUNITAKE[11], the aldehydes are released from their dinitrophenylhydrazones by heating them in a methanol–sulphuric acid mixture, and then analysed by gas chromatography.

Dinitrophenylhydrazones can be analysed by gas chromatography if they are oxidized with ozone as has already been described in preliminary communications[12–14]. The ozonisation leads to cleavage of the double bond between carbon and nitrogen in the hydrazone[15,16]. This study provides a detailed description of the methods of ozonisation and subsequent gas chromatography. Two successive chromatograms were run in the gas chromatographic analyses. In one run, the mixture of pure aldehyde hydrazones, and in the other the mixture of aldehyde hydrazones isolated by adsorption and elution technique from ethanol–water solution (Part I), were analysed after their ozonisation.

### EXPERIMENTAL

*Preparation of mixtures of aldehyde hydrazones and their ozonisation*

A mixture containing 0.2 mmole of each of the 2,4-dinitrophenylhydrazones of acetaldehyde, propionaldehyde, butyraldehyde, isobutyraldehyde, valeraldehyde, isovaleraldehyde, 2-methylbutyraldehyde and furfural, was prepared by weighing and dissolved in 100 ml of methyl formate (purum, Fluka AG). A 10-ml volume of this stock solution was evaporated to dryness and the residue R was dissolved in 1 ml of methyl formate. A dinitrophenylhydrazone mixture M, isolated by adsorption on carbon from the 4 l of 8 wt. % aqueous ethanol from which the bishydrazones had been removed, was eluted from the carbon (Part I) and dissolved in 1 ml of methyl formate. The solutions of the mixtures R and M were cooled to the temperature of a

* For Parts I–III, see refs. 1–3.

dry ice–ethanol mixture. Oxygen containing 2 vol. % of ozone was passed into them at a rate of 10–20 ml/min for a period of about half an hour and then pure oxygen was passed through the solutions for half an hour to remove any excess ozone. The solutions were then allowed to warm to room temperature and subjected to gas chromatography.

The mixtures R and M were also treated with ozone in distilled formic acid. The temperatures of the solutions were then held a few degrees above zero to avoid solidification of the solvent. The resulting solutions were also analysed by gas chromatography.

*Gas chromatography*

The column was a stainless steel tube 4 m long and 3 mm in inner diameter. The solid support was Chromosorb W (acid-washed, 60–80 mesh, Johns-Manville Products Corp.) and the liquid phase was NEGS (neopentyl glycol succinate) (Applied Science Laboratories, Inc.) containing phosphoric acid[17]. The proportions by weight of Chromosorb W, phosphoric acid (85 wt. %) and NEGS were 77:3:20. The gas chromatograph was a Perkin-Elmer Fractometer F 6/4 HF. The operating conditions were: carrier gas, helium; flow rate, 74 ml/min; inlet pressure, 2.5 kp/cm$^2$; temperature 140°; detection by flame ionisation, sensitivity 8; recorder range from 0 to 10 mV; paper speed in the recorder 1/3 in./min; injected volume 5 $\mu$l.

The carrier gas was led through a vessel containing formic acid before it passed into the gas chromatograph. The gas picked up some formic acid which prevented the trace compounds, higher homologues of formic acid, from being adsorbed on the column packing and caused these compounds to emerge as peaks with reproducible areas.

Several other columns, including a Perkin-Elmer capillary column, were also tested for their ability to separate carboxylic acids. The best results were, however, obtained in the resolution of trace amounts of carboxylic acids derived from aldehyde hydrazones on the NEGS column.

## RESULTS AND DISCUSSION

*Gas chromatograms of the carboxylic acids produced by ozonisation of the aldehyde hydrazones*

Gas chromatograms of the carboxylic acids produced by ozonisation of the mixtures M and R of aliphatic $C_2$–$C_5$ aldehyde- and furfural 2,4-dinitrophenylhydrazones are shown in Fig. 1. The mixture M was isolated from 8 wt. % ethanol by adsorption and subsequent elution; the amounts of the components in this mixture should have been equal to the amounts of the corresponding components in the reference mixture R. The possibility of cleaving the carbon-nitrogen double bond in hydrazones by ozonisation is not generally known, although this reaction has been studied in the case of dinitrophenylhydrazones of steroids[15] and its mechanism has been studied with the dinitrophenylhydrazones of acetone and several aromatic ketones[16]. In preliminary experiments the ozonisation reaction proved to be of value in gas chromatographic studies of dinitrophenylhydrazones[13,14]. Carboxylic acids are formed when the double bonds are broken by ozone[14]. The aromatic moieties of the dinitrophenylhydrazones are mainly converted into *m*-dinitrobenzene and 2,4-dini-

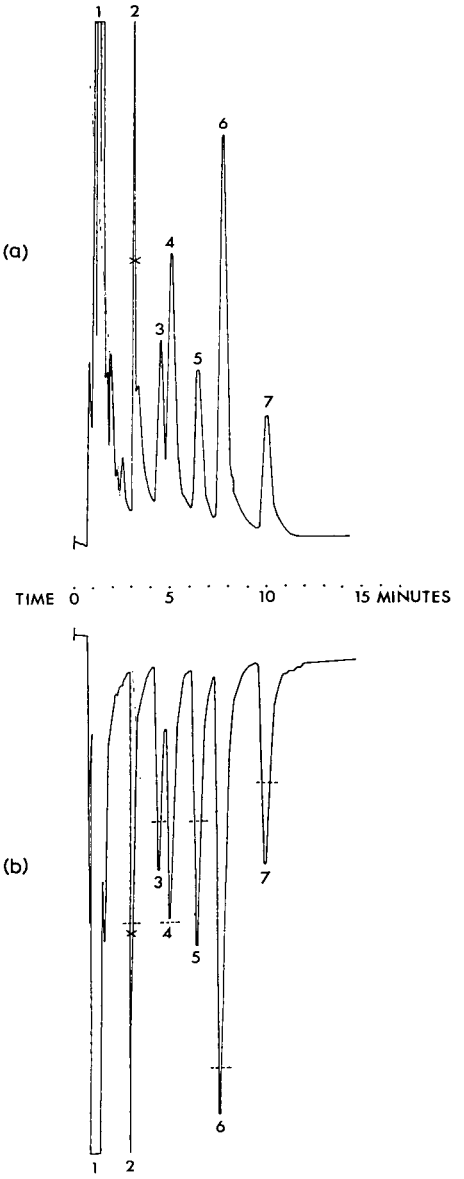


Fig. 1. Gas chromatogram of the carboxylic acids produced by ozone oxidation of the mixture M of 2,4-dinitrophenylhydrazones of aldehydes isolated by adsorption on carbon from aqueous ethanol and elution from the carbon (a), and that of the carboxylic acids produced by ozone oxidation of the reference mixture R of pure aldehyde hydrazones (b). Acetaldehyde is analysed as acetic acid, propionaldehyde as propionic acid, and so on. The dotted lines on the latter chromatogram give the heights of the corresponding peaks in the upper chromatogram. Conditions: column length 4 m, internal diameter 3 mm, liquid phase NEGS containing phosphoric acid, solid support acid-washed Chromosorb W; carrier gas helium, flow rate 74 ml/min, inlet pressure 2.5 kp/cm$^2$; temperature 140°; detection by flame ionisation, sensitivity 8; injected volume 5 $\mu$l. 1 = Solvent; 2 = acetic acid; 3 = propionic acid; 4 = isobutyric acid; 5 = butyric acid; 6 = isovaleric acid and 2-methylvaleric acid; 7 = valeric acid.

trophenol[16], but these compounds do not give rise to interfering peaks when the oxidized mixture is subjected to gas chromatography as described above. The gas chromatographic method has several advantages over the paper chromatographic method (Part III) described earlier. Thus, for example, any excess dinitrophenylhydrazine in the sample studied does not interfere with the identification of aldehydes as the corresponding carboxylic acids and hence this method can be used to analyse oxidized mixtures of dinitrophenylhydrazones isolated by adsorption on carbon from aqueous ethanol and elution from the carbon. Also several isomeric aldehydes can be identified by this method. No peak due to a carboxylic acid derived from furfural hydrazone is found in the gas chromatogram, and therefore the paper chromatographic method described earlier must be used to identify this aldehyde.

The ozonisation of the aldehyde hydrazones can be carried out in methyl formate at the temperature of the dry ice–ethanol mixture[14] or in formic acid at temperatures a few degrees above 0°. It is advantageous to use carrier gas containing formic acid in the gas chromatography of the higher homologues of formic acid or to add formic acid to the solution to be analysed before gas chromatography[14] to prevent retention in the column of acids present only in trace quantities. The chromatogram in Fig. 1 refers to the analysis of the ozonisation products of the mixtures M and R in formic acid when the carrier gas contained formic acid. Formic acid itself does not give any peak when a flame ionisation detector is used, but when several microlitres of formic acid are injected simultaneously into the gas chromatograph, several peaks are formed in the chromatogram which evidently originate in the liquid phase in the column. However, these peaks emerge before those of the carboxylic acids derived from the aldehyde hydrazones and are indicated by the word solvent in Fig. 1.

The isothermal gas chromatographic procedure employed is suitable for the identification of lower aliphatic monocarboxylic acids. However, a temperature of 140° seems to be too high for acetic acid and results in a very sharp peak extending beyond the paper. The acetic acid peaks in the gas chromatograms of the two mixtures M and R in Fig. 1 are marked by x's to indicate the peak levels if the sensitivity of the detection had been decreased from the employed 8 to 32.

If the isolation of the dinitrophenylhydrazones from the 8 wt. % aqueous ethanol had been complete, the corresponding peaks in both chromatograms of Fig. 1 should have been equal in size. To facilitate comparison, the peak heights for the components derived from mixture M are indicated by horizontal dotted lines crossing the corresponding peaks for the same components in the reference mixture R.

The peak heights are lower for all the components, except isobutyric acid, derived from mixture M than those for the components derived from reference mixture R. All the components do not seem to have been equally effectively eluted from carbon. The solvent used to elute the compounds from the carbon is naturally of importance. Better results were obtained when the carbon was treated successively with methyl formate and dichloromethane than when either solvent was used alone. An experiment was also carried out in which the pyridine–water azeotrope was used to elute aldehyde hydrazones from carbon, but the chromatogram of the carboxylic acids produced by ozonisation of the eluted aldehyde hydrazones contained a number of additional interfering peaks. The pyridine–water azeotropic mixture is, however, suitable for the elution of keto acid hydrazones after the aldehyde hydrazones have

already been eluted with methyl formate and dichloromethane, or the pyridine–water azeotropic mixture can be used to elute all the hydrazones from the carbon for the analysis of keto acid hydrazones. In the chromatogram of the carboxylic acids derived from mixture M in Fig. 1, there is a small unidentified peak adjoining the acetic acid peak, which does not, however, interfere with the identification of the major components.

When the NEGS column is employed, isovaleric acid and 2-methylbutyric acid emerge at the same time. The gas chromatographic separation of these two acids has been very little studied. Despite repeated attempts, it was not found possible to separate these two acids on a silicone oil–stearic acid column, which has been stated to effect their separation[18]. In experiments with the pure carboxylic acids, it was found that the two acids are separated on a Perkin-Elmer capillary column (50 m long, 0.25 mm internal diameter) coated inside with a (25:1, w/w) mixture of trimer acid (a $C_{54}$ tribasic acid with about 10 % $C_{36}$ dibasic acid content) and dinonylnaphthalene-disulphonic acid[19], especially when a programmed temperature rise is employed. A capillary column requires, however, that the solution of the components under study is at least one hundred times as concentrated as the solutions of the mixtures M and R (1–2 mg/ml) employed in this investigation; this is because only a low proportion of the mixture injected into the gas chromatograph enters the capillary column. When samples of the mixtures M and R were submitted to gas chromatography in the capillary column, only the solvent peaks were visible in the chromatograms. The isomeric aldehydes, isovaleraldehyde and 2-methylbutyraldehyde, can therefore only be identified as a pair by the proposed method.

In contrast to paper chromatography, gas chromatography using a NEGS column can, however, be employed to separate carboxylic acids derived from isomeric aldehydes such as butyraldehyde and isobutyraldehyde and also valeraldehyde and isovaleraldehyde or 2-methylbutyraldehyde. The peak heights of the corresponding carboxylic acids derived from mixtures M isolated by the adsorption and elution technique from identical solutions showed good agreement. Although the hydrazones are not quantitatively recovered by the adsorption and elution technique (Fig. 1), the method can be employed, in view of its reproducibility, to determine unknown quantities of aldehydes in alcoholic solutions by comparing their gas chromatograms with those obtained for standard solutions of aldehydes by the same procedure. The aldehyde hydrazone that is recovered in highest yield, by adsorption and elution, is isobutyraldehyde. Because this aldehyde is less completely precipitated as its dinitrophenylhydrazone than other aldehydes from alcoholic solutions, and as it gives a much weaker colour with fuchsin than other aldehydes[20], the method described here offers the possibility of detecting this aldehyde in fermenting liquors and their distillates more reliably than previously.

SUMMARY

A study has been made of the ozonisation of 2,4-dinitrophenylhydrazones of aliphatic $C_2$–$C_5$ aldehydes to the corresponding carboxylic acids, which were analysed by isothermal gas chromatography on a NEGS column. The completeness of the isolation was examined by comparison of the peak heights of the carboxylic acids derived from a mixture of pure aldehyde hydrazones (R) with those of carboxylic

acids derived from a mixture of aldehyde hydrazones (M) isolated by adsorption on carbon from aqueous ethanol and elution from the carbon.

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# CHROMATOGRAPHIC IDENTIFICATION OF CARBONYL COMPOUNDS*

## V. GAS CHROMATOGRAPHY OF KETO ACID METHYL ESTERS

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Various methods involving pyrolysis, heating with acids, and ozonisation[5,6] (*cf.* Part IV), have been applied as preliminary treatment in attempts to convert the keto acid methyl ester 2,4-dinitrophenylhydrazones into volatile compounds for gas chromatography. As the ozonisation method gave reproducible results, its applicability was studied in greater detail. The ozonisation severs the double bond between carbon and nitrogen in the keto acid ester hydrazones, and leads to formation of the corresponding keto acid methyl esters, which do not further decompose in the presence of ozone, and can be analysed by gas chromatography as has been described in a preliminary communication[7]. In this study, a detailed description is given of the methods of ozonisation of keto acid ester hydrazones and subsequent gas chromatography of the liberated esters. Two successive chromatograms were run in the gas chromatographic analyses. In one run, the mixture of pure keto acid methyl ester hydrazones was analysed after ozonisation. In the other, the mixture of keto acid hydrazones isolated by adsorption and elution technique from ethanol–water solution (Part I) was esterified and analysed after ozonisation.

### EXPERIMENTAL

*Esterification of 2,4-dinitrophenylhydrazones of keto acids*

For esterification, a solution containing 0.5 g of the 2,4-dinitrophenylhydrazone of a keto acid in diethyl ether (pure, Rikkihappo Oy) was cooled in an ice–water bath and treated with an excess of a solution of diazomethane in diethyl ether that had been precooled in an ice–water bath. Immediately after the solution had been thoroughly mixed by shaking, the ether was evaporated and the precipitated ester was recrystallized from a (1:4, v/v) mixture of methyl formate (guaranteed reagent, E. Merck AG) and methanol (guaranteed reagent, E. Merck AG).

*Preparation and ozonisation of mixtures of keto acid ester hydrazones*

A mixture containing 0.2 mmole of each of the pure 2,4-dinitrophenylhydrazones of the methyl esters of pyruvic, 2-oxobutyric, 2-oxoisovaleric, 2-oxoisocaproic, 2-oxo-3-methylvaleric, levulinic, 2-oxoglutaric and oxalacetic acids was prepared by weighing and dissolved in 100 ml of methyl formate (purum, Fluka AG). A 10-ml aliquot of the resulting solution was evaporated to dryness in the Rotavapor and

* For Parts I–IV, see refs. 1–4.

the residue R, containing 0.02 mmole of each component, was dissolved in 1 ml of a (1:4, v/v) mixture of dichloromethane (guaranteed reagent, E. Merck AG) and methanol (guaranteed reagent, E. Merck AG). A mixture of the same keto acid hydrazones isolated from 4 l of the 8 wt. % aqueous ethanol solution by adsorption on carbon and by selective elution (first the aldehyde hydrazones with methyl formate and dichloromethane and then the keto acid hydrazones with azeotropic pyridine–water mixture) from the carbon (Part I) was treated with methanol containing hydrogen chloride to liberate the acid hydrazones from their pyridinium salts. After evaporation of the solvent, the residue was esterified with diazomethane in diethyl ether at 0°. The resulting ester mixture was isolated and dissolved in 1 ml of (1:4, v/v) dichloromethane–methanol mixture. This mixture is designated M. The solutions of the mixtures M and R were cooled to the temperature of a dry ice–ethanol mixture, and oxygen containing about 2 vol. % of ozone was passed into both solutions at a rate of 10–20 ml/min for 2.5 h, after which pure oxygen was passed through the solution for half an hour to remove any excess ozone. After the solutions had warmed to room temperature, they were analysed by gas chromatography.

*Gas chromatography*

The column was a stainless steel tube 4 m long and 3 mm in inner diameter. The solid support was Chromosorb W (acid-washed, 60–80 mesh, Johns-Manville Products Corp.) and the liquid phase DEGA (diethylene glycol adipate) (Applied Science Laboratories, Inc.) containing phosphoric acid[8]. The proportions by weight of Chromosorb W, phosphoric acid (85 wt. %) and DEGA were 77:3:20. The gas chromatograph was a Perkin-Elmer Fractometer F 6/4 H F. The operating conditions were: carrier gas helium, flow rate 65 ml/min, inlet pressure 1.8 kp/cm$^2$; temperature programme 100–180°/2.5°/min; detection by flame ionisation, sensitivity 8; range of the recorder from 0 to 10 mV; paper speed in the recorder 1/3 in./min; injected volume 5 $\mu$l.

## RESULTS AND DISCUSSION

*Gas chromatograms of the keto acid methyl esters*

Gas chromatograms of the keto-acid methyl esters derived from keto acid methyl ester 2,4-dinitrophenylhydrazones are shown in Fig. 1. The structural isomers 2-oxoisocaproic acid and 2-oxo-3-methylvaleric acid were well separated by gas chromatography on a DEGA column. As already shown previously, the dinitrophenylhydrazones of these two acids and their esters are resolved less effectively on a thin layer[9,10]. The heights of the peaks in the gas chromatograms of mixture M are marked by horizontal dotted lines on the corresponding peaks of the gas chromatogram of the reference mixture R to facilitate comparison. The peaks of corresponding components in the two chromatograms should have been equal in height. The peaks of methyl levulinate differ the most in height. This is due to the incomplete elution of its hydrazone and possibly to its partial decomposition during the vaporisation of the eluant. The gas chromatographic method is, however, so sensitive that levulinic acid can nevertheless be identified by this method. The peaks of the methyl pyruvate in the gas chromatograms of the mixtures M and R are much closer in height than the peaks of the other methyl esters. This is due to the decarboxylation of the oxalacetic

acid present in the solution from which the M was isolated to pyruvic acid.

The methods developed for the gas chromatographic separation of keto acids are suitable only for keto monocarboxylic acids. The dinitrophenylhydrazones of 2-oxoglutaric acid and oxalacetic acid yielded on treatment with diazomethane ester derivatives from which ozone liberated esters that did not give peaks on gas chromatography. The esters in question are monoesters and they boil at temperatures too high for analysis by the gas chromatographic technique used. When the diesters formed by heating the free keto dicarboxylic acids in methanol containing hydrogen chloride and thionyl chloride[11] were analysed by the gas chromatographic method used, they gave peaks. In addition, the dinitrophenylhydrazones prepared from the dimethyl esters of the above-mentioned two keto dicarboxylic acids were found to

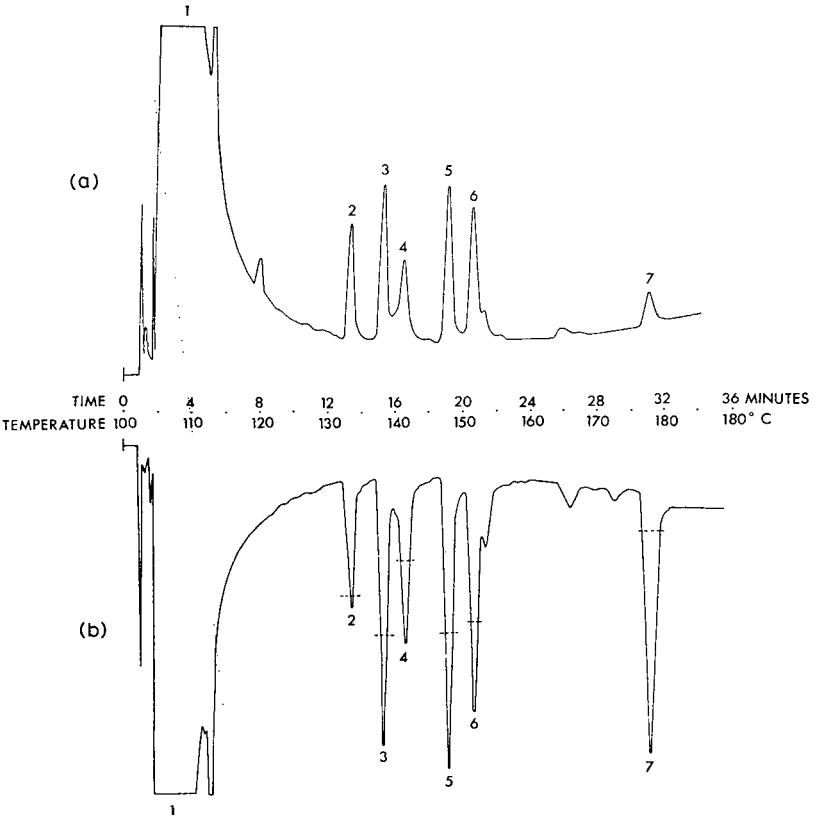


Fig. 1. Gas chromatogram of the keto acid methyl esters liberated by ozone oxidation of the mixture M of 2,4-dinitrophenylhydrazones of keto acid methyl esters (a), and that of the keto acid methyl esters liberated by ozone oxidation of the reference mixture R of pure keto acid methyl ester hydrazones (b). The dotted lines on the latter chromatogram give the heights of the corresponding peaks in the upper chromatogram. Conditions: column length 4 m, internal diameter 3 mm, liquid phase DEGA containing phosphoric acid, solid support acid-washed Chromosorb W; carrier gas helium, flow rate 65 ml/min, inlet pressure 1.8 kp/cm$^2$; temperature programme 100–180°, 2.5°/min; detection by flame ionisation, sensitivity 8; injected volume 5 $\mu$l. 1 = Solvent; 2 = methyl pyruvate; 3 = methyl 2-oxoisovalerate; 4 = methyl 2-oxobutyrate; 5 = methyl 2-oxo-3-methylvalerate; 6 = methyl 2-oxoisocaproate; 7 = methyl levulinate.

migrate on a thin layer at rates that differed from the rates of the monoester hydrazones of the keto dicarboxylic acids.

The peak heights of the keto acid methyl esters in the gas chromatograms of mixtures M which were isolated as acid hydrazones by the same adsorption and elution technique from identical solutions and then esterified and ozonisated showed good agreement. A number of additional peaks are seen in the chromatograms of the mixtures M and R in Fig. 1. In separate experiments with individual keto acid methyl ester hydrazones it was found that the small peak adjoining the peak of methyl 2-oxoisocaproate is due to a by-product formed when the dinitrophenylhydrazone of methyl pyruvate is treated with ozone and the small peaks between peaks 6 and 7 are due to by-products formed in the ozonisation of the dinitrophenylhydrazones of methyl 2-oxoisocaproate and methyl 2-oxo-3-methylvalerate. These additional peaks may sometimes be relatively large, especially when one of the decomposing compounds, for example pyruvic acid, is a strongly dominating component in the mixture of keto acids that is to be analysed.

SUMMARY

A study has been made of the ozonisation of 2,4-dinitrophenylhydrazones of eight keto acid methyl esters to corresponding methyl esters, and analysis of the latter by programmed gas chromatography on a DEGA column. The completeness of the isolation of the components from aqueous ethanol was examined by comparing the peak heights of the keto acid methyl esters liberated by ozonisation of a mixture of pure keto-acid methyl ester hydrazones (R) with those of methyl esters liberated by ozonisation of a mixture of keto acid methyl ester hydrazones (M) isolated as keto acid hydrazones by adsorption on carbon from ethanol–water solution and elution from the carbon, and then esterified.

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# CHROMATOGRAPHIC IDENTIFICATION OF CARBONYL COMPOUNDS*

## VI. THIN-LAYER CHROMATOGRAPHIC RESOLUTION OF MIXTURES OF KETO ACID 2,4-DINITROPHENYLHYDRAZONES

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Solvent mixtures containing petroleum ether, ethyl formate and propionic acid, previously developed in this laboratory for the thin-layer chromatography of keto acid 2,4-dinitrophenylhydrazones[6,7], have now been thoroughly examined in preliminary experiments by varying the ratios of the solvent components, and using silica gel, cellulose, nylon and aluminium oxide as adsorbents. However, the best results were obtained by application of the method previously employed, after slight modification. This modification involved the use of a neutral silica gel suspension instead of one of acidified silica gel for preparing the thin layers on the plates, and was used in this study. The purpose was that of studying the possible interference of the reagent used in the identification of the components; the influence of the solvents in which the hydrazones are applied to the thin layers on the separation of stereoisomers of the hydrazones; and the completeness of the isolation of the components from aqueous ethanol.

### EXPERIMENTAL

*Dissolution of keto acid hydrazones*

Two hundredths of a millimole of each of the pure 2,4-dinitrophenylhydrazones of pyruvic acid, 2-oxobutyric acid, 2-oxoisovaleric acid, 2-oxoisocaproic acid, 2-oxo-3-methylvaleric acid, levulinic acid, 2-oxoglutaric acid and oxalacetic acid were weighed, and a mixture R containing 0.02 mmole of each was prepared by weighing. The weighed individual hydrazones and their mixture R were dissolved in 10-ml volumes of dioxan (for chromatography, E. Merck AG). Also a pyridine ('Baker Analysed' Reagent, J. T. Baker Chemical Co.)–water (1:1, v/v) mixture was employed as solvent in place of dioxan. A mixture (M) of the same keto acid hydrazones isolated from 4 l of the 8 wt. % aqueous ethanol solution by adsorption on carbon and by selective elution (first the aldehyde hydrazones and then the keto acid hydrazones) from the latter (Part I) was dissolved in 10 ml of a (1:1, v/v) mixture of pyridine ('Baker Analysed' Reagent, J. T. Baker Chemical Co.) and distilled water. After samples of this solution had been taken for application to one plate, the remaining solution was evaporated to dryness in a Rotavapor and the residue M was dissolved

* For Parts I–V, see refs. 1–5.

in 10 ml of dioxan (for chromatography, E. Merck AG). A second mixture of hydrazones isolated by adsorption on carbon from the 4 l of 8 wt. % aqueous ethanol was eluted from the carbon with only the pyridine–water azeotropic mixture and contained besides the keto acid hydrazones, hydrazones of the other monocarbonyl compounds studied (Part I). This mixture M′ was dissolved in 10 ml of a (1:1, v/v) pyridine–water mixture. Solutions containing only dinitrophenylhydrazine (1 mg/ml) in dioxan and in a (1:1, v/v) pyridine–water mixture were also prepared.

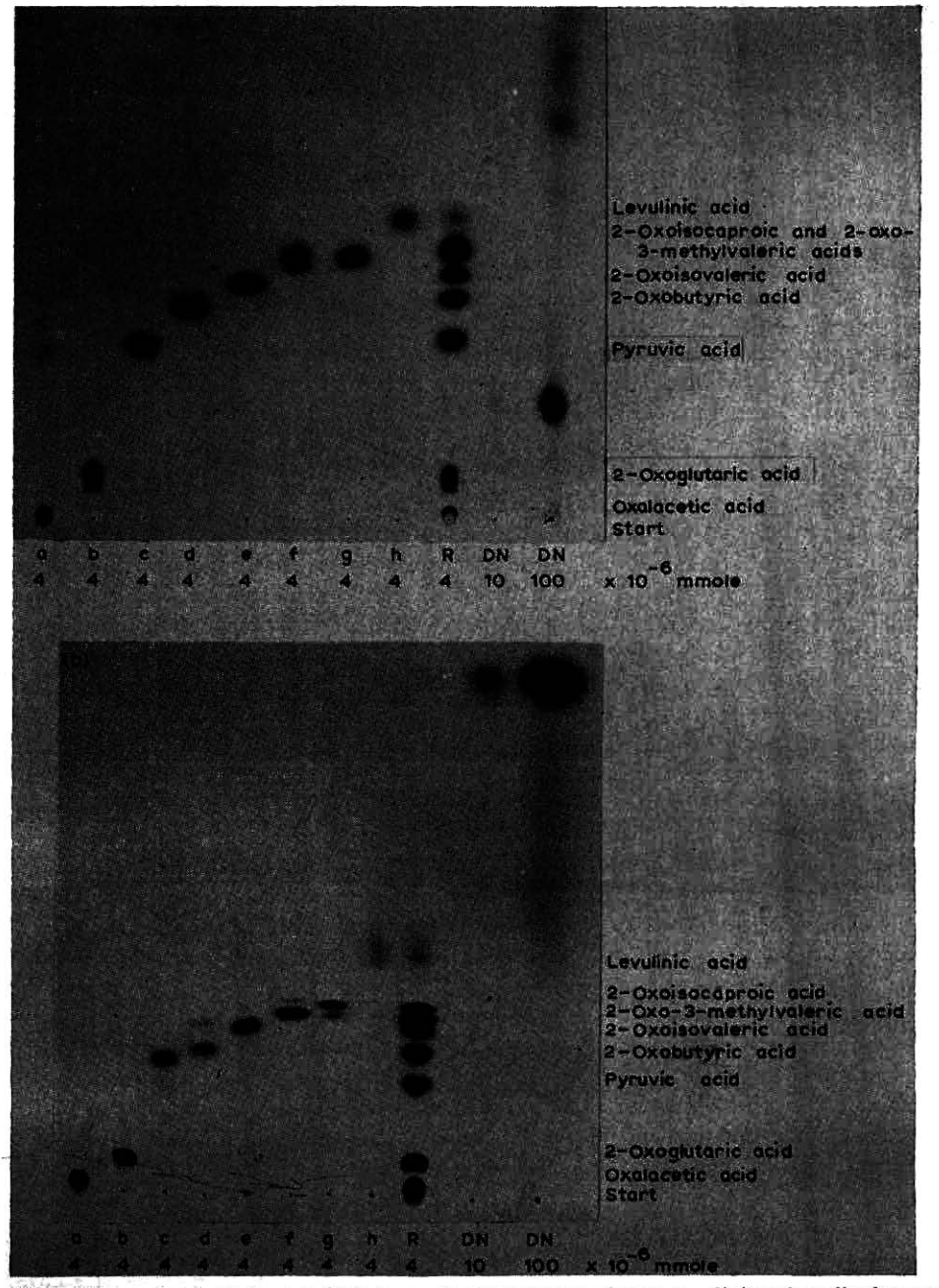
*Thin-layer chromatography*

A 2-$\mu$l volume of the dioxan solution of each keto acid hydrazone, 2 $\mu$l of the dioxan solution of the mixture R and two different volumes, 4 and 40 $\mu$l, of the dioxan solution of dinitrophenylhydrazine were applied to an activated thin layer of Silica Gel G (for thin-layer chromatography, E. Merck AG). The activation was carried out by heating at 120° for half an hour. The same compounds were applied to a second thin-layer plate, but in pyridine–water instead of in dioxan. To a third and fourth thin layer were applied 4, 2, 1, 0.5 and 0.2 $\mu$l of the solutions of the mixture M and the same volumes of the solutions of the reference mixture R, equal volumes of both mixtures side by side, in pyridine–water (Plate 3) and in dioxan (Plate 4). The mixtures M′ and R were also applied to a thin layer side by side as above. The plates were equilibrated in the vapour above the chromatographic solvent in a closed chamber for half an hour before they were resolved with a solvent composed of 26 volumes of petroleum ether (boiling range 60–80°, British Drug Houses Ltd.), 14 volumes of ethyl formate (puriss., Fluka AG) and 3 volumes of propionic acid (puriss., Fluka AG). The running time was about 3 h.

## RESULTS AND DISCUSSION

*Thin-layer chromatograms of the keto acid hydrazones*

Thin-layer chromatograms of pure 2,4-dinitrophenylhydrazones of keto acids, their mixture R and dinitrophenylhydrazine run with petroleum ether–ethyl formate–propionic acid (26:14:3) are shown in Fig. 1a and 1b. The solvent in which the dinitrophenylhydrazones, their mixture and the reagent were applied to the thin layer was dioxan in the former case (Fig. 1a) and a (1:1, v/v) pyridine–water mixture in the latter. In the former chromatogram each keto acid hydrazone gave only one spot. The dinitrophenylhydrazones of the structural isomers 2-oxoisocaproic acid and 2-oxo-3-methylvaleric acid migrated at the same rate. It has previously been found[6] that when the proportion of propionic acid in the chromatographic solvent is increased, or the propionic acid is replaced by formic acid, several keto acid hydrazones are resolved into two stereoisomeric forms which give two successive spots in the chromatogram. The two chromatograms (Fig. 1a and 1b) show that the resolution of the stereoisomers is not solely determined by the solvent mixture employed to develop the chromatograms, but also by the solvent in which the dinitrophenylhydrazones are applied to the thin layer. The hydrazones of the structural isomers 2-oxoisocaproic acid and 2-oxo-3-methylvaleric acid are seen to have separated in the chromatogram in Fig. 1b, but the smaller spots, to which they also give rise, will interfere with their identification if one of them is present in a much higher concentration than the other. Also the rate of migration of dinitrophenylhydrazine and




Figs. 1a (upper) and 1 b (lower). Thin-layer chromatograms of pure 2,4-dinitrophenylhydrazones of oxalacetic acid (a), 2-oxoglutaric acid (b), pyruvic acid (c), 2-oxobutyric acid (d), 2-oxoisovaleric acid (e), 2-oxo-3-methylvaleric acid (f), 2-oxoisocaproic acid (g), levulinic acid (h), their mixture (R) and 2,4-dinitrophenylhydrazine (DN). The chromatographic solvent was petroleum ether–ethyl formate–propionic acid (26:14:3) and the adsorbent was Silica Gel G. The solvents in which the components, their mixture and the reagent were applied to the thin layers were dioxan (Fig. 1a) and a (1:1, v/v) pyridine–water mixture (Fig. 1 b).

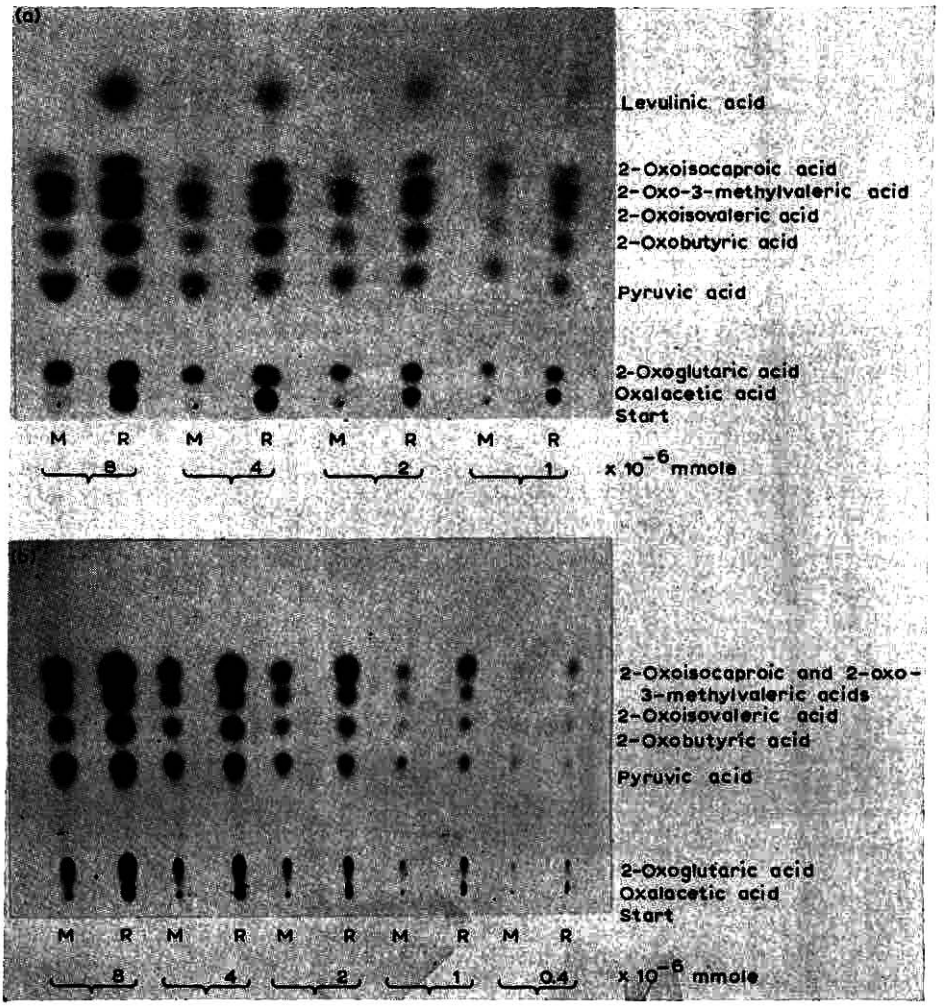
its distribution over several spots seems to depend on the solvent in which the compounds are applied to the thin layer. When the solvent is dioxan (Fig. 1a), part of the reagent migrates between pyruvic acid and 2-oxoglutaric acid hydrazones, although most of the reagent migrates before the hydrazones close behind the solvent front. When the solvent in which the compounds are applied to the thin layer is a pyridine–water mixture (Fig. 1b), all of the reagent migrates immediately behind the solvent front.

The thin-layer chromatograms in Figs. 2a and 2b are those of the keto acid hydrazone mixtures M and R. The mixture M was isolated by adsorption from 8 wt. % aqueous ethanol and R was the reference mixture. The mixtures were applied in the pyridine–water solution to the starting line on the thin-layer plate when the chromatogram of Fig. 2a was run, and in dioxan solution when the chromatogram of Fig. 2b was run. In both cases the chromatographic solvent was the petroleum ether–ethyl formate–propionic acid (26:14:3) mixture. The solvent for application of the mixtures M and R to a thin layer was exchanged by evaporating the solutions of the mixtures M and R remaining after samples of these mixtures in the pyridine–water solvent had been taken for application to one plate (Fig. 2a) to dryness in a Rotavapor and dissolving the residue in dioxan for application to the other thin-layer plate (Fig. 2b). The amounts of the mixtures applied to the thin layers were varied similarly and hence the intensities of the spots of each component should have been equal in the two parallel chromatograms if the isolation of the keto acid hydrazones by adsorption and elution had been complete. No spots due to levulinic acid hydrazone and only a weak spot due to oxalacetic acid hydrazone are seen in the chromatogram for mixture M in Fig. 2a; the other components were more completely isolated. No spot due to levulinic acid hydrazone is observed in the chromatogram for the reference mixture R in Fig. 2b either, and it must be concluded that the greater part of this compound must have decomposed when the solution of the mixture R in the pyridine–water mixture was evaporated to dryness in the Rotavapor. The decomposition products migrated close behind the solvent front. The decomposition of levulinic acid hydrazone in the evaporation stage also explains why the compound gave no spot in the chromatogram for the mixture M in Fig. 2a; this mixture was eluted with pyridine–water azeotrope from carbon and the effluent was evaporated to dryness in the Rotavapor. Experiments carried out with levulinic acid alone revealed that the low yield was not only due to the decomposition during the evaporation in the Rotavapor but also to the fact that its dinitrophenylhydrazone was less effectively eluted from carbon than the other keto acid hydrazones. The decomposition of levulinic acid hydrazone can be avoided to some extent by carrying out the evaporation of the solvent at a low pressure and by not evaporating the solution to complete dryness, but even then only traces of the levulinic acid hydrazone are found in the chromatogram.

The disappearance of oxalacetic acid hydrazone during the isolation process is due to the instability of this compound, which, as established in separate experiments, releases carbon dioxide and changes into the pyruvic acid derivative. This decarboxylation hence increases the intensity of the spot containing pyruvic acid hydrazone in the thin-layer chromatogram.

Fig. 2c shows a thin-layer chromatogram of the mixture M′ of aldehyde hydrazones and keto acid hydrazones isolated together by adsorption on carbon from

8 wt. % aqueous ethanol and elution from the carbon only with the pyridine–water azeotropic mixture (without prior elution of the aldehyde hydrazones with methyl formate and dichloromethane) and of the reference mixture R of keto acid hydrazones. In contrast to the experiments already described, all the carbonyl compounds, aldehydes, dicarbonyl compounds and keto acids mentioned previously (Part I) except pyruvic acid were initially added to the 8 wt. % aqueous ethanol, the purpose being to obtain information about the possible decarboxylation of oxalacetic acid. Both mixtures were applied to the thin layer in the pyridine–water (1:1, v/v) mixture.




Figs. 2a (upper) and 2b (lower). Thin-layer chromatogram of the mixture M of 2,4-dinitrophenylhydrazones of keto acids isolated by adsorption on carbon from aqueous ethanol and elution from the carbon, and that of the reference mixture R of pure keto acid hydrazones. The chromatographic solvent was petroleum ether–ethyl formate–propionic acid (26:14:3) and the adsorbent was Silica Gel G. The solvents in which the mixtures were applied to the thin layers were a (1:1, v/v) pyridine–water mixture (Fig. 2a) and dioxan (Fig. 2b).

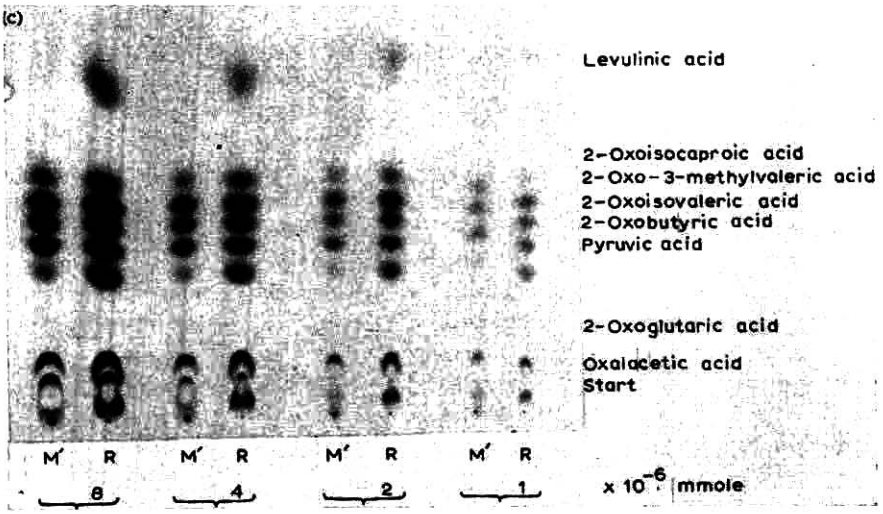



Fig. 2c. Thin-layer chromatogram of the mixture M′ of 2,4-dinitrophenylhydrazones of carbonyl compounds isolated by adsorption on carbon from aqueous ethanol and elution from the carbon with the pyridine–water azeotrope (without prior extraction of aldehyde hydrazones) and of the reference mixture R of pure keto acid hydrazones. The chromatographic solvent was petroleum ether–ethyl formate–propionic acid (26:14:3) and the adsorbent was Silica Gel G. The solvent in which the mixtures were applied to the thin layer was a (1:1, v/v) pyridine–water mixture.

The solvent employed in running the thin-layer chromatogram was the petroleum ether–ethyl formate–propionic acid (26:14:3) mixture used to obtain the chromatograms of Figs. 1a, 1b, 2a and 2b. When this chromatographic solvent mixture is used, the aldehyde hydrazones and dinitrophenylhydrazine do not interfere with the identification of the keto acid hydrazones as they all migrate as one group ahead of the keto acid hydrazones near the solvent front. The spots of the corresponding components in the mixtures M′ and R should have been equal in size and intensity. The presence of oxalacetic acid in mixture M′ is clearly evident, although the acid had undergone partial decarboxylation as there are weak spots due to pyruvic acid hydrazone (which was not initially present) in the chromatogram of the mixture.

SUMMARY

A study has been made of the resolution of a mixture of pyruvic acid, 2-oxobutyric acid, 2-oxoisovaleric acid, 2-oxoisocaproic acid, 2-oxo-3-methylvaleric acid, levulinic acid, 2-oxoglutaric acid and oxalacetic acid 2,4-dinitrophenylhydrazones, and the possible interference of 2,4-dinitrophenylhydrazine in identification of the components, by the application of thin-layer chromatography. With pure keto acid hydrazones (mixture R) as reference standard, the completeness of the isolation by the adsorption and elution technique (Part I), of these keto acids as their hydrazones (mixture M) from aqueous ethanol was examined. The partial decomposition of oxalacetic acid hydrazone was also examined by making a comparison of the intensities of the spots of the components of both the test and reference mixtures in the chromatograms.

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# CHROMATOGRAPHIC IDENTIFICATION OF CARBONYL COMPOUNDS

# IX. THE CARBONYL COMPOUNDS IN CRUDE SPIRITS*

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The total aldehyde content of crude Finnish grain spirits, as determined by the fuchsin method of HÄHNEL[1-3], and expressed as acetaldehyde, varies from 30 to 100 mg/l, and that of Finnish crude sulphite spirits from 300 to 500 mg/l. The main component of the carbonyl compounds in these two grades of spirit is acetaldehyde[4,5], and some of its higher homologues present in trace quantities have also been identified in spirits after they had first been concentrated by distillation[4,5]. In this study the carbonyl compounds in spirits were identified, without previous concentration of the components by distillation, by applying the methods developed for isolation (Part I) and chromatography (Parts II and IV). A method for quantitative estimation of the aldehydes in a spirit has also been examined.

## EXPERIMENTAL

### *Materials and isolation of the components*

The crude grain spirit studied here was produced by the Koskenkorva factory of the Finnish State Alcohol Monopoly and contained, as determined by HÄHNEL's method, 58 mg of aldehydes per l. The crude sulphite spirit examined was from a sulphite spirit factory in the centre of Finland, and contained 374 mg of aldehydes per l. Several 400-ml volumes of each grade of spirit were diluted with water to an alcohol content of about 8 wt.%. A solution of dinitrophenylhydrazine (2.5 g/l in 2*N* hydrochloric acid) was added to the diluted spirits, 60 ml to the diluted grain spirit, and 320 ml to the diluted sulphite spirit. The solutions were left to stand overnight at room temperature. An abundant precipitate (S) was separated from the diluted sulphite spirit samples, but only a small precipitate (G) from the diluted grain spirit samples. These precipitates were preserved for subsequent thin-layer chromatography. A second pair of samples of the diluted spirits was treated similarly with dinitrophenylhydrazine, and left to stand overnight. The precipitated dinitrophenylhydrazone mixtures (S and G) were collected by filtration. The filtrates were treated with carbon and filtered, and the aldehyde hydrazone mixtures ($S_A$ and $G_A$) eluted from the carbon successively with methyl formate and dichloromethane (*cf.* Part I). The precipitates isolated from these samples were kept for subsequent gas chromatography.

* For Parts I–VIII, see *J. Chromatog.*, 27 (1967) 374, 380, 384; 28 (1967) 253, 259, 263, 440, 443.

*Identification*

The first pair of precipitated hydrazone mixtures S and G were dissolved in 160 ml of pyridine, and a mixture R of pure bishydrazones of glyoxal, methylglyoxal, diacetyl and 2,3-pentanedione, containing 0.02 mmole of each, was dissolved in 250 ml of pyridine. Volumes of 4 $\mu$l of these solutions were applied side by side on an activated thin layer of Silica Gel $HF_{254}$, and the mixtures were resolved with a solvent system containing 34 volumes of benzene, 5 volumes of petroleum ether, and 1 volume of ethyl acetate (*cf.* Part II).

The second pair of dinitrophenylhydrazone mixtures S and G isolated by precipitation from the diluted crude sulphite and crude grain spirits, and precipitates $S_A$ and $G_A$ isolated from the same solutions after filtration by the adsorption and elution technique (*cf.* Part I), were dissolved in formic acid (S in 5 ml, G, $S_A$ and $G_A$ in 0.5 ml), ozonated (*cf.* Part IV) and analysed by isothermal gas chromatography on a NEGS column.

*Quantitative estimation of aldehydes in a crude grain spirit*

For the estimation of the quantities of the major aldehydes in crude grain spirit, three standard solutions (I, II, III, each 400 ml) containing freshly distilled acetaldehyde, propionaldehyde, isobutyraldehyde and isovaleraldehyde in aldehyde-free 94 wt.% ethanol were prepared. The amounts of the aldehydes in the solutions are given in Table I.

TABLE I

| | *Acetaldehyde (mmole)* | *Propionaldehyde (mmole)* | *Isobutyraldehyde (mmole)* | *Isovaleraldehyde (mmole)* |
|---|---|---|---|---|
| Solution I | 0.430 | 0.004 | 0.004 | 0.004 |
| Solution II | 0.500 | 0.007 | 0.007 | 0.007 |
| Solution III | 0.570 | 0.010 | 0.010 | 0.010 |

Every solution was diluted with water until the ethanol content was about 8 wt. %. Three 400-ml volumes of crude 94 wt.% grain spirit (from the same container) were similarly diluted with water. To each diluted solution was added the same volume (60 ml) of acid dinitrophenylhydrazine solution and the hydrazones formed were isolated by adsorption on carbon from the solutions and elution from the carbon and analysed, after ozonation, as carboxylic acids by gas chromatography.

## RESULTS AND DISCUSSION

Fig. 1 reproduces a thin-layer chromatogram of hydrazone mixtures S and G isolated by precipitation from diluted crude sulphite and crude grain spirits. The main purpose of this run was that of identifying the dicarbonyl compounds in the two grades of spirit. Diacetyl and 2,3-pentanedione were identified in both the grain and sulphite spirits. It has been reported that diacetyl is present in sulphite spirits[6–8], but apparently 2,3-pentanedione has not been identified in spirits; however, it has been found quite recently that it occurs in beer[9,10], and in whisky and brandy[11].

A second pair of dinitrophenylhydrazone mixtures S and G isolated by precipi-

tation from the diluted samples of crude spirits, and precipitates $S_A$ and $G_A$ isolated by the adsorption and elution technique from the same solutions after removal of the precipitates S and G, were analysed by gas chromatography; the results are indicated in Figs. 2 and 3. Mixture G, precipitated from the crude grain spirit, was found to contain only traces of acetaldehyde dinitrophenylhydrazone (Fig. 2, lower chromatogram of carboxylic acids produced by ozonation). The greater part of the hydrazones of acetaldehyde and other aldehydes possibly present in low concentrations thus remained in solution. These compounds ($G_A$) can, however, be recovered by adsorption on carbon from the solution and elution from the carbon and analysed after ozonation as carboxylic acids (Fig. 2, upper chromatogram). The dominating aldehydes are seen to have been acetaldehyde, isobutyraldehyde, isovaleraldehyde and/or 2-methylbutyraldehyde and propionaldehyde, whereas butyraldehyde, valeraldehyde and caproaldehyde were present in trace quantities. Neither butyraldehyde nor valeraldehyde has apparently been previously identified in grain spirits.

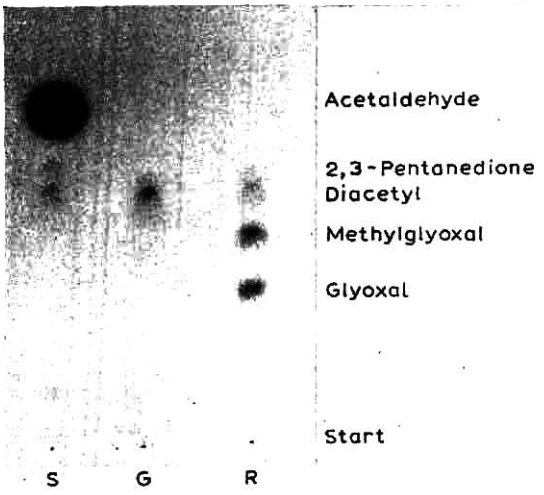


Fig. 1. Thin-layer chromatogram of mixtures S and G of 2,4-dinitrophenylhydrazones isolated by precipitation from crude sulphite (S) and crude grain (G) spirits, and of a reference mixture R of pure bishydrazones. The chromatographic solvent was benzene–petroleum ether–ethyl acetate (34:5:1) and adsorbent Silica Gel $HF_{254}$. The amount of each of the reference compounds applied to the thin layer was $3 \times 10^{-7}$ mmole.

The mixture (S) of dinitrophenylhydrazones isolated by precipitation from the diluted sample of crude sulphite spirit and the hydrazone mixture ($S_A$) isolated by the adsorption and elution technique from the same solution after removal of the precipitate S contained almost the same number of components (Fig. 3). The amount of precipitated hydrazones was so large that the volume of formic acid which was required to dissolve them for analysis was ten times the volume required to dissolve the dinitrophenylhydrazones isolated by adsorption on carbon from the filtrate and elution from the carbon. The major aldehyde components in the mixtures S and $S_A$ were found, when analysed as the corresponding carboxylic acids by gas chromatography, to be acetaldehyde, isovaleraldehyde and/or 2-methylbutyraldehyde, isobutyraldehyde and caproaldehyde. The precipitate $S_A$ was also found to contain butyraldehyde.

Peaks due to enanthic acid (heptanoic acid) and caprylic acid (octanoic acid) are seen in the chromatogram of Fig. 3. The corresponding aldehydes were not, however, identified by paper chromatography, whereas the dinitrophenylhydrazone of caproaldehyde was identified on the paper chromatogram. The enanthic and caprylic acids

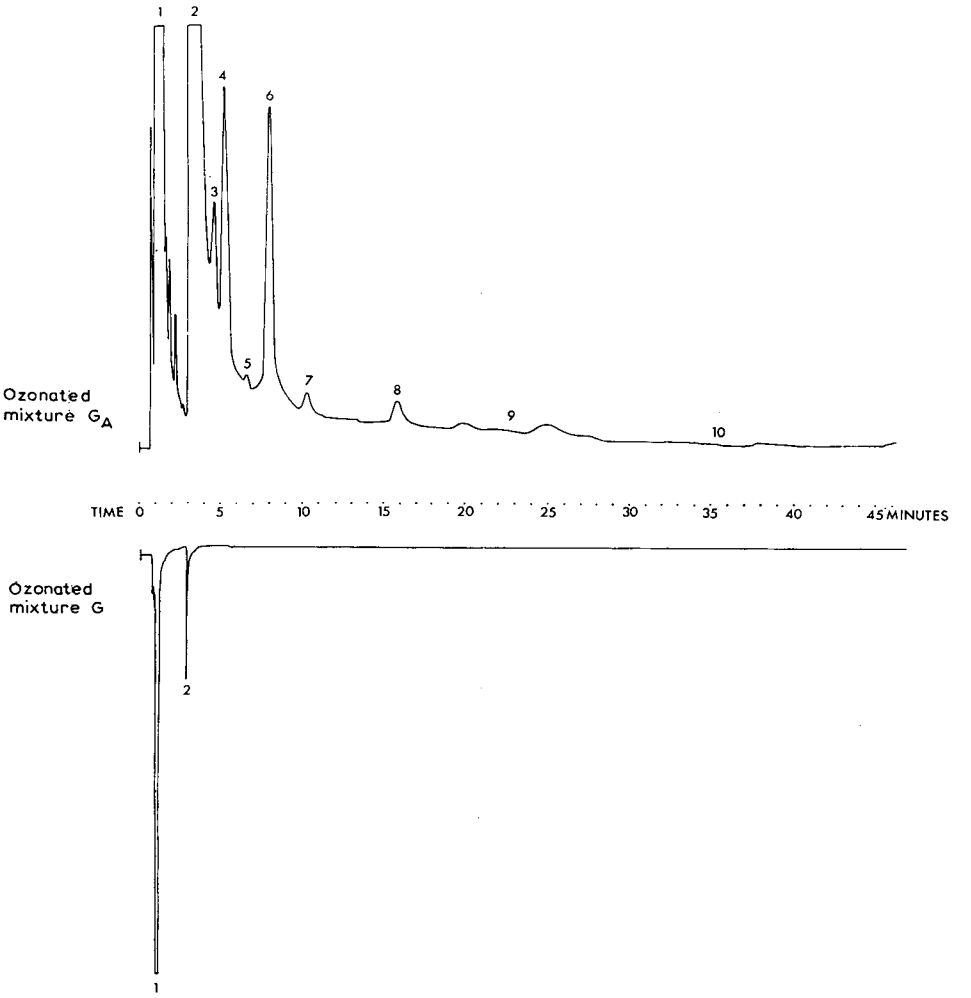


Fig. 2. Gas chromatogram of carboxylic acids produced by ozone oxidation of the 2,4-dinitrophenylhydrazones G isolated by precipitation from 400 ml of crude grain spirit after dilution with water, and that of the carboxylic acids produced by ozone oxidation of the hydrazones $G_A$ isolated by adsorption on carbon from the same solution after filtration and eluted from the carbon. Acetaldehyde is analysed as acetic acid, propionaldehyde as propionic acid, and so on. The hydrazone mixtures G and $G_A$ were each dissolved in 0.5 ml of formic acid for the ozonation and subsequent gas chromatography. Conditions: column length, 4 m; internal diameter, 3 mm; liquid phase, NEGS containing phosphoric acid; solid support, acid-washed Chromosorb W; carrier gas, helium; flow rate, 73 ml/min; inlet pressure, 2.5 kp/cm$^2$; temperature, 140°; detection by flame ionisation: sensitivity, 4; injected volume, 5 $\mu$l. 1 = Solvent; 2 = acetic acid; 3 = propionic acid; 4 = isobutyric acid; 5 = butyric acid; 6 = isovaleric acid and/or 2-methylbutyric acid; 7 = valeric acid; 8 = caproic acid (hexanoic acid); 9 = enanthic acid (heptanoic acid); 10 = caprylic acid (octanoic acid).

were evidently formed in the ozonation of the dinitrophenylhydrazones from a high-molecular weight, possibly unsaturated, carbonyl compound present in these mixtures. Butyraldehyde has not been identified positively in sulphite spirits previously. Propionaldehyde was not identified as propionic acid; the very large peak of acetic acid probably obscured its peak (Fig. 3).

In the quantitative estimation by gas chromatography of the amounts of

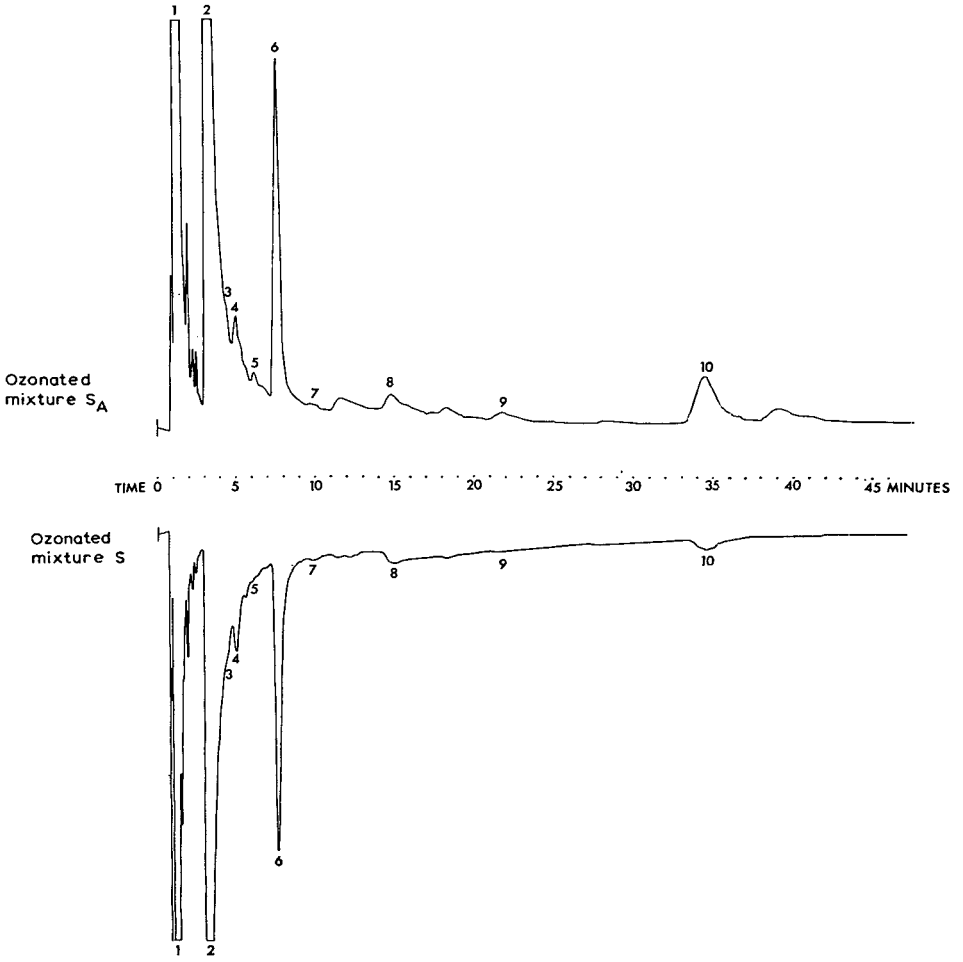


Fig. 3. Gas chromatogram of carboxylic acids produced by ozone oxidation of the 2,4-dinitrophenylhydrazones S isolated by precipitation from 400 ml of crude sulphite spirit after dilution with water, and that of the carboxylic acids produced by ozone oxidation of the hydrazones $S_A$ isolated by adsorption on carbon from the same solution after filtration and eluted from the carbon. Acetaldehyde is analysed as acetic acid, propionaldehyde as propionic acid, and so on. The hydrazone mixture S was dissolved in 5 ml and the mixture $S_A$ in 0.5 ml of formic acid for the ozonation and subsequent gas chromatography. Conditions: column length, 4 m; internal diameter, 3 mm; liquid phase, NEGS containing phosphoric acid; solid support, acid-washed Chromosorb W; carrier gas, helium; flow rate, 76 ml/min; inlet pressure, 2.5 kp/cm$^2$; temperature, 140°; detection by flame ionisation: sensitivity, 4; injected volume, 5 $\mu$l. For designations, see the legend of Fig. 2.

aldehydes in a crude grain spirit, the areas of the peaks in the chromatograms were determined by multiplying the peak heights by the peak half width[12]. To increase the areas of the peaks and the accuracy of their measurement, the chart speed of the recorder was increased to 2 in. per min from the usual 1/3 in. per min, and for the same reason the column temperature was lowered when the acetic acid produced from acetaldehyde hydrazone was analysed. The data for the standard solutions are plotted and joined by straight lines in Fig. 4. The abscissa gives the aldehyde concentration in millimoles in 400 ml of the 95 wt.% ethanol and the ordinate the area of the peak of the carboxylic acid derived from the aldehyde in square centimetres.

The points marked by symbols ×, ○ and △ in Fig. 4 represent the amounts of the components in the crude grain spirit deduced from the peak areas. Each symbol refers to one of three spirit samples. Because all the samples were taken from the same container, the distances between the symbols on each line are due to differences in the adsorption and elution. To eliminate the differences in peak areas due to differences in

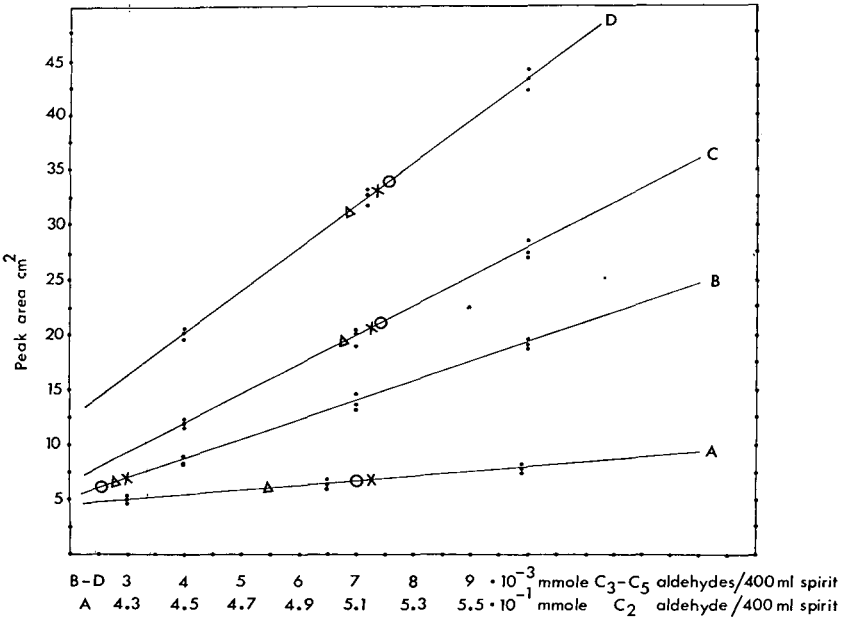


Fig. 4. Calibration lines plotting peak areas of carboxylic acids in gas chromatograms against the numbers of millimoles of aldehydes dissolved in 400-ml volumes of 94 wt.% ethanol. The lines were drawn through observed values marked by points. The aldehydes were isolated as 2,4-dinitrophenylhydrazones by adsorption on carbon from the ethanol, after its dilution with water, and eluted from the carbon and the hydrazones were then converted for gas chromatography into the corresponding carboxylic acids by treatment with ozone. Line A refers to acetic acid derived from acetaldehyde; the gas chromatograms were run using a column temperature of 110° and detector sensitivity of 688. The lines B, C and D refer to propionic acid, isobutyric acid and isovaleric acid, respectively, derived from the corresponding aldehydes; the gas chromatograms were run using a column temperature of 140° and a detector sensitivity of 8. The other conditions in the gas chromatographic runs were: column length, 4 m; internal diameter, 3 mm; liquid phase, NEGS containing phosphoric acid; solid support, acid-washed Chromosorb W; carrier gas, helium; flow rate, 74 ml/min; inlet pressure, 2.5 kp/cm²; detection by flame ionisation; recorder range from 0 to 5 mV; paper speed, 2 in./min; injected volume, 5 μl. Peak areas for carboxylic acids derived similarly from the aldehydes present in three samples of a crude grain spirit are marked by the symbols ×, ○ and △.

injected volumes every symbol in Fig. 4 gives the mean of three values from three gas chromatograms.

The crude grain spirit thus contained, as means of the three mean values (the three symbols in Fig. 4), 1.25 mmole/l (55 mg/l) of acetaldehyde, 0.018 mmole/l (1.5 mg/l) of isovaleraldehyde and/or 2-methylbutyraldehyde, 0.018 mmole/l (1.3 mg/l) of isobutyraldehyde, and 0.007 mmole/l (0.4 mg/l) of propionaldehyde.*

The areas of the peaks in the chromatograms and the corresponding concentrations of aldehydes in the solutions were subjected to regression analysis on an Elliott 803 electronic computer at the Computer Centre of the Suomen Kaapelitehdas Osakeyhtiö (Finnish Cable Works Co.), Helsinki, to determine the mean concentrations of the aldehydes in the crude grain spirit and the 99 % confidence limits of these mean concentrations. The following mean aldehyde concentrations and their 99 % confidence limits were evaluated as: acetaldehyde 55 $\pm$ 3 mg/l; isovaleraldehyde and/or 2-methylbutyraldehyde 1.5 $\pm$ 0.1 mg/l; isobutyraldehyde 1.3 $\pm$ 0.1 mg/l and propionaldehyde 0.4 $\pm$ 0.1 mg/l. The mean values are the same as the mean values obtained by the graphical method (Fig. 4).

SUMMARY

Carbonyl compounds in crude Finnish sulphite and grain spirits have been isolated by precipitation as their 2,4-dinitrophenylhydrazones, and the unprecipitated hydrazones by adsorption on carbon and elution from the latter. The dicarbonyl compounds were identified as their bishydrazones in thin-layer chromatography. Diacetyl and 2,3-pentanedione were found present in these two grades of spirit. Aldehydes were analysed by isothermal gas chromatography on a NEGS column, after ozone oxidation of the hydrazones to the corresponding carboxylic acids. Acetaldehyde proved to be the dominating component, whereas $C_3$–$C_6$ aldehydes were present in smaller quantities. The amounts of acetaldehyde, propionaldehyde, isobutyraldehyde and isovaleraldehyde and/or 2-methylbutyraldehyde in a crude grain spirit were estimated quantitatively by the gas chromatographic method mentioned, using standard solutions of these compounds for reference. The confidence limits of the values were estimated on an electronic computer.

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* The total aldehyde content expressed as acetaldehyde as measured by the fuchsin method was 58.5 $\pm$ 0.3 mg/l as the mean of ten determinations.

# GAS CHROMATOGRAPHY IN QUALITATIVE ANALYSIS

## IV. AN INVESTIGATION OF THE CHANGES IN RELATIVE RETENTION DATA ACCOMPANYING THE OXIDATION OF APOLAR STATIONARY PHASES

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### INTRODUCTION

The results of correlation trials conducted by the Gas Chromatography Discussion Group[1,2] have revealed that the reproducibility of relative retention data obtained by workers in different laboratories is markedly inferior to that of data obtained by an individual worker[3,4]. Possible reasons for the greater scatter of the former results are, slight differences in the adsorptivity of the support materials used (Celite in the first trial, Chromosorb G in the second), non-uniformity of column temperature, and with the apolar stationary phases perturbation of retention data as the result of oxidation. In order to determine the significance of these three effects in qualitative analysis, the roles of the support, column temperature, and stationary phase oxidation in retention measurements have been determined; the latter being the subject of this communication (the other phenomena will be discussed in subsequent papers).

### EXPERIMENTAL

#### *Apparatus*

Chromatograms were obtained using an apparatus consisting of Pyrex glass columns (5 ft. long, 4 mm I.D.) with a modified flame ionisation detector[5]. The columns were packed with 20 % w/w mixtures of stationary phase (for details see text) and acid washed (1 % v/v phosphoric acid) 60–72 mesh Celite (J.J's Chromatography Ltd., King's Lynn, Norfolk). The column temperature was regulated by a vapour jacket consisting of boiling water (100°). A mixture of hydrogen and nitrogen (3:1 by volume) was used as carrier gas, except in the oxidation experiments. Mixtures of the solutes of interest and suitable *n*-alkane standards in either *n*-pentane or *n*-heptane solution (~ 5 % w/v) were introduced onto the column by means of stainless steel capillary pipettes[6].

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*Measurement of relative retention data*

Relative retentions, in KOVATS' retention index units[7], were obtained from chromatograms of suitable mixtures containing *n*-alkanes as internal standards. Retention distances, measured between point of injection and peak maxima, were corrected for the column gas hold-up volume by means of the retention of methane[8]. The adjusted retentions were then used to calculate values for retention index by means of the following expression:

$$I = 100\,N + 100\,n\left\{\frac{\log_{10}R_x - \log_{10}R_N}{\log_{10}R_{N+n} - \log_{10}R_N}\right\}$$

where $R_x$, $R_N$, and $R_{N+n}$ are the adjusted retentions of the solute, and the *n*-alkane standards containing $N$ and $N+n$ carbon atoms, respectively.

## DISCUSSION AND RESULTS

Previous work carried out in the authors' laboratory has revealed that the apparent polarity, as indicated by values for retention, of apolar stationary phases frequently increases with column usage, particularly at elevated temperatures. In so far as it was found that this effect could be largely eliminated if care was taken to exclude air from the column it was tentatively ascribed to the oxidation of the liquid phase. In order to substantiate this hypothesis, squalane–Celite columns have been deliberately oxidised and the changes in polarity followed by retention measurements.

*Selection of solutes suitable for use as physico-chemical probes*

In order to select solutes which would reveal the chemical changes induced by oxidation, the retentions of compounds of a wide range of chemical types were determined using squalane containing known quantities of combined oxygen. Although the possibility of oxidising squalane to known extents was considered, this method was not used owing to the likelihood of the oxygenated groups being lost or modified during the preparation of the liquid phase–support mixtures. Instead, known mixtures of squalane and involatile, thermally stable, aliphatic oxygenated substances (namely, *n*-octadecanol and di-*n*-octyl ketone) were prepared by the following procedure:

(i) Appropriate quantities of squalane and *n*-octadecanol or di-*n*-octyl ketone (total 4.0 g in each case) were weighed into a 250 ml round bottomed flask, 30 ml of redistilled dichloromethane added and the solution warmed until the alcohol or ketone had dissolved fully.

(ii) Sixteen grams of Celite were then poured into the solution and the solvent carefully evaporated under a water pump vacuum; the flask being periodically immersed in warm water (approximately 50°) in order to compensate for the heat losses accompanying evaporation.

The columns were packed in the normal way and the final traces of solvent removed by the passage of carrier gas at 100°; the columns being deemed ready for use once a stable base-line had been obtained.

The retentions, in KOVATS' retention index units, of all the solutes examined were found to increase with the oxygen content of the mixed stationary phases, as shown in Table I. In each case the shifts of retention were found to vary linearly with

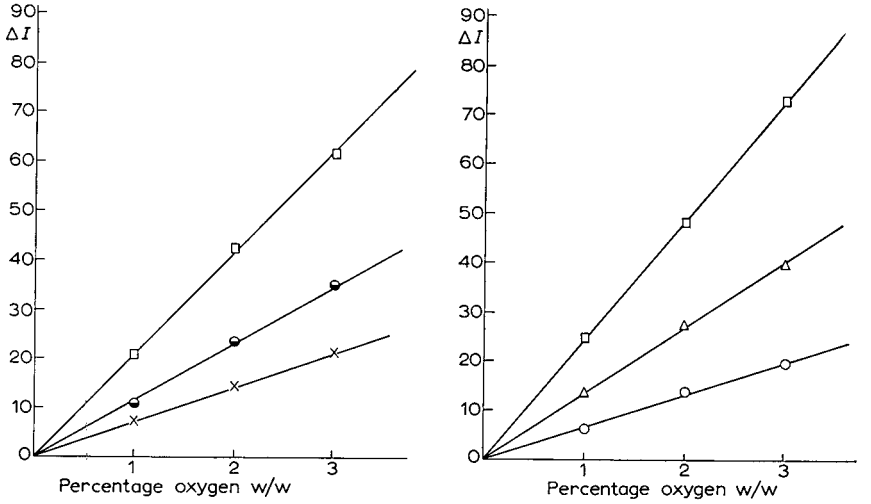


Fig. 1. Change of retention index with introduction of oxygen into a squalane column (as *n*-octadecanol). (□) = Nitrobenzene; (◒) = phenetole; (×) = *n*-butylbenzene.
Fig. 2. Change of retention index with introduction of oxygen into a squalane column (as di-*n*-octyl ketone). (□) = Nitrobenzene; (△) = 1,2-dibromoethane; (○) = toluene.

oxygen content, as illustrated by the typical examples in Figs. 1 and 2, indicating the possibility of determining the extent of oxidation of an "aged" apolar column from retention data. On this basis, the results for operator G, in the Gas Chromatography Discussion Group retention data correlation trial (see Table II in ref. 1), would suggest the presence of approximately 3 % of combined oxygen in the squalane used, provided that the groups formed on oxidation have similar retentive properties to alcoholic hydroxyl and ketonic carbonyl respectively, and that the contribution of the

TABLE I

CHANGES IN VALUES FOR RETENTION INDEX ACCOMPANYING THE INTRODUCTION OF OXYGEN, AS EITHER HYDROXYL OR CARBONYL GROUPS, INTO AN APOLAR STATIONARY PHASE

Column: 5 ft. × 4 mm 20 % w/w stationary phase, Celite at 100°.

| | *Oxygen as n-octadecanol* (% *w/w*) | | | | *Oxygen as di-n-octyl ketone* (% *w/w*) | | |
|---|---|---|---|---|---|---|---|
| | *0* | *1.0* | *2.0* | *3.0* | *1.0* | *2.0* | *3.0* |
| Toluene | 756.4 | 764.5 | 771.4 | 779.0 | 762.4 | 770.0 | 775.8 |
| 1,2-Dibromoethane | 783.9 | 798.9 | 811.3 | 821.9 | 797.2 | 811.2 | 823.3 |
| Chlorobenzene | 826.3 | 836.1 | 845.3 | 854.3 | 836.2 | 846.4 | 856.6 |
| Allyl *tert.*-butyl sulphide | 855.7 | 866.3 | 876.4 | 884.7 | 861.4 | 869.3 | 877.8 |
| Benzonitrile | 905.5 | 927.4 | 955.2 | 977.8 | 928.8 | 953.4 | 978.5 |
| Camphene | 947.4 | 949.7 | 952.4 | 955.6 | 948.2 | 949.7 | 950.3 |
| Phenetole | 953.7 | 964.3 | 976.9 | 988.5 | 961.0 | 972.6 | 982.4 |
| *trans*-2,6-Dimethyl-octa-2,6-diene | 978.4 | 982.1 | 985.6 | 988.3 | 980.1 | 982.0 | 985.4 |
| Nitrobenzene | 1008.0 | 1028.6 | 1050.2 | 1069.3 | 1032.4 | 1056.1 | 1080.8 |
| 1-Iodohexane | 1011.8 | 1019.8 | 1028.0 | 1034.8 | 1019.2 | 1026.3 | 1035.4 |
| *n*-Butylbenzene | 1035.2 | 1042.8 | 1049.5 | 1056.0 | 1040.6 | 1047.6 | 1054.5 |
| Di-*n*-butyl sulphide | 1060.0 | 1069.5 | 1078.4 | 1085.6 | 1064.8 | 1071.4 | 1078.0 |

support to retention is negligible (see Table II). The inclusion, in this investigation, of materials containing strongly polar functional groups, such as alcohols, ketones, and esters, was precluded by severe peak tailing; presumably due to an adsorption phenomenon*.

On the basis of the results given in Table I, toluene, 1,2-dibromoethane, benzonitrile, phenotole, nitrobenzene, and allyl *tert.*-butyl sulphide were chosen as physico-chemical probes for the oxidation experiments. The sulphide was later withdrawn from this list because it was found to be oxidised, presumably to either the corresponding sulphoxide or sulphone, by oxidised squalane.

*Changes of retention induced by the deliberate oxidation of a squalane–Celite column*

A fresh squalane–Celite column was prepared and the retention indices of the compounds chosen as physico-chemical probes determined at 100°, by the established procedures. The column was then oxidised, as follows:

(i) The cracked ammonia carrier gas supply ($H_2$–$N_2$, 3:1) was replaced by oxygen by the appropriate adjustment of a three-way tap (connecting the column, and the oxygen and cracked ammonia cylinders, respectively).

(ii) The oxygen was allowed to flow for approximately 10 min, during which time the rate of flow was monitored continuously by means of a soap film bubble meter (in order that the volume of oxygen passing through the column could be determined).

(iii) The oxygen flow was then replaced by the hydrogen–nitrogen mixture, and the chromatograms of suitable calibration solutions of the solutes shown in Table III and suitable *n*-alkane internal standards were obtained.

(iv) The oxygen flow was resumed and the cycle repeated.

Diatomaceous earths, which are widely employed as gas chromatographic supports, have relatively high surface areas[9] (Celite ~ 1 sq.m/g) so that the average film thickness of the liquid phase in a normal packed column is only about 0.2 $\mu$. It is therefore probable that, in the presence of oxygen at elevated temperatures, apolar stationary phases would undergo oxidation to yield oxygenated groups, which in the bulk liquid phase would give rise to increased forces of solute–solvent inter-

TABLE II

DETERMINATION OF THE EXTENT OF OXIDATION OF THE SQUALANE COLUMN USED BY OPERATOR G, IN THE G.C.D.G. CORRELATION TRIAL[a], FROM VALUES FOR RETENTION INDEX

Column: 5 ft. × 4 mm 20% w/w stationary phase, Celite at 100°.

| | *Values for retention index* | | | *Oxygen content* (% *w/w*)[a] | |
|---|---|---|---|---|---|
| | *Operator G* | *Operators C.E.H. (mean value)* | *δI* | *>C=O* | *—OH* |
| Toluene | 776 | 756 | 20 | 2.45 | 3.10 |
| Chlorobenzene | 855 | 827 | 28 | 3.05 | 2.90 |
| Di-*n*-butyl sulphide | 1079 | 1059 | 20 | 2.05 | 3.25 |

[a] The oxygen contents were determined by interpolation on the appropriate plots of retention index change *versus* percentage combined oxygen.

* Subsequent work (to be described in Part VII of this series) revealed that meaningful chromatograms could be obtained for apolar stationary phases provided low surface area, chemically inactive supports, such as Chromosorb G, were used.

action, and in turn increased values for retention index. However, when oxygen was passed through a squalane–Celite column at 100° the retention indices of all the solutes, with the exception of toluene and 1,2-dibromoethane, were found to decrease, pass through a minimum value, and then increase*. In each case, the decreases in retention were accompanied by improvements of peak symmetry, the peaks being symmetrical at the minimum and beyond. The changes in the values for retention index induced by oxidation are summarised in Table III and illustrated by the typical examples shown in Fig. 3.

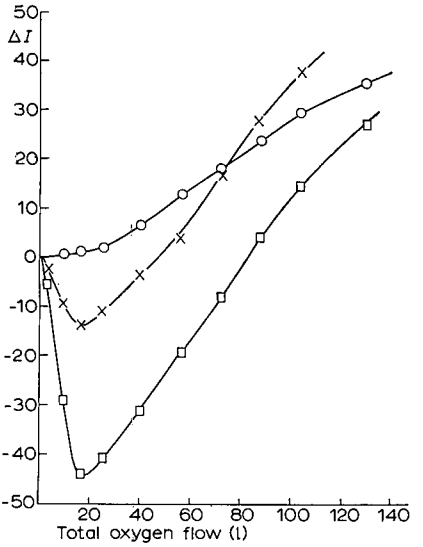


Fig. 3. Changes of retention index observed during the oxidation of a squalane column at 100°. (○) = 1,2-Dibromoethane; (□) = benzonitrile; (×) = nitrobenzene.

A likely explanation of these changes of retention is that oxidation of squalane, or impurities in the material supplied for use in gas chromatography, gives rise to strongly polar groups which are preferentially adsorbed on to the support surface, thus precluding the adsorption of the solute molecules. The gradual formation of a coherent protective film would thus give rise to the observed peak symmetry and retention changes; it being assumed that the peak asymmetry (tailing) observed in the case of the squalane column prior to oxidation was a consequence of adsorption of solute molecules upon active sites on the support surface. Once the adsorptive sites are saturated, the excess oxygenated groups would then be available for partitioning purposes, in the bulk liquid phase, thus increasing the apparent polarity of the liquid phase, consistent with the observed values for retention. On this evidence the minimum of the curve may be regarded as the retention index characteristic of the simple partitioning of the solute molecules between the mobile gas phase and pure squalane. Whilst the increases to the left of the minimum may be regarded as the contributions

* This characteristic behaviour was not detected in the case of the squalane–*n*-octadecanol and squalane–di-*n*-octyl ketone mixtures, presumably because of the relatively high proportion of oxygenated material in the mixtures. However, later work has revealed that minima do occur on the plots of retention index *versus* oxygen content provided the concentration of combined oxygen is in the region of 0.1 to 0.3 %[10].

TABLE III

CHANGES OF RETENTION ACCOMPANYING THE OXIDATION OF A SQUALANE–CELITE COLUMN AT 100°

| *Substance* | *Values for retention index* | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Toluene | 756.4* | 756.1** | 757.3 | 756.6 | 756.7 | 756.8 | 760.0 | 762.3 | 764.7 | 767.5 | 771.0 | 774.2 | 778.3 |
| 1,2-Dibromoethane | 783.9 | 783.1 | 782.9 | 783.7 | 784.4 | 784.9 | 789.7 | 796.1 | 800.7 | 806.6 | 812.4 | 818.5 | 825.7 |
| Benzonitrile | 905.5 | 935.8 | 930.6 | 906.0 | 891.8 | 895.1 | 904.5 | 916.4 | 917.8 | 939.9 | 950.1 | 962.8 | 979.8 |
| Phenetole | 953.7 | 952.7 | 952.1 | 950.4 | 949.4 | 951.0 | 954.8 | 957.9 | 964.3 | 969.3 | 974.0 | 979.7 | 987.1 |
| Nitrobenzene | 1008.0 | 1017.5 | 1015.2 | 1008.2 | 1003.7 | 1006.8 | 1014.0 | 1021.0 | 1034.7 | 1045.1 | 1055.0 | 1066.2 | 1081.3 |
| Volume of oxygen passed (l) | | Nil | 3.4 | 9.8 | 16.2 | 24.9 | 39.8 | 56.5 | 72.2 | 87.7 | 104.0 | 130.3 | 166.8 |

* Original column used in calibration work.

** New squalane–Celite column prepared using a fresh batch of Celite.

The differences between these two sets of retention data are almost certainly due to presence of more active sites on the surface of the second batch of Celite.

to the overall retention due to the adsorption of solute molecules at the liquid–solid interface. Adsorption at the gas–solid interface may be discarded, in this context, since two 10% squalane–Celite columns, one prepared by the normal slurry method and the other by mixing appropriate quantities of Celite and a 20% squalane–Celite mixture, gave identical retention and peak symmetry characteristics for a wide range of solutes, thereby revealing squalane to be sufficiently mobile to completely cover the support surface.

TABLE IV

COMPARISON OF THE RETENTION CHARACTERISTICS OF A NORMAL SQUALANE–CELITE COLUMN WITH ONE CONTAMINATED WITH SILICA GEL

Column A: 20% w/w squalane–Celite at 100°.
Column B: 20% w/w squalane–0.1% w/w silica gel–Celite at 100°.

| | *Values for retention index* | | | |
|---|---|---|---|---|
| | *Column A* | | *Column B* | |
| | *Initial* | *Minimum* | *Initial* | *Minimum* |
| Toluene | 756 | —* | 758 | 756 |
| 1,2-Dibromoethane | 783 | —* | 786 | 784 |
| Benzonitrile | 936 | 891 | 956 | 894 |
| Phenetole | 953 | 949 | 960 | 950 |
| Nitrobenzene | 1017 | 1002 | 1028 | 1004 |

* No minimum detected.

If the above arguments were correct, contamination of Celite with a highly adsorptive material, such as silica gel, would be expected to give rise to increased initial values for retention index for polar solutes, but not weakly polar solutes, but with the same minimum values on the plots of retention index *versus* oxygen flow, as a normal squalane–Celite column. Oxidation of a squalane column prepared using Celite deliberately contaminated with silica gel gave rise to this predicted behaviour, as illustrated by the data in Table IV, thus confirming our hypothesis. Accordingly there appears to be little doubt that in the case of apolar liquid phases adsorption of solute molecules at the liquid–solid interface can contribute significantly to the overall values for retention. This phenomenon will be explored further in a future paper in the present series.

SUMMARY

Oxidation of an apolar stationary phase has been shown to lead to large changes of values for retention index, which can be either negative or positive depending upon the extent of oxidation, the chemical nature of the solutes, and the adsorptivity of the support. Therefore it would seem necessary to deoxygenate apolar phases, by conventional column chromatography, prior to preparing liquid phase–support mixtures and ensure the exclusion of oxygen during the use of the column, in order to obtain reproducible retention data. Furthermore, it would seem necessary to use supports of low adsorptivity, which as far as possible are batch invariant.

The results of this work also reveal that adsorption of solutes at the liquid–solid interface can lead to increased values for retention index with concomitant peak asymmetry.

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# THE USE OF THE CHROMATOGRAPHIC EQUILIBRATION PROCEDURE FOR AIR POLLUTION STUDIES

## DETERMINATION OF MINUTE AMOUNTS OF BENZENE, CHLOROBENZENE, AND NITROBENZENE IN AIR

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### INTRODUCTION

Standard procedures for the determination of benzene in the presence of nitrobenzene in air pollution control are based on polarography, while chlorobenzene is usually determined by photometry. In pyridine, acidified with acetic acid, nitrobenzene gives a well-developed reduction wave whose height is proportional to the concentration of nitrobenzene[1–3], whereas chlorobenzene is determined by photometry of the violet complex of 1-chloro-2,4-dinitrobenzene formed in an alkaline solution of the latter in pyridine[4]. The application of gas chromatography for the analysis of air pollutants has been tackled by several authors[5–7] and seems to be one of the most promising techniques. However, the detection limits, particularly for nitrobenzene, are not satisfactory when using gas chromatography directly, due to the very low vapour pressure of this compound.

Concentration of air pollutants prior to their determination by gas chromatography is, therefore, very often unavoidable**. The most frequent concentration techniques are: condensation[8], chromatographic sorption[9], or chromatographic equilibration[10]. The purpose of the present paper is to show the advantages of chromatographic equilibration, as described by NOVÁK, VAŠÁK AND JANÁK[10], for the determination of minute concentrations of benzene, chlorobenzene and nitrobenzene in chlorobenzene and nitrobenzene producing plants.

### EXPERIMENTAL

Artificial mixtures of the above air pollutants with air were obtained by using a conventional assembly with vaporizers for obtaining saturated vapour of the individual components and mixing, in a mixing chamber, the appropriate volumes of the saturated vapours so obtained with air. The various mixtures are obtained by

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** U.S. TLV's (Threshold Limit Values) for benzene, chlorobenzene, and nitrobenzene are, respectively, 80, 350, and 5 mg/m³ (see ref. 11).

controlling the flow rates in the respective branches of the sampling assembly (contaminator) (Fig. 1).

*Concentration tubes*

The design and packing of the concentration tubes were the same as described elsewhere[10]. The concentration tubes were packed with E 301 (30 % w/w) on Celite 545 (30/60 mesh). The tubes were conditioned in the usual manner for several hours at 150°C and, afterwards, stoppered at both ends.

*Sampling and desorption*

The necessary volumes of artificially polluted air, as demanded by theory, were drawn through the concentration tubes at room temperature, which was carefully measured; the concentration tubes were then disconnected from the sampling assembly and stoppered at both ends.

The desorption was carried out in a special, electrically heated oven, maintained at a controlled temperature, and positioned adjacent to the injection port of the gas chromatographic apparatus.

*Gas chromatography of desorbed samples*

Gas chromatographic analyses were carried out with a Chrom II apparatus (Laboratory Instruments, Prague), equipped with a flame ionization detector (FID) and specially adapted for the purposes of the present paper; for details see[10].

Specific retention volumes were measured using a glass column 42 cm long, I.D. 7 mm, packed with 6.00 g of E 301 (30 % by weight) on Celite 545 (30/60 mesh) packing material. The temperature of the column was varied from 30°C to 83°C (*cf.* Table I).

The relative molar response of benzene, chlorobenzene, and nitrobenzene was measured using a stainless steel column 85 cm long, I.D. 6 mm, packed with 5 % by weight of E 301 on Chromosorb W (30/60 mesh) at 65°C. The inlet pressure was 0.175 kp/cm², the chart drive 40 mm/min, and the weight of packing material 7.55 g.

Standard solutions were prepared by weighing the appropriate amounts of individual components and solvent. All solutions were stored in well-stoppered flasks in a refrigerator.

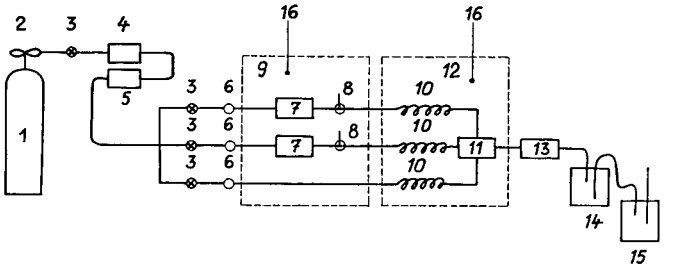



Fig. 1. Contaminator assembly. 1 = Air cylinder; 2 = two-stage reducer; 3 = needle valves; 4 = charcoal filter; 5 = molecular sieve; 6 = pressure gauges; 7 = vaporizers; 8 = stopcocks (T); 9 = thermostatted bath kept at elevated temp.; 10 = cooling coils; 11 = mixing chamber; 12 = cooling bath kept at room temp.; 13 = concentration tube; 14 = volumetric bottle; 15 = levelling bottle; 16 = thermometers.

## RESULTS AND DISCUSSION

The main prerequisite of the method used[10] is to achieve equilibrium conditions in the concentration tube. At equilibrium, the concentration of a given component in the gaseous phase, $C_G$, is equal to the concentration of the same in the sample analysed; this concentration, therefore, can be calculated from $C_G = 273\, m/V_g(T)GT$, where $m$ is the mass of the component trapped in the tube ($m$ can be determined by using a conventional GC procedure), $V_g(T)$ is the specific retention volume of the component as measured on the tube packing at the temperature of sampling, $T$, and $G$ is the weight of the sorbent liquid. The above relation can be used to calculate the concentration efficiency, as compared with direct injection of sample. The height of the peak of a component, $h$, can be expressed as

$$h = A \cdot n \cdot v \cdot C_G/V_R \tag{1}$$

where $n$ is the number of theoretical plates of the chromatographic column, $V_R$ is the retention volume of the component as measured on this column, $A$ is a proportionality constant involving specific features of the instrument and of the substance under analysis, and $v$ is the volume injected.

When using a concentration tube, the amount of substance sorbed in the sorbent liquid is equivalent to its amount contained in its retention volume, and depends upon the tube packing and on the conditions of the determination,

$$V_R = V_g(T)GT/273 \tag{2}$$

As there is an upper limit, $v_{max}$, to the volume $v$ directly injected, the ratio

$$v_{max}273/V_g(T)GT \tag{3}$$

determines the extent to which the actual concentration range amenable to measurements can be decreased when using the equilibration technique as compared with direct injection.

Let us suppose that the maximum injected volume, $v_{max}$, of the gaseous sample is 10 ml and the weight of sorbent in the concentration tube is about $10^{-1}$ g. If the specific volume of the component under analysis is about $10^3$ ml/g, then the minimum detectable amount of this compound becomes $10^1$ times less than on direct injection of the sample. At room temperature and in E 301, the specific retention volume of nitrobenzene is of the order of $10^4$ ml/g. Thus, we can increase the sensitivity of determination for nitrobenzene by two orders of magnitude while using an unsophisticated sampling device, and considerably shortening the time necessary for carrying out the actual sampling, because a relatively quick withdrawal of the sample has been proved to be of considerable importance. The volume of nitrobenzene which has to be drawn through the concentration tube is less than about 5 l (with a very fair safety margin for attainment of equilibrium in the tube), which at a flow rate of 5 ml/min represents approximately 17 min or rather less.

The specific retention volumes of benzene, chlorobenzene, and nitrobenzene were measured at several temperatures ranging from 30° to 83°C in order to determine their

temperature dependence. The respective plots were linear, thus making feasible extrapolation to lower temperatures by using equations of the type: $\log V_g(T) = A/T - B$ The data are summarized in Table I.

TABLE I

SPECIFIC RETENTION VOLUMES OF BENZENE, CHLOROBENZENE AND NITROBENZENE AT TEMPERATURES FROM 15°–83°C

E 301 (30% by weight) on Celite 545 (30/60 mesh).
a = Extrapolated values; b = measured values.
Equations for $\log V_g$: benzene, $\log V_g = 1{,}867.4/T - 3.746$; chlorobenzene, $\log V_g = 2{,}291.5/T - 4.434$; nitrobenzene, $\log V_g = 2{,}858.5/T - 5.415$.

| $t$ (°C) | $T$ (°K) | $1/T \times 10^3$ | $V_g$ (ml/g) | | |
|---|---|---|---|---|---|
| | | | $C_6H_6$ | $PhCl$ | $PhNO_2$ |
| 15 | 288 | 3.472 | 547.1[a] | 3,332[a] | 32,360[a] |
| | | 3.466 | 533.2 | 3,228 | 31,100 |
| 16 | 289 | 3.460 | 519.6 | 3,127 | 29,990 |
| | | 3.454 | 506.4 | 3,030 | 28,750 |
| 17 | 290 | 3.448 | 493.6 | 2,936 | 27,640 |
| | | 3.442 | 481.2 | 2,846 | 26,580 |
| 18 | 291 | 3.436 | 469.1 | 2,768 | 25,570 |
| | | 3.431 | 457.3 | 2,674 | 24,600 |
| 19 | 292 | 3.425 | 446.0 | 2,592 | 23,660 |
| | | 3.419 | 434.9 | 2,513 | 22,770 |
| 20 | 293 | 3.413 | 424.1 | 2,437 | 21,910 |
| | | 3.407 | 413.6 | 2,364 | 21,090 |
| 21 | 294 | 3.401 | 403.4 | 2,292 | 20,300 |
| | | 3.396 | 393.5 | 2,224 | 19,540 |
| 22 | 295 | 3.390 | 383.9 | 2,157 | 18,810 |
| | | 3.384 | 374.6 | 2,093 | 18,120 |
| 23 | 296 | 3.378 | 365.5 | 2,031 | 17,450 |
| | | 3.373 | 356.6 | 1,971 | 16,810 |
| 24 | 297 | 3.367 | 348.0 | 1,912 | 16,190 |
| | | 3.361 | 339.7 | 1,856 | 15,600 |
| 25 | 298 | 3.356 | 331.5 | 1,802 | 15,030 |
| 30 | 303 | 3.300 | 259.3[b] | 1,370[b] | —[b] |
| 39 | 312 | 3.200 | 175.5 | 847 | — |
| 40 | 313 | 3.195 | — | — | 5,140 |
| 50.5 | 323.5 | 3.091 | — | — | 2,596 |
| 51 | 324 | 3.086 | 99.6 | 423 | — |
| 60.5 | 333.5 | 2.999 | — | — | 1,481 |
| 63 | 336 | 2.976 | 64.7 | 237 | — |
| 69 | 342 | 2.924 | — | — | 852 |
| 77 | 350 | 2.857 | 38.3 | 132 | — |
| 83 | 356 | 2.809 | — | — | 415 |

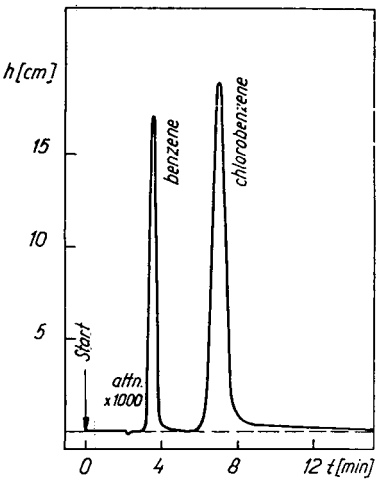
These $V_g$ values were used for the calculation of the concentration, $C_G$, of the respective pollutants. The mass of the individual compounds was calculated from chromatographic measurements. As the relative molar response of the FID to chlorobenzene and nitrobenzene was not known, it had to be determined. Careful measurements have shown that the relative molar responses for chlorobenzene and nitrobenzene were, respectively, 0.678 and 0.524, when using mesitylene as the reference compound. In other words, the contribution of chlorine and nitro substituents to the total effective carbon number was, respectively, + 0.1 $C_{eff}$ and — 1.3 $C_{eff}$.

The evaluation of chromatograms obtained for samples of artificially polluted air was performed by the internal standard technique, using a mesitylene standard; in some cases, xylene was used instead. Fig. 2 illustrates a chromatogram obtained for a mixture of benzene and chlorobenzene with air. The concentrations of the compounds were 500 and 450 mg/m³, respectively. The volume of air drawn through the concentration tube was 1.0 l. At a flow rate of approximately 5 ml/sec the sampling lasted approximately 3.5 min. Fig. 3 represents a chromatogram of benzene and nitrobenzene as pollutants, obtained in a similar manner. In this case the concentration of benzene was 400 mg/m³ and that of nitrobenzene 10 mg/m³.

The chromatograms show that the determination of the trace components mentioned can be performed at concentrations which are considerably lower than their TLV's; *e.g.* for a nitrobenzene determination to give fair results, it would be possible to work at concentrations 100 times less than the TLV. This finding prompted an analytical study of the TLV's[12].

As can be seen from Fig. 3, benzene and nitrobenzene can be determined simultaneously in one run; the fact that the content of the more volatile benzene is greater by a factor of 40 does not present any problems. This example shows the main advantage of the equilibration method over other concentration methods. The masses of individual substances become equilibrated and a gas chromatogram of the mixture consists of peaks of comparable areas, which can be evaluated very accurately, since the lower the vapour pressure of the substance under investigation, the larger its partition coefficient and, consequently, the greater the concentration effect. The choice of a non-polar stationary phase eliminated the influence of water vapour present in the usually moist air samples, as the partition coefficient of water, on E 301, is nearly zero.

For the sake of comparison, the chromatogram illustrated in Fig. 4 has been



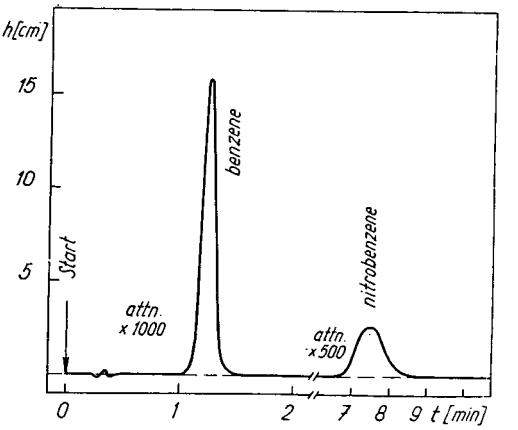




Fig. 2. Determination of chlorobenzene in the presence of benzene. Benzene 500 mg/m³; chlorobenzene 450 mg/m³; FID, E 301 (30% w/w) on Celite 545; concentration tube.

Fig. 3. Determination of nitrobenzene in the presence of benzene. Nitrobenzene 10 mg/m³; benzene 400 mg/m³; FID; E 301 (30% w/w) on Celite 545; concentration tube.

obtained by directly injecting a 5 ml sample taken from the contaminator without previous concentration. The agreement between the concentration expected, *i.e.* as calculated from vapour pressure and dilution data, and a sample equilibrated by the method used, varied within 25 relative %. These variations were due probably to non-equilibrium conditions in the contaminator.

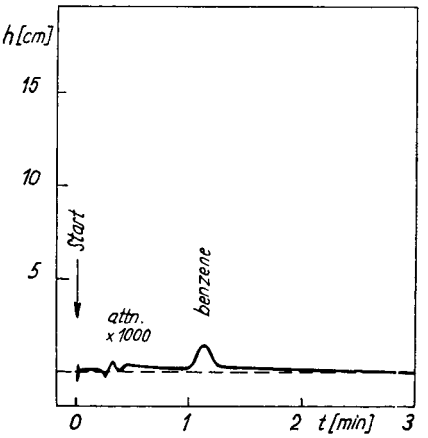



Fig. 4. Determination of benzene and nitrobenzene. Concentrations same as in Fig. 3; direct injection of 5 ml sample from contaminator; other conditions same as in Fig. 3.

Care must be taken also in extrapolating the $V_g$ data. In order to speed up the determinations of $V_g$, we used, in a preliminary experiment, a column packed with 5 % by weight of E 301 on Chromosorb W (30/60 mesh). Upon comparing the $V_g$ values so obtained with $V_g$ data obtained on a 30 % by weight E 301 on Celite 545 column, it was found that they did not tally. Neither did the use of freshly prepared packing materials for both columns make the $V_g$ values coincide.

The actual amounts of individual components of dilute gaseous mixtures directly withdrawn from the contaminator were calculated from the vapour pressure data taken from literature[13] and summarized, together with the respective concentration data for saturated vapours of pure compounds in Table II.

## CONCLUSIONS

The applicability of the chromatographic equilibration technique for air pollution control has been verified for the systems benzene–chlorobenzene, benzene–nitrobenzene, and for a ternary mixture of the three compounds. For field assays, invaluable advantages of the method used, as has already been emphasized, are:

(1) Water does not interfere with the determinations when non-polar phase is used for the concentration-tube packing.

(2) The extent to which individual components are trapped is proportional to their partition coefficients, *i.e.* usually inversely proportional to their volatility. Thus, the least volatile compounds that are present in the atmosphere usually at lowest concentrations, are most efficiently accumulated.

TABLE II

VAPOUR PRESSURES AND CONCENTRATION OF SATURATED VAPOURS OF BENZENE, CHLOROBENZENE AND NITROBENZENE

| *Temperature* | | *Benzene* | | *Chlorobenzene* | | *Nitrobenzene* | |
|---|---|---|---|---|---|---|---|
| *t* (°C) | *T* (°K) | $p \times 10^{-2}$ (*atm*) | $g/l \times 10^{-1}$ | $p \times 10^{-3}$ (*atm*) | $g/l \times 10^{-2}$ | $p \times 10^{-4}$ (*atm*) | $g/l \times 10^{-3}$ |
| 15 | 288 | 7.734 | 2.556 | 9.057 | 4.314 | 2.579 | 1.344 |
| 16 | 289 | 8.130 | 2.678 | 9.626 | 4.569 | 2.737 | 1.421 |
| 17 | 290 | 8.544 | 2.805 | 10.229 | 4.839 | 2.908 | 1.505 |
| 18 | 291 | 8.974 | 2.936 | 10.863 | 5.121 | 3.066 | 1.581 |
| 19 | 292 | 9.422 | 3.072 | 11.534 | 5.419 | 3.250 | 1.670 |
| 20 | 293 | 9.889 | 3.213 | 12.238 | 5.730 | 3.447 | 1.765 |
| 21 | 294 | 10.374 | 3.359 | 12.982 | 6.057 | 3.632 | 1.853 |
| 22 | 295 | 10.758 | 3.472 | 13.766 | 6.402 | 3.816 | 1.941 |
| 23 | 296 | 11.404 | 3.668 | 14.580 | 6.762 | 4.026 | 2.041 |
| 24 | 297 | 11.950 | 3.830 | 15.458 | 7.140 | 4.237 | 2.140 |
| 25 | 298 | 12.518 | 3.999 | 16.371 | 7.536 | 4.474 | 2.252 |
| 26 | 299 | 13.107 | 4.173 | 17.332 | 7.952 | 4.658 | 2.337 |
| 27 | 300 | 13.719 | 4.353 | 18.342 | 8.388 | 4.882 | 2.441 |
| 28 | 301 | 14.354 | 4.540 | 19.404 | 8.844 | 5.092 | 2.538 |
| 29 | 302 | 15.013 | 4.733 | 20.520 | 9.321 | 5.316 | 2.641 |
| 30 | 303 | 15.780 | 4.958 | 21.691 | 9.821 | 5.592 | 2.769 |

(3) During sampling, care need not be taken of the exact volume drawn through the concentration tube, thus eliminating the use of clumsy-to-operate sampling devices.

(4) The sampling procedure is very versatile and extremely simple, thus rendering the method suitable for field practice. Also the transport of the pocket-size tubular samples is convenient.

(5) The overall time necessary to prepare and analyse samples of benzene in the presence of chloro- or nitrobenzene is about 30 to 40 min as compared with standard procedures, where a complete analysis takes several hours. The equilibration method is therefore of considerable advantage in studying micro-climatic conditions and momentary surges of exhalates.

ACKNOWLEDGEMENT

One of us, M.S., wishes to express his gratitude to Dr. V. KLEINBAUER for obtaining permission for him to undertake this investigation.

SUMMARY

The equilibration technique has been used for the determination of benzene in the presence of nitrobenzene and chlorobenzene.

The advantages of this technique as compared with standard procedures used in air pollution studies, as well as its relative simplicity, make it suitable for routine work.

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# STRUCTURAL CORRELATIONS TO SOLUTE ELUTION AND MOLECULAR SENSITIVITY DATA IN THE ELECTRON CAPTURE ANALYSIS OF ISOMERIC CHLOROPHENYL *m*-FLUOROSULFONYLBENZOATES

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An earlier study reported the gas chromatography of *m*-fluorosulfonylphenyl carbamates[1] in which it was noted that the solutes underwent thermal lysis to *m*-fluorosulfonylaniline and respective phenols during analysis. The thermal instability of the aryl carbamoyl grouping has been experienced in several other investigations[2–5]. It was of interest to compare the analytical stability of this moiety to the aryl ester linkage *via* chromatography of *m*-fluorosulfonylbenzoate esters and to investigate the gas chromatographic behavior of this related class of derivatives.

## EXPERIMENTAL

The *m*-fluorosulfonylbenzoates were synthesized by reaction of *m*-fluorosulfonylbenzoyl chloride with various phenols in pyridine medium. Melting points were determined on a Fisher-Johns melting point apparatus. Gas chromatography was performed on a 3 ft. by 0.125 in. coiled Pyrex glass column packed with 4 % Dow-11 silicone on 40–60 mesh HMDS-pretreated Chromosorb W, and housed in an Aerograph Hy-FI Model 600-B (Varian Aerograph, Walnut Creek, Calif., U.S.A.) containing a Model 600-D electrometer and an electron capture detector (250 mC titanium tritide). Experimental conditions are given in the footnotes to Table I.

## RESULTS AND DISCUSSION

The analytical results obtained in this study are discussed in two parts, *viz.*: (A) chromatographic behavior and (B) delineation of electron capture sensitivity. Relationships between molecular structure of solutes and their chromatographic behavior have been reported for paper[6,7], alumina impregnated paper[8], thin-layer[9–12] and alumina column[13] chromatography; and in detailed studies by KOVATS[14,15] and other investigators[16–18] for gas chromatography.

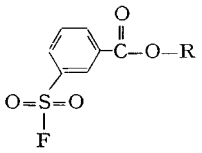
### *A. Chromatographic behavior*

In view of the thermal lability of *m*-fluorosulfonylphenyl carbamates reported earlier[1], it was of interest to observe that the structurally-related aryl *m*-fluorosulfonylbenzoates did not degrade during gas chromatographic assay. This would appear consistent with the greater bonding energy associated with a C–O bond (*ca.* 81 kcal/mole) in contrast to that of a C–N bond (*ca.* 62 kcal/mole)[19].

The analytical results are presented in Table I. The elution data may be utilized to obtain linear relative contributions of moieties in the manner reported previously for carbamate chromatographic interpretations[20]. This was achieved in the present study by determining logarithmic differences of the relative elution values of the isomeric chlorophenyl derivatives with phenyl *m*-fluorosulfonylbenzoate (standard; relative elution of 1.00). The linear contributions of the isomeric aryl chloro substituents thus obtained, are shown in Table II. The relative importance of the various substitutions to the chromatographic data is indicated in the last column of the table.

TABLE I

GAS CHROMATOGRAPHY OF *m*-FLUOROSULFONYLBENZOATES



| *Compound No.* | *R* | *Mol. wt.* | *M.p.* | *Relative elution*[a] | *N/ft.*[b] | *Relative sensitivity*[a] |
|---|---|---|---|---|---|---|
| 6 | $\phi$ | 280 | 43– 44 | 1.00 | 34.7 | 1.00 |
| 10 | *o*-Cl$\phi$ | 314 | 68– 69 | 1.63 | 55.4 | 0.751 |
| 7 | *m*-Cl$\phi$ | 314 | 47– 48 | 2.14 | 53.6 | 0.964 |
| 9 | *p*-Cl$\phi$ | 314 | 78– 79 | 2.15 | 59.0 | 0.809 |
| 2 | 2,3-diCl$\phi$ | 349 | 101–102 | 3.36 | 58.0 | 1.39 |
| 5 | 2,5-diCl$\phi$ | 349 | 85– 86 | 2.86 | 52.4 | 1.22 |
| 1 | 2,6-diCl$\phi$ | 349 | 129–130 | 2.34 | 42.4 | 1.60 |
| 8 | 3,4-diCl$\phi$ | 349 | 74– 75 | 4.44 | 49.4 | 0.927 |
| 3 | 3,5-diCl$\phi$ | 349 | 110–111 | 3.59 | 42.6 | 1.36 |
| 11 | 2,4,5-triCl$\phi$ | 383 | 79– 81 | 5.06 | 50.4 | 0.505 |
| 4 | 2,4,6-triCl$\phi$ | 383 | 118–119 | 3.38 | 46.6 | 1.33 |

[a] Relative to phenyl *m*-fluorosulfonylbenzoate. Typical sensitivity: 49.4 mm$^2$ (peak area)/ng.
[b] Theoretical plates calculated by: $N = 16[t_r^0/b]^2$, where $t_r^0$ = corrected retention time of the peak maximum in mm of chart and $b$ = peak base calculated from $b = 2$ peak area (mm$^2$)/peak height (mm) following calculation of peak area *via* triangulation (height and width at height/2).

TABLE II

RELATIVE LINEAR RETENTION CONTRIBUTIONS OF ISOMERIC ARYL CHLORO SUBSTITUENTS

| *Substituents* | *Linear contribution* | *Relative contribution* |
|---|---|---|
| 2-Chloro (*ortho*) | 0.212 | 1.00 |
| 3-Chloro (*meta*) | 0.330 | 1.56 |
| 4-Chloro (*para*) | 0.332 | 1.57 |
| 2,6-Dichloro | 0.369 | 1.78 |
| 2,5-Dichloro | 0.456 | 2.15 |
| 2,3-Dichloro | 0.526 | 2.48 |
| 2,4,6-Trichloro | 0.529 | 2.50 |
| 3,5-Dichloro | 0.555 | 2.62 |
| 3,4-Dichloro | 0.647 | 3.05 |
| 2,4,5-Dichloro | 0.704 | 3.32 |

It is of interest to note that, while the linear contributions should contain an additive feature, they obviously do not (*e.g.* the 3-chloro substituent value [0.330] and the 4-chloro value [0.332] do not add to the value for the 3,4-dichloro substituent value [0.647]). Hence, one must not only consider values for the presence or absence of groups, but the group interactions which must occur in di- and tri-substituted rings and which might influence the molecular chromatographic retention.

TABLE III

RETENTION EQUATIONS FOR INTERACTION ESTIMATION

| *Compound No.* | *Equation*[a] |
|---|---|
| 10 | $a = 0.212$ |
| 7 | $b = 0.330$ |
| 9 | $c = 0.332$ |
| 1 | $(2a)e = 0.369$ |
| 5 | $(a + b)f = 0.456$ |
| 2 | $(a + b)d = 0.526$ |
| 4 | $(2a + c)h = 0.529$ |
| 3 | $(2b)e = 0.555$ |
| 8 | $(b + c)d = 0.647$ |
| 11 | $(a + b + c)g = 0.704$ |

[a] Presence of chlorine atoms: $a$ = positions 2 or 6; $b$ = positions 3 or 5; $c$ = position 4. Interactions: (1) Di-substituted compounds: $d$ = attached to adjacent ring carbons; $e$ = 1 ring carbon between points of attachment; $f$ = 2 ring carbons between points of attachment. (2) Tri-substituted compounds: $g$ = 0 and 1 ring carbons between points of attachment; $h$ = 1 and 1 ring carbons between points of attachment.

Utilizing the linear contribution values of Table II, retention equations were written in an attempt to discern an estimation of the contribution of group interaction towards chromatography for the di- and trichloro substituted derivatives. These may be seen in Table III. The designations of *a–c* represent the physical presence of the chloro substituents; while those of *d–h* indicate the various interactions between chlorine atoms on the same ring, and are singularly identified in the footnote to Table III. The interaction product values obtained by solution of the equations are as follows:

$d = 0.971$ (Compound 2)
$d = 0.978$ (Compound 8)
$e = 0.871$ (Compound 1)
$e = 0.841$ (Compound 3)
$f = 0.842$ (Compound 5)
$g = 0.806$ (Compound 11)
$h = 0.699$ (Compound 4)

The agreement for the *d* and *e* replicates is quite remarkable when one considers the crudeness of this interpretative approach. Further, a rather interesting and reasonable generalization may be made concerning the spatial spread between ring chlorine atoms for both di- and trichloro derivatives: the interaction towards retention effects increases as the chlorine atom substitution sites get further apart. It should be readily apparent from the approach utilized, that an interaction product value of 1.000 would imply that no interaction was present. The graphic trend of the interaction product values may be seen in Fig. 1.

*B. Delineation of electron capture sensitivity*

An approach towards a delineation of relative quantitative contributions of molecular moieties to the electron capturing capacity of the molecule has been reported recently in an interpretation of the electron capture analysis of pesticides[21]. This technique has been employed in the present study for the aryl *m*-fluorosulfonylbenzoate derivatives.

A total of 6 moieties and 3 interaction designations were coded *A* through *H* and are given in Table IV. Linear equations for the coded moieties and interactions

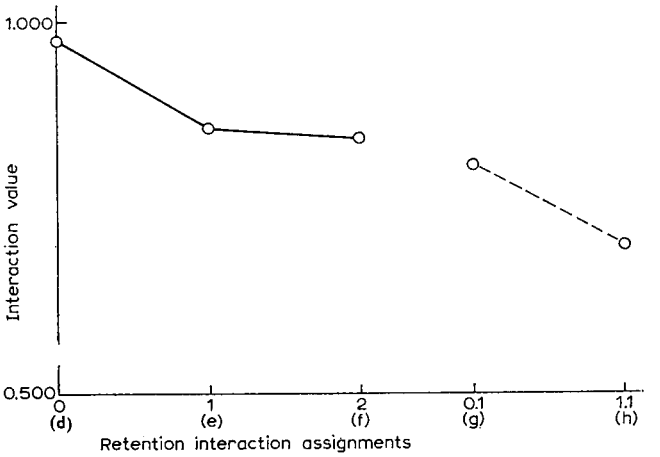
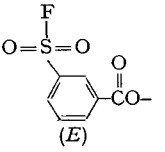



Fig. 1. Interaction factors in di- and tri-substituted phenyl *m*-fluorosulfonylbenzoates.

TABLE IV

CODE DESIGNATIONS FOR MOLECULAR MOIETIES AND INTERACTIONS

| *Compound No.* | *Aryl chlorine* | | | *Phenyl ring* (*D*) |  (*E*) | *Interactions*[a] | | | *Relative sensitivity* |
|---|---|---|---|---|---|---|---|---|---|
| | *o* (*A*) | *m* (*B*) | *p* (*C*) | | | (*F*) | (*G*) | (*H*) | |
| 1 | 2 | 0 | 0 | 1 | 1 | 0 | 0 | 1 | 1.60 |
| 2 | 1 | 1 | 0 | 1 | 1 | 0 | 0 | 0 | 1.39 |
| 3 | 0 | 2 | 0 | 1 | 1 | 1 | 0 | 0 | 1.36 |
| 4 | 2 | 0 | 1 | 1 | 1 | 2 | 0 | 1 | 1.33 |
| 5 | 1 | 1 | 0 | 1 | 1 | 0 | 1 | 0 | 1.22 |
| 6 | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 1.00 |
| 7 | 0 | 1 | 0 | 1 | 1 | 0 | 0 | 0 | 0.964 |
| 8 | 0 | 1 | 1 | 1 | 1 | 0 | 0 | 0 | 0.927 |
| 9 | 0 | 0 | 1 | 1 | 1 | 0 | 0 | 0 | 0.809 |
| 10 | 1 | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 0.751 |
| 11 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 0.505 |

[a] Interactions are designated according to the number of ring carbon atoms located between the points of ring attachment for the chlorine atoms, as determined from position 2 to position 6, as: one ring carbon atom between two attached chlorine atoms (*F*); two ring carbons (*G*); three ring carbons (*H*).

are given in Table V. Regression weights (coded moieties and interactions) were calculated which minimized the difference between the observed and predicted relative sensitivities (as in the earlier study[21]) by means of a multiple linear regression program using a Control Data Corporation Model 3600 computer. It should be pointed out that the use of such a program as a model for interpretative purposes involves the degree of validity in the assumptions that (1) the overall electron capturing capacity of the molecule is a linear function of electron capturing groups in the molecule; and (2) that the contribution of a given type of moiety is roughly the same in one compound as it is in other compounds containing it. The computed regression weights may be inspected in Table VI. The implications are inconsistent with those obtained in the earlier study in which it was found that the *para* substitution site for an aryl chloro substituent afforded the greatest sensitivity. In addition, in the light of the acknowledged potential of aryl chlorine atoms for electron capture analysis, it was surprising to obtain less sensitivity for several chlorophenyl derivatives than for the phenyl derivative itself (Table I). It might be suggested that the presence of an electron-withdrawing group such as the fluorosulfonyl substituent, *meta* to a carbonyl grouping, could result in sufficient electronic inductive distortion away from the phenyl ring, thereby enhancing the electron capturing ability of that portion of the benzoate molecule to a degree which is not overcome by the addition of one chloro substituent.

TABLE V

LINEAR EQUATIONS OF MOIETY AND INTERACTION CONTRIBUTIONS TO MOLECULAR SENSITIVITY ON ELECTRON CAPTURE

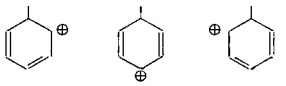
| *Compound No.* | *Equation* | |
|---|---|---|
| 1 | $2A + D + E + H$ | = 1.60 |
| 2 | $A + B + D + E$ | = 1.39 |
| 3 | $2B + D + E + F$ | = 1.36 |
| 4 | $2A + C + D + E + 2F + H$ | = 1.33 |
| 5 | $A + B + D + E + G$ | = 1.22 |
| 6 | $D + E$ | = 1.00 |
| 7 | $B + D + E$ | = 0.964 |
| 8 | $B + C + D + E$ | = 0.927 |
| 9 | $C + D + E$ | = 0.809 |
| 10 | $A + D + E$ | = 0.751 |
| 11 | $A + B + C + D + E + F + G$ | = 0.505 |

TABLE VI

MOLECULAR VALUES

| *Code* | *Moiety/interaction* | *Value* | *Standard error* |
|---|---|---|---|
| *A* | *ortho* aryl Cl | + 0.0241 | 0.219 |
| *B* | *meta* aryl Cl | + 0.263 | 0.156 |
| *C* | *para* aryl Cl | — 0.231 | 0.195 |
| *D* | phenyl ring | 0.000 | 0.000 |
| *E* | *m*-$FSO_2$-benzoxylate radical | 0.000 | 0.000 |
| *F* | 1 interstitial ring C atom | — 0.103 | 0.167 |
| *G* | 2 interstitial ring C atoms | — 0.172 | 0.266 |
| *H* | 3 interstitial ring C atoms | + 0.720 | 0.434 |

The relative sensitivity values for the *ortho*, *meta* and *para* monochloro derivatives conceivably then indicate a slight decline in molecular sensitivity by counteracting to some degree the electronic distortion of the phenyl ring, rendering it less positive (and less electron capturing) than the unsubstituted phenyl derivative. This counteraction is least for the *meta* chloro derivative, as might be explained by the hybrid unsubstituted phenyl structures:



Further interpretation of the data becomes increasingly difficult and necessarily cautious in view of the limited data on hand, excepting the expected trend of general enhancement of molecular sensitivity for the dichloro derivatives. Substitution of the values given in Table VI back into the equations (Table V) afforded a check on the validity of the procedure adopted for delineation of molecular sensitivity. The results are given in Table VII.

TABLE VII

RELATIVE SENSITIVITY DATA: PREDICTED *vs.* EXPERIMENTAL

| *Compound No.* | *Predicted* | *Experimental* |
|---|---|---|
| 1 | 1.68 | 1.60 |
| 2 | 1.20 | 1.39 |
| 3 | 1.34 | 1.36 |
| 4 | 1.25 | 1.33 |
| 5 | 1.03 | 1.22 |
| 6 | 0.92 | 1.00 |
| 7 | 1.18 | 0.96 |
| 8 | 0.95 | 0.93 |
| 9 | 0.68 | 0.81 |
| 10 | 0.94 | 0.75 |
| 11 | 0.70 | 0.51 |

Several qualifying remarks should be mentioned. The relative sensitivities of the moieties depend upon the frequency with which they appear in the compounds employed in their computation. The relative sensitivity for a moiety appearing in every compound in the calculations would be zero (as may be seen for two moieties in Table VI).

Caution should be exercised in excessive interpretation of the data because of the small number of compounds employed. Further experiments are presently in progress which will involve a large number of diverse compound classes to more fully evaluate the efficacy of this analytical approach towards a quantitative delineation of molecular sensitivity values. Fortified with properly qualified moiety values, it might be then possible to estimate the sensitivity of compounds possessing electron capturing ability *a priori* from structural considerations alone.

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SUMMARY

This study describes the chromatographic behavior and relative electron capturing ability of a number of *m*-fluorosulfonylbenzoate aryl esters. The discussion attempts to relate various portions of the ester molecules to the analytical results which were obtained. This was done by logarithmic differences for chromatographic interpretations, and by multiple linear regression analysis of the electron capture sensitivity data.

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# WATER DEACTIVATED MAGNESIA AS A CHROMATOGRAPHIC ADSORBENT

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## INTRODUCTION

Magnesia (magnesium oxide or hydroxide) has been used as a chromatographic adsorbent principally in the separation of the carotenoids (see review of RAGAZZI *et al.*[1]), with only occasional application to other sample types (*e.g.* alkaloids[1], porphyrins-). By comparison with such adsorbents as silica, alumina or charcoal, magnesia is scarcely used at all today in adsorption chromatography. The lack of interest in magnesia as adsorbent can be traced to several factors: no obvious advantages over the more common adsorbents; reports by different workers that different batches of magnesia are highly variable; the availability of most commercial chromatographic magnesias as fine powders rather than in larger particle sizes which are more popular for column chromatography; the early failure[3] of magnesia in thin-layer chromatography (TLC) applications (but note later success with magnesia in TLC[1]). Despite these apparent shortcomings of magnesia, a number of isolated observations in the literature emphasize its uniqueness with respect to other polar adsorbents. Most polar adsorbents strongly adsorb compounds containing polar substituents such as hydroxyl or keto, and it is possible to separate different compound groups according to the type and number of polar substituents. The presence of one or two vinyl groups in such compounds frequently has only a slight effect on their relative adsorption. STRAIN[4] has noted, however, that the relative adsorption of the carotenes on magnesia is determined largely by the number of olefinic double bonds, with polar groups playing a less important role. Similarly NICHOLAS[2] has shown that the relative adsorption of the porphyrins on most polar adsorbents increases with increasing number of ester substituents on the porphyrin molecule, while for adsorption on magnesia a reverse separation sequence is observed. SCHWARTZ AND PARKS[5] were able to separate homologous carbonyl compounds *as a class* from corresponding monounsaturated compounds on magnesia, a separation which does not appear possible on other polar adsorbents and which again demonstrates the preferential adsorption of unsaturated compounds on magnesia. Finally SABACKY *et al.*[6] have reported that ethyl ether and acetone are weaker solvents (*i.e.* give larger sample adsorption) for adsorption on magnesia than is benzene, while on other polar adsorbents ether and acetone are always stronger solvents than is benzene. Again this can be interpreted as preferential adsorption of an unsaturated compound (*i.e.* benzene) on magnesia since solvent strength has been related to solvent adsorption energy[7].

The purpose of the present study was to evaluate the chromatographic proper-

ties of magnesia in terms of a previous theoretical model for other polar adsorbents (*e.g.* refs. 8, 9 and preceding papers in that series), and to compare the types of separations which can be obtained on magnesia relative to other adsorbents.

## EXPERIMENTAL

The major part of the present investigation was based on an adsorbent composed of two parts by weight of Sea Sorb 43 "Adsorptive Magnesia" (Fisher Scientific) plus one part of Celite 545 (Johns Manville). The dry adsorbent mixture was first blended with water, dried on an air filter, and heated overnight in air at 130°. Limited studies were also carried out on a granular magnesia (West Vaco) which was ground to 100–200 mesh and heated as above. Unless otherwise specified, all of the data reported in following sections refer to the Sea Sorb Magnesia–Celite mixture rather than to the West Vaco magnesia. Dry adsorbent was further activated by heating in air for 16 h at various temperatures. Water deactivated adsorbent samples were prepared from activated adsorbent by adding liquid water and equilibrated in closed containers for 24 h or longer. The experimental data reported in following sections refer generally to adsorbent with a final activation at 300°, with the indicated amount of added water (as % $H_2O$–MgO); this material (Sea Sorb Magnesia–Celite) had a BET surface area of 90 $m^2/g$ prior to deactivation by water.

The measurement of equivalent retention volumes $\underline{R}^o$ (ml/g) for various sample compounds was carried out as previously[10]. Sample was charged to prewet columns and used columns were normally discarded after one separation. All measurements refer to separation in the linear isotherm region (linear elution adsorption chromatography, LEAC). The linear capacity[11] of 3 % $H_2O$–MgO was approximately $2 \cdot 10^{-5}$ g/g.

## IRREVERSIBLE SAMPLE ADSORPTION ON MAGNESIA

It was observed at the beginning of the present study that irreversible adsorption of aromatic samples on magnesia can occur under certain conditions. This effect, which has been referred to as "chemisorption" previously[12], can severely limit the usefulness of a separation technique. Typically it was found that elution of a sample such as anthracene or phenanthrene from fresh columns of 3 % $H_2O$–MgO by (dry) pentane gave quantitative sample recoveries, but low recoveries resulted if the process was repeated a second time on the same column. Recovery during the second elution from a given column varied with sample size; *e.g.* for anthracene a sample size of $8 \cdot 10^{-7}$ g/g gave 4 % recovery, rising to 33 % recovery for $8 \cdot 10^{-6}$ g/g sample. Water wet pentane increased sample recovery as shown in Table I. Only with water saturated pentane was quantitative sample recovery achieved, however. These observations suggest that elution with dry pentane removes water from the magnesia surface, and that the resulting dry surface can irreversibly hold the sample by strong adsorption or chemical reaction. A similar effect has been noted by BRIERLY AND SMITH[13] in the separation of rotenone on alumina, and several other workers have noted the drying of water deactivated adsorbents by column washing. The unusual aspect of the present phenomenon involving magnesia is the ease with which it occurs. Water is normally removed from alumina and silica only by fairly polar solvents[7,9,14], although extensive washing of heavily deactivated alumina by pentane

TABLE I

SAMPLE RECOVERY IN THE ELUTION OF ANTHRACENE FROM 3 % $H_2O$–MgO BY PENTANE CONTAINING VARYING AMOUNTS OF WATER[a]

| *Pentane water content*[b] (% *saturation*) | *Sample recovery* (%) | |
|---|---|---|
| | *First elution* | *Second elution*[c] |
| 0 | 100 | 4 |
| 30 | 100 | 11 |
| 60 | 100 | 35 |
| 100 | 100 | 100 |

[a] Sample size $8 \cdot 10^{-7}$ g/g.

[b] Solvent prepared by mixing indicated proportion of water saturated pentane with dry pentane.

[c] Repeated separation after initial elution, fresh sample charged.

results in a gradual dehydration of the adsorbent[10]. Possibly this reflects a lower affinity of the magnesia surface for water and a higher affinity for aromatic molecules, relative to other polar adsorbents. Such an explanation agrees with the various observations cited in the Introduction.

Samples with large retention volumes ($\underline{R}° > 20$) gave incomplete sample recoveries even for initial elution from a fresh column. For example, the pentane elution from 3 % $H_2O$–MgO of anthracene ($\underline{R}° = 6.7$), pyrene ($\underline{R}° = 29.6$) and chrysene ($\underline{R}° = 174$) gave recoveries, respectively, of 100, 80, and 3 %. Further elution of the column to which chrysene was charged, using benzene solvent, gave only 35 % additional sample recovery, despite the fact that benzene is a much stronger solvent. This behavior makes it impractical to use dry solvents in the study or practical application of magnesia in adsorption chromatography. However, use of water wet solvents eliminated this problem. Consequently all of the following studies were carried out using 100 % water saturated solvents unless otherwise stated. Where $\underline{R}°$ values were determined using both dry and water wet solvents, it was found that $\underline{R}°$ values for a given compound and solvent did not vary by more than 10–15 % (*i.e.* elution by dry and wet solvents from fresh columns is quite similar). Whereas water wet solvents on other adsorbents[7,9] generally give lower $\underline{R}°$ values than do dry solvents, both higher *and* lower $\underline{R}°$ values were observed on magnesia with wet solvents. All of the present studies were carried out with water immiscible solvents. It is not known how much (if any) water would have to be added to such solvents as acetone or the lower alcohols to avoid the problem of irreversible sample adsorption on magnesia. The West Vaco magnesia appeared to give less of a problem with irreversible sample asorption than did the Sea Sorb magnesia.

At first glance it appears odd that the problem of irreversible adsorption on magnesia has escaped previous notice in the literature. However most previous separations on magnesia have involved either column or plate development, where extensive washing of the adsorbent *in front of the sample* does not occur. In unreported studies LIJINSKY[15] has noted that the pentacyclic aromatic hydrocarbons are held on magnesia so tightly, following normal elution of the lower aromatics, that they can be recovered only by chemical destruction of the adsorbent. In conclusion

it seems apparent that in elution from columns of magnesia it is generally wise to use water saturated solvents.

## SAMPLE RETENTION VOLUME AS A FUNCTION OF SEPARATION CONDITIONS

### *Adsorbent activation temperature*

All polar adsorbents undergo similar surface changes upon being heated (*i.e.* activated) at moderate temperatures. Activation at temperatures below 150° removes varying amounts of physically adsorbed water from the adsorbent surface, and adsorbent activity increases with increasing activation temperature. At temperatures above 150° more drastic changes in adsorbent surface structure begin to occur: surface hydroxyls react to form oxide groups and liberate water, and other forms of chemically bound water are similarly lost. This process can lead either to an increase or decrease in adsorbent activity, depending upon the importance of surface hydroxyls as adsorption sites. In the case of silica, surface hydroxyls are strong adsorption sites, and above 150° adsorbent activity decreases with higher activation temperature[16]. Alumina, on the other hand, shows a continuing increase in adsorbent activity as activation temperature is increased from room temperature to 1000°[17]. The effect of activation temperature on the activity of magnesia was briefly studied, using the pentane elution of styrene from samples of activated adsorbent. Under these conditions the adsorption isotherm was markedly nonlinear for at least some of the adsorbent samples, and the experimental results are only qualitatively significant. Adsorbent activity was observed to decrease continuously with higher activation temperature over the interval 130–500°. Equivalent retention volumes (ml/g) for styrene ($8 \cdot 10^{-6}$ g/g) eluted from adsorbent activated at the following temperatures were: 130° (12.5), 200° (10.4), 300° (8.4) and 500° (6.6). KISELEV *et al.*[18] have noted a similar decline in the activity of magnesia with higher temperature of activation. Interestingly the elution band obtained with the 500° band was quite symmetrical and sharp while the corresponding bands for the lower temperature adsorbents were quite broad and tailing (compare Fig. 1). Quite limited data suggest that 500° water deactivated magnesias preserve these differences to some degree and are less subject to irreversible sample adsorption. Activation of magnesia at 500° may therefore give a chromatographic adsorbent of generally better performance than is the case for acti-

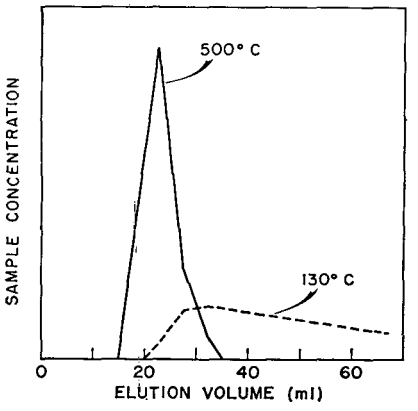


Fig. 1. Effect of magnesia activation temperature on band shape; elution of styrene ($8 \cdot 10^{-6}$ g/g) by pentane.

vation at lower temperatures. The decrease in activity of magnesia with increasing activation temperature suggests (but does not prove) that surface hydroxyls are important adsorption sites.

*Adsorbent activity versus water deactivation*

Addition of water to an activated adsorbent generally results in adsorption onto the strongest surface sites, resulting in a loss in the surface available for adsorption of sample molecules and a decrease in average surface activity. The results of water deactivation may be interpreted in terms of changes in two adsorbent parameters: the adsorbent surface volume $V_a$ and the surface activity function $\alpha$. A previous relationship (*e.g.* refs. 7, 8) gives sample $\underline{R}^\circ$ values as a function of these two adsorbent parameters:

$$\log \underline{R}^\circ = \log V_a + \alpha \, f(S,E) \qquad (1)$$

For a particular adsorbent type (*e.g.* silica, magnesia, etc.), the function $f(S,E)$ depends only upon the sample and eluent corresponding to $\underline{R}^\circ$. Eqn. 1 therefore describes the variation of $\underline{R}^\circ$ with adsorbent activity. Eqn. 1 has been corroborated for previous adsorbents, and the parameters $V_a$ and $\alpha$ have been determined as a function of adsorbent water content, by determining $\underline{R}^\circ$ values for various sample–eluent combinations on several adsorbent samples of differing water content (*e.g.* ref. 10). This procedure is impractical in the case of magnesia, primarily because of the problem of irreversible sample adsorption on adsorbents of reduced water content. A further complication is a quite rapid increase in $\underline{R}^\circ$ values with decreasing magnesia water content. This precludes the possibility of comparing $\underline{R}^\circ$ values for the same sample and solvent over the full range of adsorbent activity. These problems were circumvented to some extent by noting that $f(S,E)$ is a linear function of carbon number for the unsubstituted aromatic hydrocarbons, as discussed in a later section. Assuming that the adsorbent parameters $V_a$ and $\alpha$ are smooth functions of adsorbent activity, the data of Table II could then be used to derive $V_a$ and $\alpha$ *versus* adsorbent water content in a straightforward manner. The resulting adsorbent parameters are tabulated in Table III and plotted *versus* % $H_2O$–MgO in Fig. 2. The parameter

TABLE II

VARIATION OF RETENTION VOLUME WITH MAGNESIA WATER CONTENT[a]

| *Sample* | *Log* $\underline{R}^\circ$[b] | | | | |
|---|---|---|---|---|---|
| | *0.0 % $H_2O$–MgO*[c] | *0.5 % $H_2O$–MgO*[c] | *1.5 % $H_2O$–MgO*[c] | *3.0 % $H_2O$–MgO* | *6.7 % $H_2O$–MgO* |
| Styrene | 1.03 | 0.24 | | | |
| Naphthalene | | 1.02 | 0.24 | | |
| Acenaphthylene | | | 1.04 | 0.41 | |
| Phenanthrene | | | | 1.11 | 0.49 |
| Fluoranthene | | | | | 1.03 |
| Chrysene | | | | | 1.83 |

[a] Adsorbent activated at 300° before addition of water.
[b] Pentane elution.
[c] Dry pentane used.

TABLE III

ADSORBENT PROPERTIES OF WATER DEACTIVATED MAGNESIA[a]

| % $H_2O$–MgO | Log $V_a$ | $\alpha$ | $R_s$[b] | | |
|---|---|---|---|---|---|
| | | | *Styrene* | *Acenaphthylene* | *Fluoranthene* |
| 0.0 | —2.2 | 1.00 | 1.03 | | |
| 0.5 | —2.97 | 1.00 | 0.24 | | |
| 1.0 | —3.44 | 1.00 | | 1.33 | |
| 1.5 | —3.76 | 0.99 | | 1.04 | |
| 2.0 | —3.95 | 0.98 | | 0.73 | |
| 3.0 | —4.18 | 0.95 | | 0.41 | 1.91 |
| 4.0 | —4.32 | 0.93 | | 0.14 | 1.63 |
| 5.0 | —4.39 | 0.91 | | | 1.41 |
| 6.0 | —4.44 | 0.88 | | | 1.19 |
| 7.0 | —4.49 | 0.86 | | | 1.00 |

[a] 2:1 MgO–Celite activated at 300°.
[b] $R^\circ$ value for indicated sample; pentane elution.

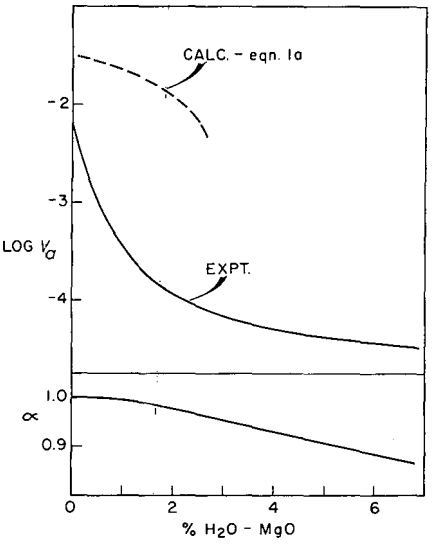


Fig. 2. Adsorbent parameters for water deactivated magnesia.

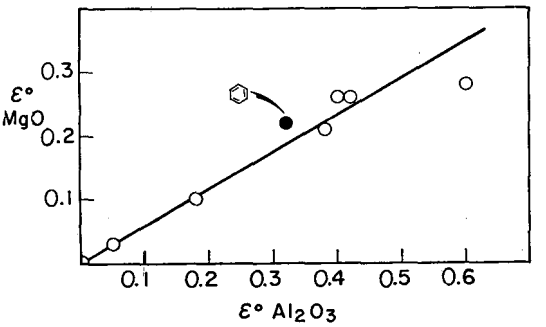


Fig. 3. Eluent strengths of various solvents on magnesia *versus* alumina.

$V_a$ can also be calculated from the BET surface area of the activated adsorbent (90 $m^2/g$) and the added water (% $H_2O$) in terms of a previous relationship[19]:

$$V_a \text{ (ml/g)} = 0.00035 \text{ surface area} - (\%\ H_2O). \tag{1a}$$

Comparison of calculated values of $V_a$ from eqn. 1a (dashed curve of Fig. 2) with experimental values (solid curve of Fig. 2) shows a large discrepancy between these two plots. The experimental $V_a$ values are considerably smaller, and this may be the result of restricted access to part of the adsorbent surface; *i.e.* nitrogen molecules may be able to adsorb in small pores which cannot accommodate the larger sample molecules of Table II. A microporous adsorbant structure might also account for the chemisorption of aromatics on magnesia. Alternatively the linear relationship between sample molecule carbon number and log $\underline{R}^\circ$ may break down for smaller sample molecules (compare adsorption energy of aromatic hydrocarbons on silica *versus* alumina[19]). Whatever the reason for this anomaly involving $V_a$ values for magnesia, the practical consequences are unimportant and the adsorbent parameters of Table III can be used with eqn. 1 to predict the effect of adsorbent activity on $\underline{R}^\circ$ values. Because of the problem of irreversible sample adsorption, 3–7 % $H_2O$–MgO appears to represent an optimum adsorbent activity range. Adsorbent linear capacity is also expected to be greater in this range, relative to more active adsorbent samples[11]. Table III presents $\underline{R}^\circ$ values for standard sample–solvent systems ($R_s$ values) which can be used to measure the chromatographic activity (as % $H_2O$–MgO) of a magnesia sample[19].

*Role of the solvent*

For elution from other polar adsorbents (*e.g.* ref. 19) the effect of the solvent on sample retention volumes can be expressed in terms of eqn. 2, which is derivable from eqn. 1:

$$\log \underline{R}^\circ = \log V_a + \alpha (S^\circ - \varepsilon^\circ A_s). \tag{2}$$

Here $S^\circ$ is the adsorption energy of the sample in a standard chromatographic system (activated adsorbent, pentane solvent), $\varepsilon^\circ$ is the eluent strength of the solvent, and $A_s$ is the adsorbed volume of the sample molecule (proportional to the area a sample molecule requires upon the adsorbent surface). In most cases values of $A_s$ can be calculated[19] for a given sample molecule. Eqn. 2 can be simplified for purposes of examining the solvent effect *per se*, by defining the retention volume $R_p$ of a sample molecule eluted by pentane:

$$\log \underline{R}^\circ = \log R_p - \alpha\varepsilon^\circ A_s. \tag{2a}$$

For elution from 6.3 % $H_2O$–MgO, values of $\alpha\varepsilon^\circ$ for several solvents and solvent binaries were obtained from $\underline{R}^\circ$ values of the samples shown in Table IV (using eqn. 2a). These $\alpha\varepsilon^\circ$ values are also shown in Table IV, along with values of $A_s$ and $R_p$ for each sample. $R_p$ values were either measured directly (pentane solvent) or else calculated from an $\underline{R}^\circ$ value for another solvent using eqn. 2a. The resulting $\alpha\varepsilon^\circ$ values for the various solvent binaries could also be calculated from a previous relationship[19] between the $\alpha\varepsilon^\circ$ values of a solvent mixture ($\alpha\varepsilon^\circ_{AB}$) and the corresponding values for the pure solvents composing that mixture ($\alpha\varepsilon^\circ_A$ and $\alpha\varepsilon^\circ_B$):

$$\alpha\varepsilon^\circ_{AB} = \alpha\varepsilon^\circ_A + \frac{\log X_B\, 10^{\alpha n_b (\varepsilon^\circ_B - \varepsilon^\circ_A)} + 1 - X_B}{n_b} \tag{3}$$

TABLE IV

ELUTION STRENGTH OF VARIOUS SOLVENTS AND SOLVENT BINARIES: ELUTION FROM 6.3 % $H_2O$–MgO[a]

| *Solvent* | *Sample* | $\alpha\varepsilon^\circ$ | |
|---|---|---|---|
| | | *Exptl.* | *Calc.*[b] |
| $CCl_4$ | Chrysene[c] | 0.091 | |
| Ethyl ether | 1,2,3,4-Dibenzanthracene[d] | 0.184 | |
| Benzene | 1,2,3,4-Dibenzanthracene[d] | 0.189 | |
| Chloroform | 2-Aminochrysene[e] | 0.224 | |
| Cyclopentane | Fluoranthene[f] | 0.026 | |
| 10 % v Ether–pentane | Anthracene[g] | 0.052 | 0.047 |
| 25 % v Ether–pentane | Pyrene[h] | 0.092 | 0.091 |
| 5 % v Benzene–pentane | Pyrene[h] | 0.037 | 0.041 |
| 20 % v Benzene–pentane | Benzanthracene[i] | 0.089 | 0.100 |
| 50 % v Benzene–pentane | Benzanthracene[i] | 0.156 | 0.149 |
| 5 % v $CH_2Cl_2$–pentane | Chrysene[c] | 0.043 | 0.049[k] |
| 15 % v $CH_2Cl_2$–pentane | Chrysene[c] | 0.098 | 0.106[k] |
| 35 % v $CH_2Cl_2$–pentane | 1,2-Benzpyrene[j] | 0.163 | 0.163[k] |
| 70 % v $CH_2Cl_2$–pentane | 2-Aminochrysene[e] | 0.220 | 0.209[k] |
| 10 % v Methyl acetate–pentane | Chrysene[c] | 0.093 | 0.097[l] |
| 25 % v Methyl acetate–pentane | 1,2-Benzpyrene[j] | 0.153 | 0.154[l] |
| 40 % v Methyl acetate–pentane | 1,2,3,4-Dibenzanthracene[d] | 0.187 | 0.183[l] |

[a] Water saturated solvents.
[b] Eqn. 3.
[c] Log $R_p$ 1,80, $A_s$ 12
[d] Log $R_p$ 3.12, $A_s$ 14
[e] Log $R_p$ 4.1 , $A_s$ 13.5
[f] Log $R_p$ 1.13, $A_s$ 11
[g] Log $R_p$ 0.39, $A_s$ 10
[h] Log $R_p$ 1.05, $A_s$ 11
[i] Log $R_p$ 1.77, $A_s$ 12
[j] Log $R_p$ 2.50, $A_s$ 13
[k] Assumes $\alpha\varepsilon^\circ$ for $CH_2Cl_2$ equals 0.230.
[l] Assumes $\alpha\varepsilon^\circ$ for methyl acetate equals 0.244.

TABLE V

THE ELUENT STRENGTH OF VARIOUS SOLVENTS ON MAGNESIA: COMPARISONS WITH VALUES FOR ALUMINA

| *Solvent* | $\varepsilon^\circ$ | | |
|---|---|---|---|
| | *MgO (exptl)* | $Al_2O_3$[a] | *MgO (calc.)*[b] |
| Pentane | 0.00 | 0.00 | 0.00 |
| Cyclopentane | 0.03 | 0.05 | 0.03 |
| $CCl_4$ | 0.10 | 0.18 | 0.10 |
| Ethyl ether | 0.21 | 0.38 | 0.22 |
| Benzene | 0.22 | 0.32 | 0.19 |
| $CHCl_3$ | 0.26 | 0.40 | 0.23 |
| $CH_2Cl_2$ | 0.26 | 0.42 | 0.24 |
| Methyl acetate | 0.28 | 0.60 | 0.35 |

[a] Values of Table II, ref. 7.
[b] Calculated for elution from MgO, as 0.58 times value for $Al_2O_3$.

Here $X_B$ refers to the mole fraction of the stronger solvent component B in the binary solvent, and $n_b$ refers to the area or $A_s$ value of component B. The agreement between experimental $\alpha\varepsilon^\circ$ values for the binary solvents of Table IV and values calculated from eqn. 3 is quite satisfactory: $\pm 0.007$ units standard deviation. Values of $\alpha\varepsilon^\circ$ for the solvents $CH_2Cl_2$ and methyl acetate were calculated from the best fit of the corresponding binary data to eqn. 3.

In Table V the derived $\varepsilon^\circ$ values for the pure solvents of Table IV are summarized, along with values of $\varepsilon^\circ$ for elution from alumina. As seen in Fig. 3 the alumina and magnesia $\varepsilon^\circ$ values are approximately proportional. (Magnesia $\varepsilon^\circ$ values equal 0.58 times alumina values). This permits the estimation of other magnesia $\varepsilon^\circ$ values from corresponding values for alumina[7]. Table V compares $\varepsilon^\circ$ values for magnesia with values calculated in this fashion from the alumina $\varepsilon^\circ$ values. The standard deviation of the calculated and experimental $\varepsilon^\circ$ values is $\pm 0.03$ units.

The data of Table V and Fig. 3 do not suggest that benzene is an anomalously strong solvent on magnesia, relative to other adsorbents, as suggested by the data of SABACKY *et al.*[6]. It instead appears that ethyl ether and acetone may have appeared as weak solvents in the latter study because of adsorbent drying by solvent during separation. The resulting increase in adsorbent activity would of course reduce sample $\underline{R}^\circ$ values, which is equivalent to the solvent appearing weaker than it actually is. SABACKY *et al.* and other workers have questioned the general value of eluotropic series or relative solvent strength values as in Table V. In general it is claimed that such series or relative values are inapplicable to different adsorbents and/or different sample types. It has now been established that essentially the same series of relative $\varepsilon^\circ$ values and the same eluotropic series exists for alumina[7], Florisil[12], silica[9] and magnesia (Table V). While some variations in apparent eluent strength with sample type have been noted (*e.g.* refs. 7, 8), these have been related to specific interactions between sample, adsorbent and/or solvent which can be predicted in advance. Hence the concept of a single eluotropic series which is valid for all polar adsorbents and most sample types seems quite acceptable as a first approximation and should continue to find use by practical chromatographers.

The application of eqns. 2 and 2a to adsorption on silica (and Florisil to a lesser extent) is complicated by the fact that sample $A_s$ values in some case do not correspond to simple molecular size[19]. Strongly adsorbing substituents appear to contribute much more to $A_s$ than their actual size would suggest. This effect was briefly examined for adsorption onto magnesia. Table VI presents $\underline{R}^\circ$ values for a few samples which give anomalous $A_s$ values on silica, and apparent $A_s$ values (on magnesia) are derived. Comparison of these magnesia $A_s$ values in Table VI with the normal values for alumina and the anomalous values for silica shows that the magnesia $A_s$ values agree much more closely with the alumina values than with the silica values. We tentatively conclude that the anomalous $A_s$ effect does not apply to adsorption on magnesia, and that $A_s$ values for different sample molecules can be calculated in the same way as for alumina[19].

## RETENTION VOLUME AS A FUNCTION OF SAMPLE STRUCTURE

Previous papers (*e.g.* refs. 8, 19) have developed a general theory for the dependence of sample retention volume on sample structure in adsorption chromato-

TABLE VI

APPARENT SAMPLE $A_s$ VALUES FOR ADSORPTION ON MAGNESIA; 6.3% $H_2O$–MgO

| *Sample* | *Log $\underline{R}^\circ$* | | | *$A_s$* | | |
|---|---|---|---|---|---|---|
| | *Pentane* | *5% v $CH_2Cl_2$–pentane* | *15% v $CH_2Cl_2$–pentane* | *MgO*[a] | *$Al_2O_3$*[b] | *$SiO_2$*[b] |
| *m*-Nitroacetophenone | 1.82 | 1.17 | 0.53 | 13.2 | 10.5 | 22.4 |
| 1,4-Dinitronaphthalene | 0.83 | 0.41 | | 9.8 | 13.0 | 22.4 |
| 2-Aminonaphthalene | 1.74 | | 0.74 | 10.2 | 9.5 | 16.0 |
| $\varepsilon^\circ$ | 0.000 | 0.043 | 0.098 | | | |

[a] Calculated from above $\underline{R}^\circ$ values and Eqn. 2a.
[b] Calculated as in ref. 19.

graphy. The standard adsorption energy $S^\circ$ of a sample molecule expresses the major contribution of sample structure to $\underline{R}^\circ$. $S^\circ$ is related to the adsorption energies $Q^\circ_i$ of the different groups $i$ which constitute the sample molecule:

$$S^\circ = \sum^{i} Q^\circ_i - f(Q^\circ_k) \sum^{i \neq k} Q^\circ_i \qquad (4)$$

Here $S^\circ$ is given as the sum of group adsorption energies for every group $i$ in the sample molecule, minus an adsorption energy loss due to localization of certain sample types on strong adsorbent sites. The localization function $f(Q^\circ_k)$ in eqn. 4 depends upon the adsorption energy $Q^\circ_k$ of the strongest adsorbing group $k$ in the sample molecule. Relative separation order on different adsorbents is largely determined by the values of $Q^\circ_i$ for different sample groups $i$ and by the relative extent of sample localization.

Retention volume data for a variety of sample types were collected and reduced to values of $S^\circ$ by means of eqn. 2. These data are summarized in Table VII. Table VII permits a fairly detailed examination of the role of sample structure in determining $R^\circ$ values on magnesia.

*Aromatic hydrocarbons*

The $S^\circ$ values for the aromatic hydrocarbons of Table VII are plotted *versus* the number of aromatic carbon atoms in each molecule in Fig. 4. The various points fall on top of a straight line through the origin. This correlation is predicted by eqn. 4 when no sample localization occurs (*i.e.* $f[Q^\circ_k]$ equal zero). A value of $Q^\circ_i$ for an aromatic carbon atom (—C=) adsorbed on magnesia can be derived, equal 0.40. The adsorption of the aromatic hydrocarbons onto alumina, Florisil and "X" sieve is similar in this respect[20]. The aromatic hydrocarbons adsorb on silica with localization, and as a result the $S^\circ$ value for a nonfused aromatic such as dibenzyl is much larger than is $S^\circ$ for fused aromatics of similar aromatic carbon number (*e.g.* acenaphthylene). As seen in Table VII and Fig. 4, this is not the case for the adsorption of hydrocarbons onto magnesia. We therefore conclude that localization of hydrocarbons on magnesia does not occur.

TABLE VII

RELATIVE SAMPLE ADSORPTION AS A FUNCTION OF SAMPLE STRUCTURE: $S°$ VALUES FOR DIFFERENT SAMPLES (6.3% $H_2O$–MgO)

| *Sample* | *Solvent*[a] | *Log* $\underline{R}°$ | $S°$ | |
|---|---|---|---|---|
| | | | *Exptl.*[b] | *Calc.*[c] |
| Acenaphthylene | P | —0.32 | 4.8 | 4.8 |
| Dibenzyl | P | 0.34[d] | 4.1 | 4.8 |
| Anthracene | P | 0.39 | 5.6 | 5.6 |
| Phenanthrene | P | 0.47 | 5.7 | 5.6 |
| Fluoranthene | P | 1.13 | 6.4 | 6.4 |
| Pyrene | P | 1.05 | 6.3 | 6.4 |
| Chrysene | P | 1.80 | 7.2 | 7.2 |
| Triphenylene | P | 1.76 | 7.1 | 7.2 |
| Benzanthracene | P | 1.77 | 7.2 | 7.2 |
| 1,2-Benzpyrene | 15% M–P | 1.23 | 8.0 | 8.0 |
| 1,2,3,4-Dibenzanthracene | 35% M–P | 0.84 | 8.7 | 8.8 |
| Methyl benzoate | P | —0.5 | 4.5 | 5.0 |
| Acetophenone | P | 0.37 | 5.5 | 5.3 |
| Aniline | P | 0.68 | 5.9 | 5.8 |
| Phenol | 35% M–P | 1.31 | 7.9 | 7.4 |
| Phenylacetone | P | 0.74 | 6.0 | 5.7 |
| 1-(1-Thiapropyl)-naphthalene | P | 0.12 | 5.3 | 5.0 |
| 2-Methoxynaphthalene | P | 0.08 | 5.2 | 5.2 |
| 1-Nitronaphthalene | P | 0.47 | 5.7 | 5.8 |
| Methyl-2-naphthoate | P | 0.73 | 6.0 | 6.1 |
| 2-Acetonaphthone | P | 1.18 | 6.5 | 6.4 |
| 1-Naphthaldehyde | P | 0.70 | 5.9 | 6.2 |
| 2-Aminonaphthalene | see Table VI | — | 7.1 | 6.9 |
| 2-Aminoanthracene | 35% M–P | 0.97 | 8.4 | 8.0 |
| Pyridine | P | 0.63 | 5.8 | 5.7 |
| Isoquinoline | P | 1.10 | 6.4 | 6.7 |
| Quinoline | P | 1.00 | 6.3 | 6.2 |
| Phenanthridine | P | 1.85 | 7.3 | 7.3 |
| 1-Azapyrene | 35% M–P | 0.49 | 7.7 | 7.9 |
| 7,8-Benzoquinoline | 5% M–P | 0.71 | 6.4 | 6.5 |
| Acridine | 15% M–P | 0.60 | 6.9 | 7.2 |
| *m*-Nitroacetophenone | see Table VI | — | 7.0 | 6.8 |
| *p*-Nitroaniline | 35% M–P | 1.05 | 8.2 | 7.3 |
| *m*-Nitromethylbenzoate | 5% M–P | 0.85 | 6.7 | 6.5 |
| *m*-Nitroanisole | P | 0.52 | 5.7 | 5.6 |
| 2,5-Dimethoxyacetophenone | 15% M–P | 0.35 | 6.7 | 7.2 |
| Dimethylisophthalate | 15% M–P | 0.34 | 6.9 | 6.9 |
| *p*-Dinitrobenzene | 5% M–P | 0.63 | 6.4 | 6.2 |
| 2,4-Dinitroanisole | 15% M–P | 0.98 | 7.6 | 7.3 |
| *m*-Nitrobenzaldehyde | 5% M–P | 0.96 | 6.7 | 6.6 |
| *p*-Methoxyacetophenone | 5% M–P | 0.84 | 6.5 | 6.3 |
| 1,4-Dinitronaphthalene | see Table I | — | 6.1 | 7.2 |

[a] P = pentane; 5% M–P = 5% v methylene chloride–pentane; 15% M–P = 15% v methylene chloride–pentane; 35% M–P = 35% v methylene chloride–pentane.
[b] From $\underline{R}°$ values and eqn. 4.
[c] From eqn. 4 and alumina data, as described in the text.
[d] Value for 1.5% $H_2O$–MgO.

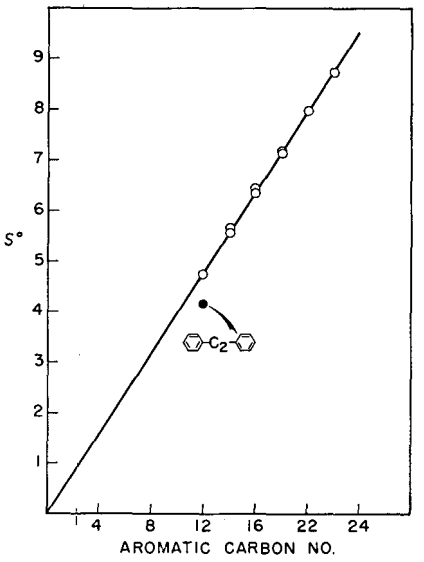


Fig. 4. Dependence of hydrocarbon $S°$ values on aromatic carbon number; data of Table VII.

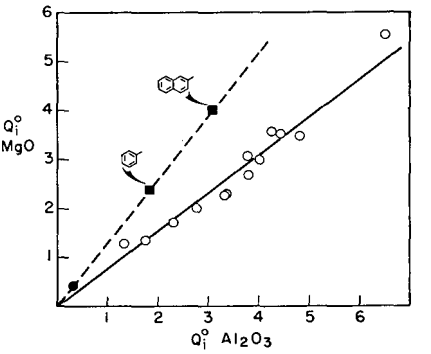


Fig. 5. Group adsorption energies on magnesia *versus* alumina; data of Table VIII.

TABLE VIII

SAMPLE GROUP ADSORPTION ENERGIES $Q°_i$ ON MAGNESIA *versus* ALUMINA

| *Group* | $Q°_i$ | | | $f(Q°_k)$ | |
|---|---|---|---|---|---|
| | *MgO (exptl.)*[a] | $Al_2O_3$[b] | *MgO (calc.)*[c] | $Al_2O_3$[d] | *MgO*[e] |
| —C= | 0.40 | 0.31 | 0.24 | 0.00 | 0.00 |
| Ar)—SR | 1.26 | 1.32 | 1.02 | 0.00 | 0.00 |
| Ar)—OR | 1.34 | 1.77 | 1.36 | 0.10 | 0.08 |
| Ar)—$NO_2$ | 2.00 | 2.75 | 2.12 | 0.31 | 0.21 |
| AR)—$CO_2R$ | 2.26 | 3.32 | 2.56 | 0.39 | 0.26 |
| Ar)—CHO | 2.29 | 3.35 | 2.58 | 0.35 | 0.23 |
| Ar)—COR | 3.03 | 3.74 | 2.88 | 0.44 | 0.29 |
| Ar)—$NH_2$ | 3.50 | 4.41 | 3.40 | 0.45 | 0.30 |
| Ar)—OH | 5.54 | 6.5 | 5.01 | 0.45 | 0.30 |
| —N=[f] | 3.50 | 4.8 | 3.70 | 0.45 | 0.30 |
| —N=[g] | 2.99 | 4.0 | 3.08 | 0.45 | 0.30 |
| —N=[h] | 2.68 | 3.8 | 2.93 | 0.35 | 0.30 |
| —N=[i] | 1.71 | 2.3 | 1.77 | 0.22 | 0.15 |
| Ar)—$CH_2COR$ | 3.57 | 4.28 | 3.30 | | |

[a] Derived from $\underline{R}°$ values of Table VII for monosubstituted aromatics.
[b] Ref. 8
[c] 0.77 times value for alumina.
[d] Ref. 21.
[e] 0.67 times alumina value.
[f] As in pyridine.
[g] As in quinoline.
[h] As in acridine.
[i] As in 7,8-benzoquinoline.

*Substituted aromatics*

$Q°_i$ values for different groups $i$ can be derived most readily from $\underline{R}°$ data for the monosubstituted benzenes ($C_6H_5$-$i$) or paraffins (R-$i$). Extensive data for compounds of this type could not be obtained in the present study because of experimental limitations. Alternatively group $Q°_i$ values can be derived from compounds such as the monosubstituted naphthalenes or other more complex sample molecules if we know the value of the localization function $f(Q°_k)$. Examination of the data of Table VII suggests that the localization function for adsorption on magnesia is given approximately as 0.67 times the corresponding value for adsorption on alumina. This fits the same pattern already noted for adsorption on silica and Florisil[19], where the respective localization functions are 0.4 and 0.65 times the alumina values. Using this approximation to $f(Q°_k)$ on magnesia, the $Q°_i$ values of Table VIII were derived from the $S°$ values of Table VII for the monosubstituted aromatics (*e.g.* acetophenone, 1-nitronaphthalene, acridine). The previous convention[19] with respect to not counting the delocalization of the benzene ring attached to the localizing group was followed in this procedure. Comparison of these magnesia $Q°_i$ values with corresponding values for alumina (Table VIII and circles in Fig. 5) shows a close correspondence. The magnesia values can in fact be calculated from values for alumina within a standard deviation of $\pm 0.25$ units, using the relationship: $Q°_i$ (MgO) equals 0.77 $Q°_i$ ($Al_2O_3$). With the exception of acidic and basic sample molecules, this similarity of group adsorption energies on different polar adsorbents now appears to be quite typical. Similar quantitative correlations between $Q°_i$ values on different adsorbents have already been noted for alumina, silica and Florisil[22] and BROCKMANN[23] has reported the same qualitative sequence of group adsorption energies on alumina, silica, magnesia, calcium sulfate and copper sulfate. The $Q°_i$ value for the one acidic group of Table VIII, aromatic hydroxyl, is relatively larger on magnesia than on alumina, compared to neutral sample groups. This confirms that magnesia, like alumina[22], is a basic adsorbent which preferentially adsorbs acidic samples.

The correlation of Fig. 5 does not suggest a large preference for the adsorption of a single aromatic carbon on magnesia relative to alumina. However the dashed curve in Fig. 5 for aromatic nucleic of varying size emphasizes the preferential adsorption of aromatic rings on magnesia relative to alumina (and other adsorbents), confirming the observations collected in the Introduction.

The overall applicability of eqn. 4 for adsorption on magnesia, and the similarity of $Q°_i$ values on magnesia *versus* alumina, was further checked as follows. All $Q°_i$ values were assumed equal to 0.77 times the alumina values, except for aromatic carbon ($Q°_i$ equal 0.40); $f(Q°_k)$ was assumed equal to 0.67 times the value for the same substituent on alumina (Table VIII); $S°$ values for the compounds of Table VII were calculated using eqn. 4. The resulting 42 calculated $S°$ values are shown in Table VII. The standard deviation of calculated from experimental values was found to be $\pm 0.3$ units. This agreement is not significantly worse than in similar calculations of $S°$ for other adsorbents, and confirms our ability to calculate $S°$ values on magnesia by means of eqn. 4.

The variability of different magnesia samples was briefly studied with respect to changes in sample separation order (*i.e.* relative $\underline{R}°$ values). $\underline{R}°$ values for several of the compounds of Table VII (Sea Sorb MgO) were redetermined on a sample of the West Vaco magnesia under approximately the same separation conditions (same

TABLE IX

SAMPLE RETENTION VOLUMES ON 3 % $H_2O$–MgO (WEST VACO)[a]; PENTANE ELUENT

| *Sample* | *Log* $\underline{R}°$[b] |
|---|---|
| Acenaphthylene | —0.35 |
| Phenanthrene | 0.34 |
| Fluoranthene | 1.07 |
| Acetophenone | —0.06 |
| Aniline | 0.47 |
| 1-Nitronaphthalene | 0.23 |
| 2-Acetonaphthone | 0.86 |
| Methyl-2-naphthoate | 0.40 |
| Quinoline | 0.54 |
| Isoquinoline | 0.55 |
| *m*-Nitroanisole | 0.09 |

[a] Adsorbent heated to 300° and 3 % water added.
[b] Corrected to 67 % w MgO basis (2:1 MgO–Celite) by subtraction of 0.18.

eluent, similar adsorbent activity). These values are reported in Table IX. A linear relationship between the latter log $\underline{R}°$ values and corresponding values from Table VII is predicted by eqn. 1. As seen in Fig. 6 the anticipated relationship between log $R°$

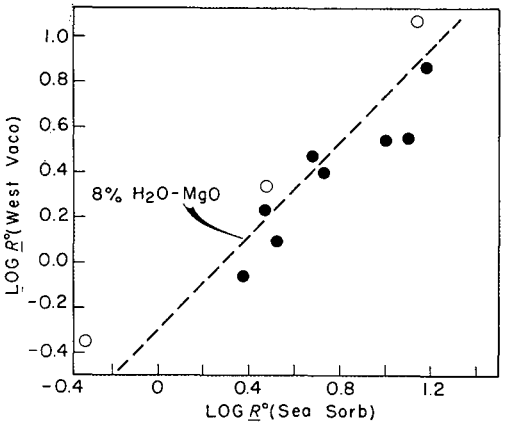


Fig. 6. Log $\underline{R}°$ data of Table IX *versus* corresponding values from Table VII. (○) Hydrocarbons; (●) substituted aromatics.

values on the two adsorbents is observed approximately. The dashed curve of Fig. 6 is that predicted for a sample of 8 % $H_2O$–MgO (Table III). The standard deviation of the points of Fig. 6 from the dashed curve is ±0.18 log units. Considering the different sources of these two magnesia samples, (and corresponding differences in their processing) the scatter of points in Fig. 6 does not indicate any marked variation in the adsorptive properties of the two adsorbents. The data of Fig. 6 for the aromatic hydrocarbons (open circles) tend to lie significantly higher than corresponding data for the substituted aromatics (closed circles). This suggests that the preferential adsorption of aromatic rings on magnesia (relative to other sample groups) is somewhat intensified on the West Vaco magnesia relative to Sea Sorb magnesia.

PRACTICAL APPLICATIONS OF SEPARATION ON MAGNESIA

The correlational model developed in this and preceding papers does not have as its primary aim the precise calculation of $\underline{R}°$ values in chromatographic systems of possible interest. In fact the experimental data collected and correlated to date suggest that such a goal is in general beyond the capability of any relatively simple theory of adsorption chromatography. A more reasonable, and in fact more useful goal of a general theory of adsorption chromatography is the classification of different separation systems with regard to *general* differences in sample $\underline{R}°$ values. In this way we can tell how an unsatisfactory separation can be improved by systematic changes in separation conditions. Our initial guess of separation conditions for a particular sample can also be made more intelligent. The primary role of adsorbent type, in this connection, is in the selective adsorption of certain sample types by various adsorbents. Thus basic adsorbents such as alumina and magnesia selectively retain acidic samples[22], and for some separation problems this factor might be decisive in the selection of adsorbent type. A previous paper[12] has examined certain other general differences in separation on alumina, silica and Florisil. In the present paper we have seen that separation on magnesia is for the most part quite similar to separation on alumina, with the one exception that olefinic and aromatic groups are preferentially held on magnesia, relative to other sample groups. Because magnesia is less convenient to work with than is alumina, separations on magnesia should be restricted to those cases where it is advantageous to have selective adsorption of unsaturated molecules. Certainly the separation of compounds differing only in the total number of carbon-carbon double bonds can be better carried out on magnesia than on such adsorbents as alumina, silica or Florisil. Silver impregnated adsorbents are of course competitive with magnesia for selective retention of unconjugated olefins.

For quite complex samples which are composed of many compounds of differing type, it is frequently found that no single adsorbent can provide adequate separation of all compounds or compound types. It is then advantageous to make use of a two dimensional chromatographic system, where initial separation on one adsorbent yields fractions which are each further separated on a second adsorbent (or by another chromatographic method, *e.g.* partition). In using this technique it is important that the selectivity of each adsorbent type chosen be complementary with respect to the chosen sample. One example is provided by certain compound type separations of petroleum. On alumina the alkyl substituted naphthalenes, benzothiophenes, sulfides, and diphenyl alkanes separate together as a single fraction. On silica as adsorbent aromatic types are localized, with the result that polycyclic aromatics are less strongly adsorbed relative to other compound types. Consequently it is possible to further separate the above group of compounds from an alumina separation by reseparation on silica: the naphthalenes and benzothiophenes will be separated as an initial fraction from the diphenyl alkanes plus sulfides. Or the same alumina fraction might be reseparated on magnesia, which selectively retains all aromatic types: an initial sulfide fraction and a final naphthalene–benzothiophenes–diphenyl alkane fraction would result in this case. These various possibilities are illustrated in Fig. 7 for an actual petroleum sample. A typical gas oil (200–350° boiling range) was first separated on alumina (Fig. 7a) to give a concentrate "A" (cross hatched) of naphthalenes, diphenyl alkanes, benzothiophenes and sulfides. Ultraviolet (U.V.) absorbance

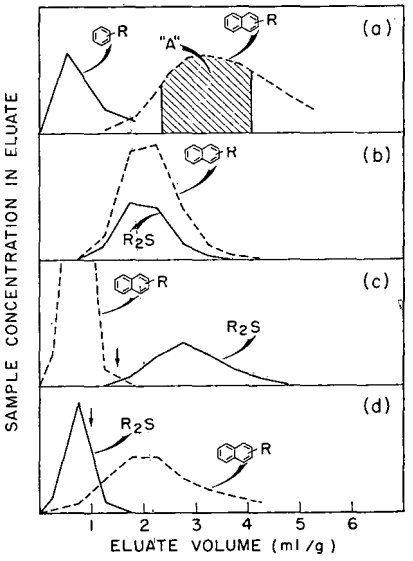


Fig. 7. Successive separation of a petroleum gas oil on alumina, silica, and magnesia. (a) = Initial separation on alumina; (b) = reseparation of cross hatched fraction "A" from (a) on alumina; (c) = reseparation of fraction "A" from (a) on silica; (d) = reseparation of fraction "A" from (a) on magnesia.

of the eluate is plotted in Fig. 7a, in order to distinguish the latter group of compounds from the initially eluting benzene derivatives. Reseparation of fraction "A" on alumina was followed by U.V. and iodine complex absorbance to distinguish sulfides from naphthalenes plus benzothiophenes (Fig. 7b). No further separation of these two compound groups resulted, as expected. In Fig. 7c reseparation of the initial alumina fraction on silica is shown, and as predicted the sulfides (plus undetected diphenyl alkanes) are cleanly separated from the naphthalenes plus benzothiophenes, which elute first. Using the cutpoint indicated by the arrow in Fig. 7c, 99% of each compound type was recovered in its respective fraction. Finally in Fig. 7d the corresponding reseparation on magnesia is shown. Again the sulfides are cleanly separated (94% in each fraction) from the aromatic types, but the elution order is now reversed relative to separation on silica (as predicted). Progressive separation on alumina, silica and magnesia could give almost complete breakdown into sample types, with only the naphthalenes and benzothiophenes remaining unresolved. Use of a fourth, sulfur selective adsorbent (*e.g.* ref. 24) might then provide complete compound type separation.

## SUMMARY

Linear elution adsorption chromatography on water deactivated magnesia has been used to evaluate the unique characteristics of this adsorbent. Water is washed from the magnesia surface by all solvents quite readily, and the resulting dry surface irreversibly adsorbs aromatic compounds. Complete sample recovery is possible, however, using water wet solvents. With respect to the effect of the solvent on sample separation order (retention volume values) and the relationship of sample adsorption to sample structure, magnesia resembles alumina rather closely. The major difference between these two adsorbents is a stronger relative adsorption of the carbon–carbon double bond on magnesia. Magnesia has a general tendency to

adsorb olefins and aromatics more strongly than other adsorbents, leading to some useful separation possibilities with magnesia alone or in combination with other adsorbents.

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# COLUMN CHROMATOGRAPHY AND SPECTROSCOPY IN THE ANALYSIS OF AIRBORNE POLYCYCLICS

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## INTRODUCTION

The measurement of polycyclic hydrocarbons in air samples, for some fifteen years, has been based mainly on ultraviolet absorption measurements made after chromatographic separations of air sample extracts on alumina (or silica gel) columns. The work of WEDGWOOD AND COOPER[1], COOPER[2] and COMMINS[3] may be cited as representative. The heights of characteristic ultraviolet peaks have been measured above a somewhat arbitrary base line[2,3] and the peak heights found have been interpreted as proportional to the concentration of a particular hydrocarbon. Measurements may be made on individual chromatographic fractions and the individual values combined to give the total for a particular hydrocarbon. For sensitivity reasons, it may be necessary to combine fractions and reduce the volume of the composite fraction so that the concentration will be high enough to be analysable by ultraviolet absorption. Originally, ultraviolet measurements were made by manual spectrophotometers. A great deal of the tedium associated with manual measurement has been eliminated by the now widespread use of ultraviolet recording spectrophotometers. These instruments are, in fact, commonly used as both the monitoring and measuring instruments. Similarly, recording fluorimeters are now coming into use, and with their sensitivity so much greater than that obtainable from ultraviolet instruments, eluate fractions, "empty" to ultraviolet, prove to contain measurable amounts of polycyclics by fluorescence. It is worthy of note that as early as 1943, WEIL-MALHERBE[4] worked out the chromatography of benzo[*a*]pyrene (B*a*P) on alumina and silica gel, using a home-made fluorescence instrument. Fourteen years later in 1957, CAHNMANN[5] using a recording ultraviolet spectrophotometer, established the behaviour of benzo[*a*]pyrene on deactivated silica gel. It would be almost unthinkable now, to do column chromatography without the aid of some kind of automatic recording spectroscopy.

An integrated approach is illustrated by gas chromatography where, under ideal conditions, the air sample extract may be separated into a series of individual peaks which can be measured on the recorder chart chromatogram. DUPIRE[6] separated and measured tar oil fractions by gas chromatography. LIBERTI *et al.*[7] measured polycyclic hydrocarbons in three samples of dust by gas chromatography. Their method involves concentration of 100 ml of cyclohexane extract to 5 ml, partition of the polycyclics between solvents and final concentration of sample, under reduced pressure, to about 10 $\mu$l. They obtain beautiful chromatograms, but

from our experience, we would expect almost total loss of anthracene and phenanthrene, and loss of a great deal of both pyrene and fluoranthene during the concentration process. This concentration step is presumably necessary on sensitivity grounds. Similar high evaporative losses of anthracene, phenanthrene, pyrene and fluoranthene are cited by GRIMMER AND HILDEBRANDT[8]. WILMSHURST[9] analysed polynuclear arenes by gas chromatography after going through a conventional chromatographic step "to concentrate the polynuclear arenes", and also perhaps to clean up, or lower the background of the sample before gas chromatographic analysis.

WILMSHURST admits there may be some ambiguity connected with some of the peaks found. As he says "chrysene and benz[*a*]anthracene... were detectable subsequent to the peak". DE MAIO AND CORN[10] measured polycyclics in ten composite samples of Pittsburgh air, but again found it necessary to concentrate the benzene extract to attain a concentration which was usable with the gas chromatograph.

It must be pointed out here that it is quite feasible to analyse polycyclics in prepared extracts by fluorescence, without concentration. In fact, it is usually desirable to dilute and re-analyse at lower concentrations to check that concentration quenching is not at work. The sensitivity of ultraviolet is certainly less than fluorescence and it is sometimes necessary to concentrate to get a usable response. An illustration of this is the measurement of perylene in air samples where perylene structure may not appear in the spectrum until the extract has been concentrated[11]. In the gas chromatographic work cited, concentration was used by all workers. A rough comparison of the monitoring sensitivities would be fluorescence, ultraviolet and gas chromatography, in that order.

The best analytical approach would be to combine gas and liquid column chromatography with fluorescence and ultraviolet instrumentation. For example, unlike liquid chromatography, gas chromatography separates benzo[*k*]fluoranthene (B*k*F) cleanly from benzo[*a*]pyrene, but it does not separate B*a*P from benzo[*e*]-pyrene (B*e*P). Provided B*k*F is absent, it is possible, however, to measure B*a*P by fluorescence and B*e*P by ultraviolet in an eluate containing both[11]. B*a*P and B*k*F can both be measured by fluorescence in an extract or eluate containing a mixture of these two[12]. Efforts are being made to combine the best features of all, but for the present, gas chromatography will not be further discussed, and the emphasis will be on the older liquid column chromatography with spectroscopic identification and measurement of compounds.

The sampling and preparation history of an air sample has perhaps as much to do with the final total accuracy of the values obtained as the actual analytical method. For example, the solvent used to extract the air sample is not a matter of indifference. Extraction should be rapid and complete and substances interfering with the subsequent analysis should not be extracted, ideally. This optimum situation has not yet been achieved, and in the following actual sampling errors and losses will not be discussed.

Several practical analytical difficulties which are encountered are lack of sufficient instrumental sensitivity, apparent losses of hydrocarbon during chromatography and the background. These problems will now be considered.

## REAGENTS AND APPARATUS

*Alumina adsorbent*

Type H 100–200 mesh of Peter Spence is used without any washing treatment. It is heated overnight at 145° and deactivated by the addition of 1.8 % water.

*Cyclohexane*

Technical grade cyclohexane of British Drug Houses is percolated through a bed of active carbon, Pittsburgh Chemical BPL 12 X 30. The product obtained should show less than 0.5 p.p.h.b. fluorescence calculated as quinine base.

*Ether*

Ethyl ether, fluorimetric grade from Hartmann-Leddon, Philadelphia, Pa., is used without further treatment.

*Chromatographic columns*

A glass tube 1.0 cm I.D. and 40.0 cm long is fitted with a teflon plug stopcock. The column is filled to a depth of 12 cm with a slurry of the deactivated alumina in cyclohexane.

*Instrumentation*

For ultraviolet absorption a Bausch & Lomb spectrophotometer No. 502 with fixed slits was used, as well as a Cary 14 recording spectrophotometer. For fluorimetric measurements a modified Aminco-Bowman spectrophotometer was used with a 1 P28 photomultiplier tube and slit arrangement No. 2.

## EXPERIMENTAL

Before doing any experiments we can theorise, knowing the systems we are dealing with, that apparent low recovery of polycyclics may be caused by one or a combination of the following:

(1) Lack of sensitivity of the measuring technique,
(2) Irreversible adsorption on the column,
(3) Tailing on the column,
(4) Incomplete column separations,
(5) Decomposition on the column.

Each of these possibilities was investigated separately and appropriate experiments were performed to see which of these premises might be true.

With any instrumental technique used to measure consecutive eluates, a certain minimum concentration is necessary to get a measurable response. For concentrations below this limit a zero value is obtained. With a detection limit of 0.010 absorbance units we see from Table I that to detect B*a*P by ultraviolet absorption we require a concentration of 0.7 $\mu$g per 1 ml, or in a cuvette of 3 ml volume, a total amount of 2.1 $\mu$g. This is based on measurement at the characteristic B*a*P peak at 401–403 nm. It is interesting to note that precisely the most important hydrocarbon in the group, B*a*P, has the least favourable detection limit. On the other hand, B*a*P is a strong fluorescence emitter and is therefore well suited for measure-

TABLE I

CONCENTRATIONS DETECTABLE BY ULTRAVIOLET MEASUREMENT
For detection limit of 0.010 absorbance units.

| *Hydrocarbon* | *Concentration* | | *Wavelength (nm)* |
|---|---|---|---|
| | *μg/ml* | *μg/3 ml* | |
| Pyrene | 0.04 | 0.12 | 325–340 |
| Coronene | 0.06 | 0.18 | 335–342 |
| Fluoranthene | 0.05 | 0.15 | 284–290 |
| Benzo[*e*]pyrene | 0.075 | 0.22 | 323–337 |
| Anthanthrene | 0.04 | 0.12 | 422–430 |
| Benzo[*g,h,i*]perylene | 0.01 | 0.03 | 376–393 |
| Benzo[*k*]fluoranthene | 0.10 | 0.30 | 395–405 |
| Benzo[*a*]pyrene | 0.70 | 2.1 | 397–406 |

ment by fluorescence[12]. Comparing sensitivities available from ultraviolet and fluorescence for B*k*F ultraviolet measurements may be made over a range of 0 to 10 μg/ml and fluorescence measurements over a range of 0–0.1 μg/ml. Rated conservatively, there is a sensitivity differential of more than 100 times in favour of fluorescence measurements. A practical illustration of the difference in usable sensitivity is shown by Fig. 1, in which B*a*P was measured in the same eluate fraction of an air sample by both techniques. An easily measured response is given by fluorescence but ultraviolet absorption shows nothing measurable. It is easy to see that the use of the wrong measuring technique might give the false impression that B*a*P was absent. Apparent low analytical recoveries may also be explained as due to the remaining four factors. Since these factors are all column-dependent, they were investigated together.

Fifty micrograms of ten different polycyclics were chromatographed separately on the alumina column previously described, using cyclohexane as eluting agent. Quantitative measurements were made on the eluates by ultraviolet or fluorescence,

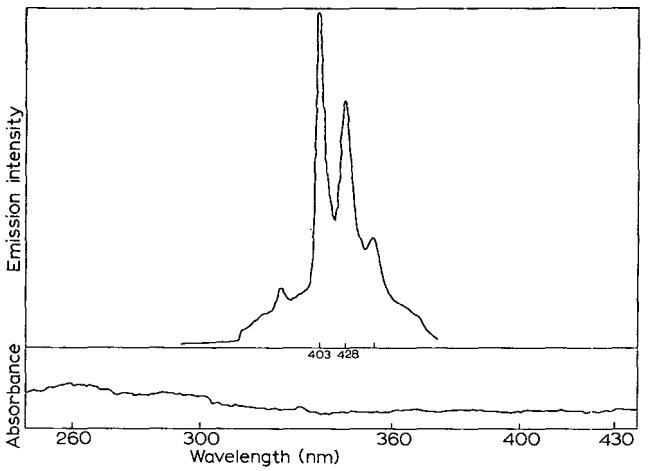


Fig. 1. Practical comparison of fluorescence and ultraviolet sensitivities based on same sample.

depending upon the concentration of a particular hydrocarbon in the eluate. All the eluates containing a particular compound were combined, a quantitative measurement was made and this was compared with the value given by the same amount of the same standard not chromatographed. Table II gives the percentage recovery for 50 $\mu$g of each of the ten polycyclic hydrocarbons listed. Within experimental error,

TABLE II

RECOVERY FROM THE COLUMN

| *No.* | *Compound* | *µg found* | | *Recovery (%)* | |
|---|---|---|---|---|---|
| | | *U.V.* | *Fluorescence* | *U.V.* | *Fluorescence* |
| 1 | Naphthalene | 50.8 | — | 101.5 | — |
| 2 | Fluorene | 49.6 | — | 99.3 | — |
| 3 | Phenanthrene | 51.2 | — | 102.5 | — |
| 4 | Anthracene | 49.9 | — | 99.9 | — |
| 5 | Pyrene | 49.6 | 51.2 | 99.2 | 102.3 |
| 6 | Fluoranthene | 48.3 | 49.2 | 96.6 | 98.4 |
| 7 | Triphenylene | 49.9 | 48.4 | 99.8 | 97.1 |
| 8 | Chrysene | — | 47.0 | — | 94.0 |
| 9 | Benz[*a*]anthracene | — | 47.0 | — | 94.0 |
| 10 | Benzo[*a*]pyrene | — | 52.5 | — | 105.0 |

recovery is complete and it is clear that there is no decomposition on the column, and that the adsorption process is reversible for the particular column conditions used. It is to be noted that the separations were performed using only cyclohexane. If a more polar solvent such as ether were used in admixture we should certainly still recover all the polycyclic.

To see whether the cause of apparent low recovery might be incomplete separation or tailing, the ten polycyclics were chromatographed together on an alumina column. Cyclohexane and increasing quantities of ethyl ether were used to elute as shown in Table III.

TABLE III

TYPICAL ELUTION

| *Volume (ml)* | *Solvent* |
|---|---|
| 0–315 | Pure cyclohexane |
| 315–415 | 0.5% ether |
| 415–470 | 1.0% ether |
| 470–690 | 1.5% ether |
| 690–740 | 2.0% ether |
| 740–840 | 3.0% ether |
| 840–860 | 4.0% ether |

Each eluate fraction was scanned in the ultraviolet. The chromatogram obtained is shown in Fig. 2. The shape of the curves indicates that tailing is negligible for all practical purposes. (See also chromatograms of GRIMMER AND HILDEBRANDT[8].) It is interesting to note the poor separation of the two groups, fluorene, phenanthrene and anthracene and triphenylene, benz[*a*]anthracene and chrysene. Meanwhile

Table IV shows that recovery is fairly good at the wavelengths used for "base line" measurements. Recovery varies from a low of 83 % for fluorene to a high of 110 % for phenanthrene. As can be seen, incomplete separation does not explain low recovery because the effect can be either positive or negative. Considering standards only, singly or in mixture no evidence has been found for low recoveries or column losses. Standard solutions, of course, lack the background which is present in an air sample.

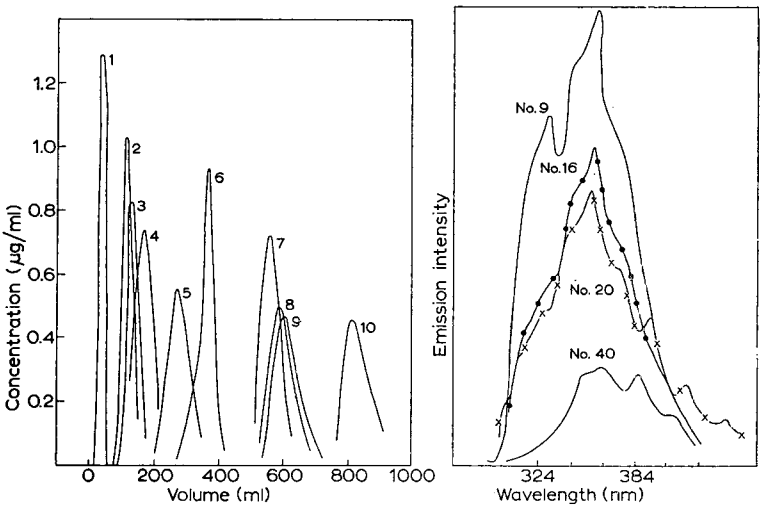


Fig. 2. Chromatogram of standard mixture of ten polycyclic hydrocarbons.

Fig. 3. Variation in compound X with chromatographic development.

Apparent recovery losses which cannot now be attributed to the column might, for the sake of argument, be attributed to the hiding of peaks by the background, leading to negative measurement errors. Accordingly, an air sample extract was chromatographed on an alumina column and fluorescence emission spectra were

TABLE IV

SEPARATION OF A MIXTURE

| *No.* | *Compound* | *Found* | *U.V. recovery (%)** | *Wavelength (nm)* |
|---|---|---|---|---|
| 1 | Naphthalene | 51.5 | 103.0 | |
| 2 | Fluorene | 41.5 | 83.0 | 296–305 |
| 3 | Phenanthrene | 54.8 | 109.7 | 287–301 |
| 4 | Anthracene | 50.9 | 101.9 | 371–381 |
| 5 | Pyrene | 46.6 | 93.2 | 325–340 |
| 6 | Fluoranthene | 48.9 | 97.9 | 284–290 |
| 7 | Triphenylene | 52.1 | 104.2 | 245–252 |
| 8 | Benz[*a*]anthracene | 47.3 | 94.6 | 283–293 |
| 9 | Chrysene | 46.8 | 93.6 | 263–273 |
| 10 | Benzo[*a*]pyrene | 47.3 | 94.7 | 375–390 |

* Mean recovery 98.6 %.

recorded for each eluate fraction using 289 nm as the exciting wavelength. This wavelength was chosen for three reasons: (a) it is a good excitation wavelength for the material which seems to be the cause of the background; (b) most of the aromatics present should be excited at this wavelength; and (c) this wavelength is less subject to concentration interference than wavelengths in the 200–270 nm region. It was soon discovered that each eluate apparently contained varying amounts of the same compound "X" as may be seen from Fig. 3. The concentration of X starts at a maximum decreasing continuously with successive eluate fractions. The predominant peak at 360 was preceded by small peaks as shown by fraction 9. In fraction 20, the peak at 330 has almost disappeared, the intensity at 360 has dropped sharply and at least three new peaks have appeared at wavelengths above 360. At no time, in these experiments, were spectra for known pure compounds obtained. The peak at 360 nm was always present. From these results the background might be described as:

(1) One compound emitting at 360 present in much higher concentration than any of the others,

(2) A mixture of hydrocarbons having a common structure like derivatives of naphthalene or phenanthrene,

(3) Overloading of the column.

Using gas chromatography on a silicone oil column at 225° with a hydrogen flame detector, it was found that gas chromatograms of the aromatic fraction of an air sample extract showed numerous peaks, each component present in approximately equal amounts. This would seem to rule out No. 1 as a working hypothesis.

We already know from past experience that tailing on alumina of 1.8 % water content is negligible for all practical purposes. Nor can incomplete separation of the polycyclics explain the background effect. Exciting the ten component standard mixture at 289 we find that there is no peak at 360 nm in the emission spectrum.

This leaves us with the last premise that the background may be due to column overloading. Results obtained by fluorescence measurements on air sample eluates indicate that the spectrum of the so-called background is almost identical to the spectrum of an air sample extract *not* chromatographed. Moreover a curve of log background against elution volume has the shape of a typical decay curve. Fluorescence measurements suggest that there is no separation of this background material before 400 ml. On the other hand, we know from ultraviolet measurements that a separation of fluoranthene and pyrene is already taking place. The curve of log fluorescence intensity *versus* eluate volume is identical to a curve of 1/carbon number *versus* log retention volume. Lastly the shapes of the curves vary depending upon the exciting wavelength, see Fig. 4. This is an indication that the absorption is different and that a separation could be taking place, since as the emission wavelength is increased, the size of the molecule becomes bigger.

## CONCLUSIONS

The use of fluorescence is mandatory in the measurement of polycyclic hydrocarbons in air samples. Without its use, the analyst would be seriously handicapped with regard to sensitivity. With respect to the special problem of B*a*P analysis, this is best carried out by fluorescence rather than ultraviolet absorption[12,13]. From our

experience we feel that much of the data on B*a*P-in-air concentrations in the literature may be seriously in error. Before the mutual interference of B*a*P and B*k*F were pointed out by SAWICKI published measurements probably reflect B*k*F as well as B*a*P[14,15].

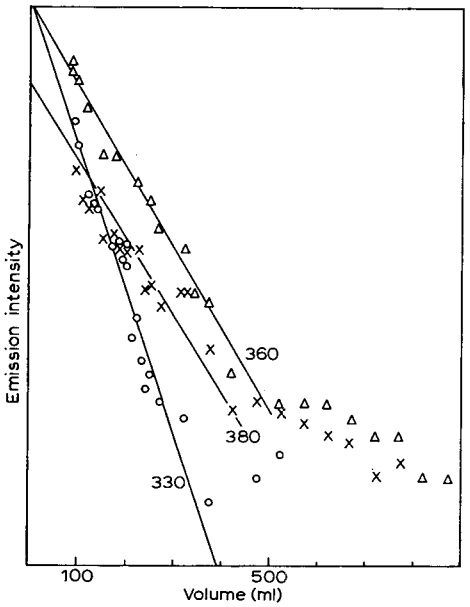


Fig. 4. Log emission intensity of compound X *versus* eluate volume for various emission wavelengths.

We can see no evidence for losses on the chromatographic column and accordingly no need to correct for such losses. It is possible that apparent losses may be due to interference from the background. Our present feeling is that the background may be due to overloading of the column or, on occasion, to incomplete separation of a mixture of hydrocarbons having a common structure. These hydrocarbons are likely to be of the two to three ring type. It must be remembered that overloading of the column and incomplete separation are different effects, but the influence on the chromatogram will be the same. A permanent condition is the fact that the aromatic fraction being analysed is only a very small portion of the total air sample. Our column is considerably shorter than that used by CLEARY[16], with equally good separations, more quickly obtained.

In air sample analysis two interfering effects may be due to the background. Firstly, certain peaks are certainly obscured, which limits the certainty of identification. Secondly, the values obtained must be less than the true value if we exclude the possibility of other interferences. Work is in progress to identify the background and to evaluate the degree of analytical error associated with it.

SUMMARY

In the authors' experience, no column losses occur during the liquid column chromatography of polycyclic hydrocarbons as found in air samples.

Monitoring of the numerous column eluates is most rapidly performed by recording instruments based on ultraviolet or fluorescence. These instrumental techniques complement one another but fluorescence is far superior in sensitivity. In the special problem of the analysis of benzo[*a*]pyrene, fluorescence must be used since ultraviolet results are quite unreliable.

The constant presence of an unknown material, compound X, has been demonstrated in air samples. This may be a lubricating oil residue derived from automobile exhaust.

Work is in progress to identify the "background" and to assess its effect in air sample analysis.

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# AUTOMATED PROTEIN CHROMATOGRAPHY

## A COMPARATIVE STUDY OF SOME BALEEN S-CARBOXYMETHYL-KERATEINES

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### INTRODUCTION

The demonstration that the baleen of the Sei whale (*Balaenoptera borealis*) is mineralised with apatite-like deposits, closely similar in form and composition to bone mineral[1] and that these deposits are intracellular and variable in quantity from cell to cell[2] has raised new questions for biological calcification and for keratin chemistry.

During the maturation of related mineralised epidermal tissues such as tooth enamel, the total protein content is not only strikingly diminished, but there are changes in the proportions of the constituent proteins[3,4]. It is important, therefore, to establish if similar variations in protein composition occur in baleen cells of widely varying mineral content. Unfortunately, the chemical study of keratinised tissues is made difficult by the insoluble nature of the proteins, although soluble preparations from keratinised tissues (S-carboxymethyl-kerateines) may be obtained by reduction with mercaptoethanol and subsequent alkylation of the free sulphydryl groups. Such preparations may be fractionated by chromatography on columns of DEAE-cellulose in solutions containing 8 *M* urea using step-wise elution with increasing potassium chloride concentrations[5–7].

Baleen fringe fibres may be disintegrated by trypsin treatment into suspensions of single intact cells, and subsequently density fractionated by centrifugation in organic solvents, yielding fractions varying in ash content from some 2 % to 35 % by weight[8,2], providing materials suitable for the study of variation in the proportions of the solublised proteins with the degree of mineralisation of the cells. Whilst it is known that the fractions obtained from "kerateine" preparations by chromatography are generally heterogeneous, this procedure provides a method of detecting gross differences in protein proportions with the degree of mineralisation. In the course of these studies an automated apparatus for protein chromatography has been developed and its accuracy in the analysis of heterogeneous protein preparations examined.

### EXPERIMENTAL

*Preparation of protein extracts*

The method used was based on that of THOMPSON AND O'DONNELL[7]. Samples of

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the dried, density fractionated Sei whale baleen cells (1 g) were suspended in 8 *M* urea/0.01 *M* Tris buffer (100 ml, pH 7.4) in 250 ml stoppered flasks. 2-Mercaptoethanol (1 ml redistilled) was added and the pH adjusted to 10.5 with potassium hydroxide solution (5 *N*) using a pH-stat (Radiometer Ltd., Copenhagen). The flasks were flushed with nitrogen, stoppered and shaken at room temperature overnight. A solution of sodium iodoacetate (3 g) in 0.05 *M* Tris buffer (10 ml, pH 8.5) was added and the pH maintained at 8.5 on the pH-stat until alkylation was completed (*ca.* 15 min).

The solublised proteins (some 50 % of initial weight) were separated from the insoluble residue by centrifugation (3,000 *g*, 30 min at 5°), and the extracts were dialysed free of urea and salts, freeze dried and stored at 2°. In this manner soluble protein preparations were obtained from baleen cell samples of 2.8 %, 14.2 % and 27 % ash weight.

*DEAE-cellulose*

Whatman brand powder (DE. 50) in the chloride form was prepared as described by PETERSON AND SOBER[9] and finally equilibrated with urea buffer (0.01 *M* Tris, 8 *M* urea; pH 7.4).

It was found that the flow resistance of columns of this material in the urea buffer was inconveniently high and the difficulty was overcome by mixing the DEAE-cellulose with an equal weight of Celite (30–80 mesh, "for gas chromatography", British Drug Houses Ltd.).

*Urea*

Urea; Laboratory Reagent Grade (British Drug Houses Ltd.) was found to contain impurities with a significant ultra violet absorption in the 230–300 mμ region. Urea (A.R. Grade) was therefore used.

*Tris*

Tris [tris-(hydroxymethyl)-aminomethane], Laboratory Reagent Grade was obtained from British Drug Houses Ltd.

All other reagents were of A.R. quality.

## CHROMATOGRAPHIC APPARATUS

The comparative, quantitative analysis of S-carboxymethyl-kerateines by chromatography has not been reported previously, although it has been shown that step-wise elution of these preparations from DEAE-cellulose yields discrete fractions[7].

The known tendency of solubilised keratin preparations to aggregation in solution[10] and the extreme complexity of the chromatographic fractions obtained, as demonstrated by starch gel electrophoresis[7], suggested that rigorously reproducible conditions would be required if useful quantitative data were to be obtained. To this end an automated step-wise elution system was developed.

The apparatus employed is shown diagrammatically in Fig. 1 and comprised:

(*a*) *Recording equipment*

A Uvicord flow cell spectrophotometer (L.K.B. Ltd.) reading at 257 mμ coupled to a chopper bar chart recorder (L.K.B. Ltd. Type 6520A) driven at 6 cm/h was used.

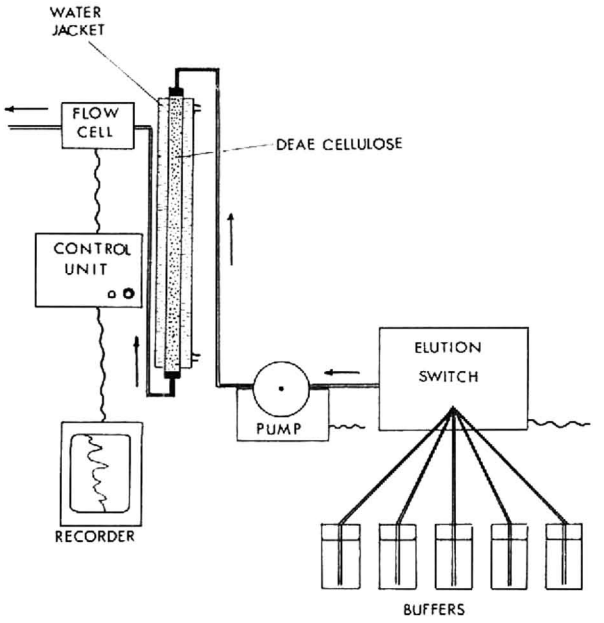



Fig. 1. Diagram of apparatus.

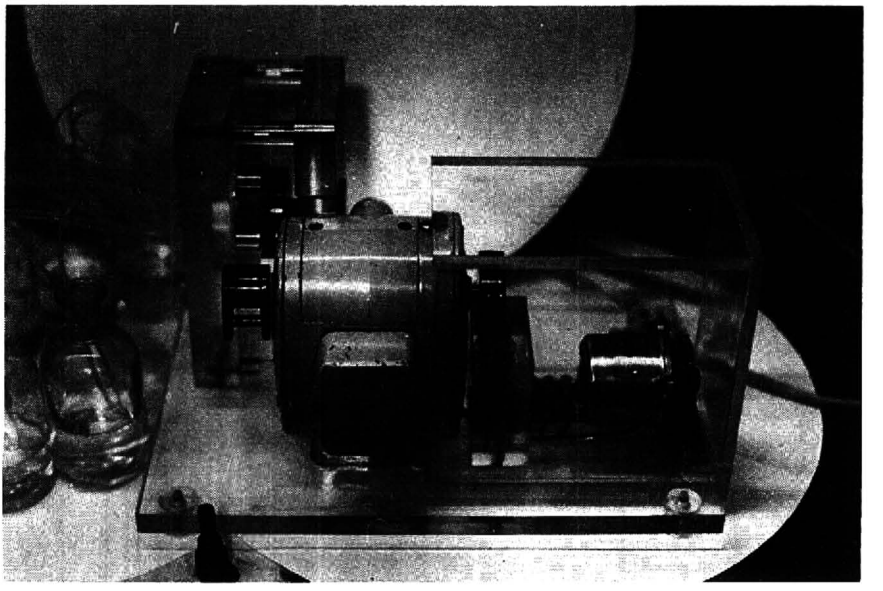


Fig. 2. Elution switch mechanism (see text for description).

*(b) Chromatographic column*

The older type of "cone and socket" chromatographic columns are unsuitable for flow analysis systems and a 30 × 0.6 cm flanged column with high pressure connections was obtained from Technicon Instruments Ltd. (Chertsey, Surrey). The "dead space" above the packing was largely eliminated by packing with Celite (30–80 mesh), after application of the sample. Temperature control was maintained by a water jacket connected to the mains supply; (*ca.* 10°). This column was entirely satisfactory in use and was easy to re-pack when required.

*(c) Peristaltic pump*

A simple peristaltic pump was used which fitted the range of pump tubes available from Technicon Ltd. With a 0.02 in. internal diameter pump tube and a rotor speed of 18 r.p.m. the flow rate was maintained at 32–35 ml per h. Pump tubes became distended after 24 h running and were therefore changed after every three chromatographic runs.

*(d) Elution switch mechanism*

A variety of devices for the automatic delivery of solutions to chromatographic columns have been described. Many of these have been designed for use with gravity-fed systems in which the passage of the meniscus from one eluant is "sensed" by an electrode system which activates transfer to the next eluant[11–14]. This system has been termed "depletion sensing"[13]. In systems in which the eluant flow rates are determined by metering pumps, chromatographic reproducibility may be attained by the use of time-determined eluant changes[15,16]. Generally, these mechanisms have made use of solenoid-operated valve assemblies dependent on complex electronic devices for their activation.

However, KARL *et al.*[17] have recently described the use of four and five channel mechanical switching mechanisms based on the "Geneva Mechanism" principle for the programmed elution and regeneration of automated amino-acid analyser columns. For the purpose of the automated step-wise elution chromatography, described in this paper, the use of the "Geneva Mechanism" has been extended to the time programmed application of as many as sixteen eluant changes. The apparatus is simple, extremely robust and has been in constant use over a period of a year. Details of the construction of the apparatus are shown in Figs. 2 and 3.

A geared motor (Drayton Type R.Q., 250 V, 50 ~, 4 r.p.m.) drives a sixteen position "Geneva Mechanism" which is coupled to a sixteen channel P.T.F.E. cylindrical tap (7) rotating it through one position for each movement of the mechanism. In this manner each of the 1/16 in. diameter holes of the tap mechanism is located exactly. The Drayton motor is activated by a micro-switch (19), operated by a sector cam driven by a synchronous motor (Sangamo-Weston, 1 r.p.h.). Interchange of the sector cams allows any time period for the elution steps of up to one hour, giving a total elution time of sixteen hours; alternative timing mechanisms allow elution/regeneration/loading sequences of longer duration and complexity to be used.

The Drayton motor is coupled also to a second cam (14) which switches off the motor after one rotation, leaving the indexing cam (21) in position to make the next change. Each changing operation, from channel to channel, is thus of only some 5 sec duration. The input and output channels of the tap barrel are tapered to accept

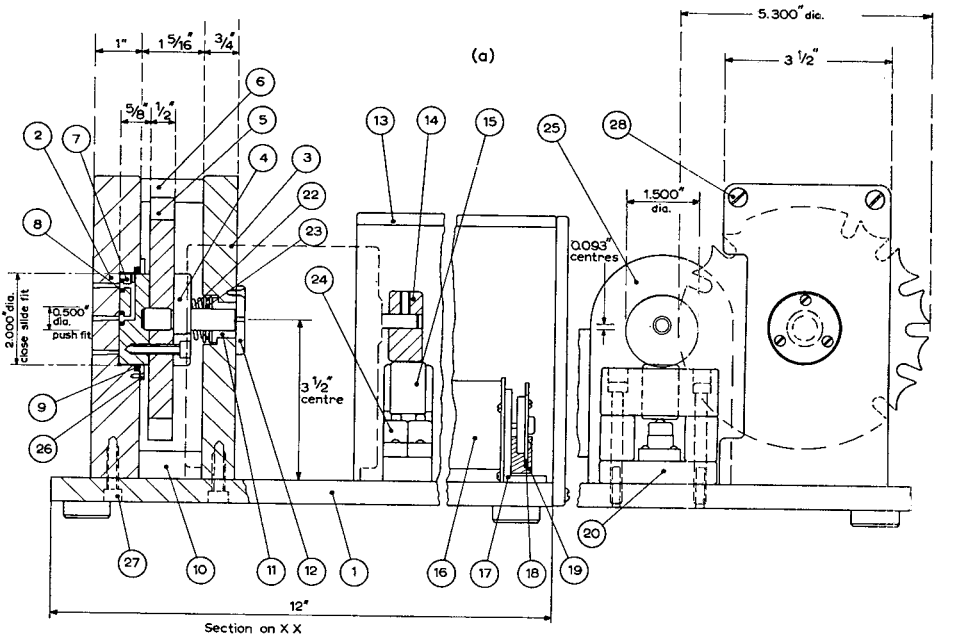



TABLE I

KEY TO NUMBERS IN FIG. 3a

| *No.* | *Item* | *Material* |
|---|---|---|
| 1 | Base plate | Perspex |
| 2 | Valve plate | Perspex |
| 3 | Back plate | Aluminium alloy |
| 4 | Spindle | Brass |
| 5 | Indexing wheel | Perspex |
| 6 | Support bar | Perspex |
| 7 | Valve spiggot | P.T.F.E. |
| 8 | "O" ring | Neoprene |
| 9 | "O" ring | Neoprene |
| 10 | Support plate | Perspex |
| 11 | Bush | Brass |
| 12 | Cover plate | Brass |
| 13 | Cover | Perspex |
| 14 | Switch cam | Brass |
| 15 | Switch plunger | Brass |
| 16 | Synchronous motor | 1 r.p.h. "Sangamo-Weston" |
| 17 | Angle bracket | Brass |
| 18 | Micro-switch cam | Brass |
| 19 | Micro-switch | 240 V, 2 A |
| 20 | Plunger switch bracket | Perspex |
| 21 | Indexing cam | Brass |
| 22 | Spring | Stainless steel |
| 23 | Thrust bearing | ½ in. |
| 24 | Castelco push-pull switch | 250 V, 1 A |
| 25 | Drayton motor | 4 r.p.m. |
| 26 | Flange plate | Brass |
| 27 | Screw | Brass, ¼ in. Whit |
| 28 | Screw | Brass, 2 B.A. |
| 29 | Foot pad. | Perspex |

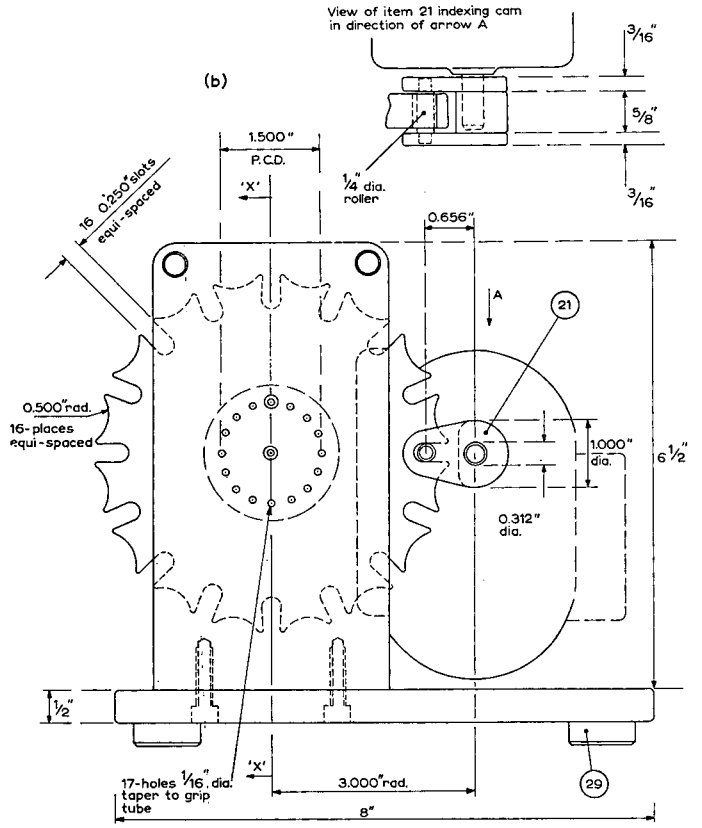



Fig. 3. Details of the construction of the elution switch. (a) and (b) Design of the apparatus. For the numbers, see Table I. (c) Wiring diagram. A = Junction box; B = Drayton type R.Q. motor, 4 r.p.m., 200–250 V, 50 ~ ; C = Castelco 200–250 V, 1 A push-push switch; D = Sangamo-Weston type S7 synchro-motor, 200–250 V, 50 ~ ; E = micro-switch operated by cam (item 18 of Fig. 3a); F = 2-off Castelco 200–250 V, 1 A, push-push switches, operated by cam and plunger (items 14 and 15 of Fig. 3a).

2 mm O.D. polyethylene tubing with which the connections to the buffer containers and metering pump may be made.

This system of eluant switching has the advantage of simplicity and reliability and may readily be extended to any number of channels. EVELEIGH AND THOMPSON[18] have described recently a multichannel mechanism using the peristaltic pump principle although their method of obtaining intermittent change is by timed pulses to the activating motor.

*Application of the sample*

Solutions of the freeze-dried total S-carboxymethylated baleen preparations were made up in Tris/urea buffer at a concentration of 50 mg/ml. It was found that the solution of such preparations was incomplete, and a slight residue remained insoluble, even in the 8 *M* urea solution. The solutions were, therefore, clarified by centrifugation at 25,000 *g*, 30 min at 5°.

0.4 ml (20 mg protein) was applied to the column with a pasteur pipette, the upper surface of the ion exchanger being protected with a circle of filter paper; Whatman No. 541 (No. 1 paper tends to disintegrate in the urea solution). The sample was washed into the column with nitrogen (15 lb./sq. in.). "Starting buffer" (0.01 *M* Tris, 8 *M* urea, pH 7.4) was then layered on to the column and displaced with Celite until the dead space was filled. The upper pressure connection was then made, care being taken to exclude air bubbles, and the joint sealed with two Thomas clips (Technicon Ltd.).

*Elution of the proteins*

The column was eluted with a step-wise gradient of increasing concentration of potassium chloride in Tris/urea buffer, dispensed automatically to the column with the apparatus described. Significant "step-gradient" mixing being eliminated by the limited "dead space" above the column. Regeneration of the exchanger was carried out on the column using 1 *M* potassium chloride in Tris/urea buffer. The column was then equilibrated with Tris/urea "starting buffer" (*ca.* 50 ml) before the application of the next sample.

*Recovery of protein from the column*

Recovery was determined from the absorption at 280 mμ of the total pooled eluant from the column. The protein concentration being read from a calibration plot for baleen S-carboxymethyl-kerateine in urea buffer. Recoverties were in the range 91–95 % of the sample weight.

*Quantitative comparison of the chromatograms*

The chromatograms were analysed by tracing the chart profile on to writing paper of good quality, cutting carefully round the outline and estimating the proportion present in each fraction by weight. It was found that this procedure gave reproducible analyses and was a satisfactory method for the analysis of chromatograms with non-gaussian peaks. The reproducibility of the method is shown for duplicate tracings and "cuts" from a typical analysis (Table II).

TABLE II

QUANTITATIVE ANALYSIS OF A CHROMATOGRAM. DUPLICATE "CUTS"

| *Fraction* | *Cut 1* | | *Cut 2* | |
|---|---|---|---|---|
| | *Weight (mg)* | *% total* | *Weight (mg)* | *% total* |
| 1 | 8.3 | 4.3 | 9.1 | 4.6 |
| 2 | 61.8 | 31.9 | 62.4 | 31.5 |
| 3 | 42.1 | 21.8 | 42.6 | 21.5 |
| 4 | 38.9 | 20.1 | 39.3 | 19.9 |
| 5 | 42.3 | 21.9 | 44.5 | 22.5 |
| Totals: | 193.4 | | 197.9 | |

## RESULTS

*The effect of variation in the elution sequence*

It is generally considered that the resolution of complex mixtures of macromolecules by ion exchange column chromatography is achieved most readily by the use of a continuous gradient of ionic strength or pH (for a review of this topic, see SNYDER[19]). However, it has been shown[20] that the elution of proteins from ion exchange materials without "trailing" occurs only at $R_F$ values close to unity, a condition which is met by step-wise elution procedures. O'DONNELL AND THOMPSON[6] showed that the elution of both $\alpha$- and $\gamma$-keratoses[21] of wool from DEAE-cellulose in buffers containing 8 $M$ urea, with a continuous gradient of potassium chloride, failed to produce separation of discrete fractions, whereas the application of a step-wise gradient produced fractions which could be shown to differ in their amino acid composition.

In a similar fractionation of S-carboxymethyl-kerateine-A from wool[7] using the step-wise procedure, the fractions obtained were found, by starch gel electrophoresis, to be very heterogeneous, although marked variation in the proportions of fractions and of the constituents within each fraction was found. The fractionation of soluble keratin preparations by these methods is essentially arbitrary, and it has been suggested[6] that the number of fractions obtained is closely related to the number of elution steps applied to the system. This is verified by the present experiments on baleen kerateines. The effect of variation in the number of elution steps is shown in Fig. 4.

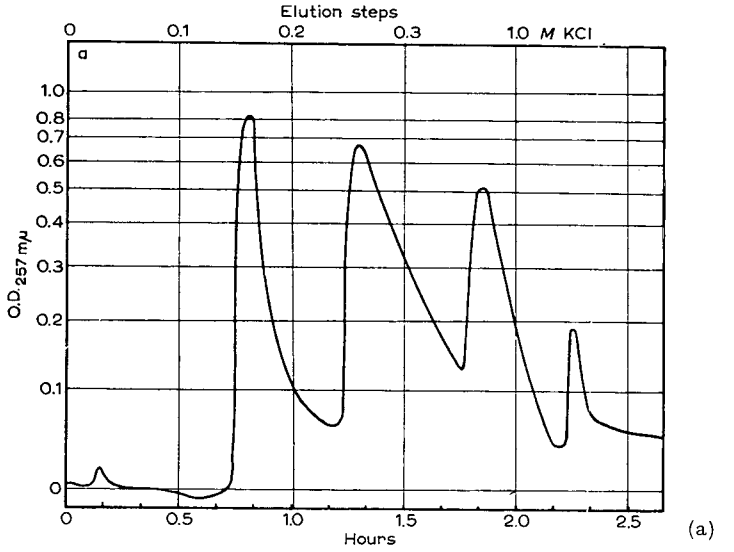
Fig. 4a showing a five step elution is similar to the result obtained in a five step elution of wool $\alpha$-keratose (O'DONNELL AND THOMPSON[6]) who obtained three major and two minor fractions. Fig. 4b illustrates the result of adding a further step to the elution sequence, showing a more complex pattern with indications of heterogeneity in some of the peaks. Fig. 4c is an example of the application of a fourteen step sequence yielding at least thirteen distinct peaks. These data are in close agreement with those of THOMPSON AND O'DONNELL[22] for wool kerateines and it is clear that the number of fractions obtained will depend on the number of elution steps applied. The equivocal results obtained in gradient elution experiments appear to result from the complexity of S-carboxymethyl-kerateine preparations.

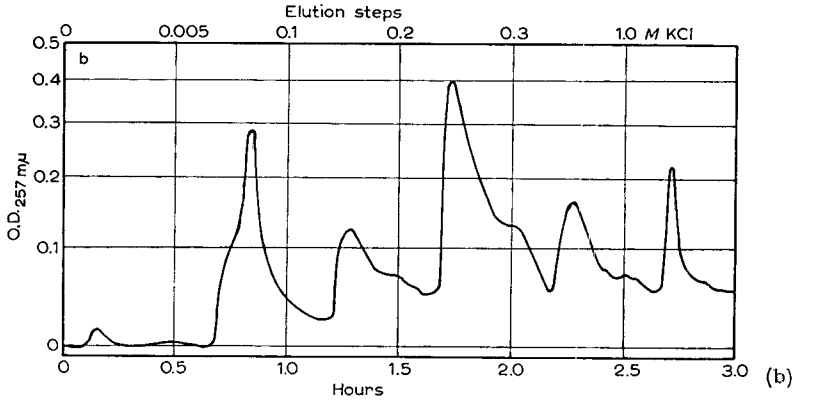
*Accuracy of the analytical system*

The accuracy of reproducibility of the chromatographic analysis was determined by the repeated analysis of a sample of baleen kerateine preparation, using an eight step elution sequence of 0, 0.1, 0.15, 0.2, 0.3, 0.4, 0.5 and 1 $M$ potassium chloride in Tris/urea buffer. A typical chromatogram is shown in Fig. 5. It can be seen that eight principal "peaks" are produced. The data for thirteen analyses of the same preparation of material are presented in Table III.

*Comparison of S-carboxymethyl-kerateine preparations from baleen density fractions*

Since the complexity of the chromatogram obtained from these soluble keratin preparations is a function of the number of elution steps applied, in order that the chromatograms should not be so complex as to render comparison of preparations unduly difficult, a standard elution sequence of six steps in the potassium chloride concentration of 0, 0.05, 0.1, 0.2, 0.3, 1.0 $M$ was adopted. A typical chromatogram is



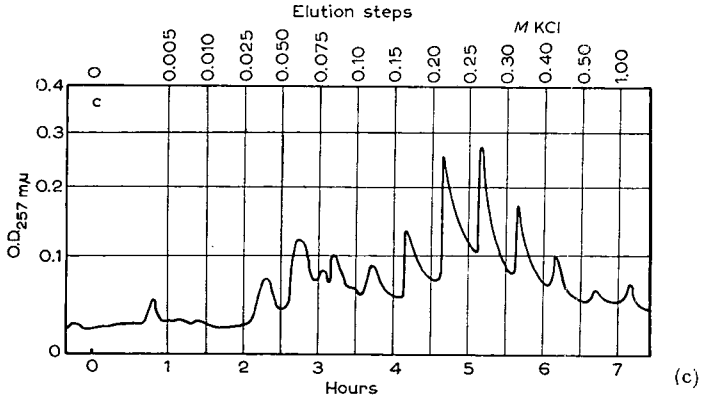




Fig. 4. (a) Chromatogram of baleen kerateine preparation using a five step elution programme. (b) Chromatogram of baleen kérateine preparation using a six step elution programme. (c) Chromatogram of baleen kerateine preparation using a fourteen step elution programme.

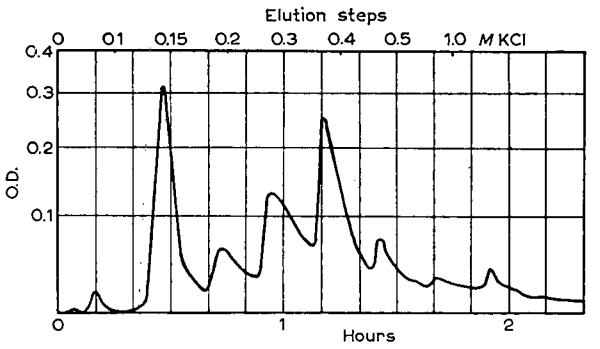



Fig. 5. Baleen kerateine preparation chromatographed using an eight step elution sequence.

TABLE III

MULTIPLE ANALYSES OF BALEEN KERATEINE

| | *Chromatogram fractions** | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | *1* | *2* | *3* | *4* | *5* | *6* | *7* | *8* |
| 1 | 2.89 | 23.04 | 12.10 | 18.60 | 21.04 | 9.07 | 6.18 | 7.08 |
| 2 | 4.28 | 22.13 | 11.90 | 19.16 | 20.60 | 8.14 | 6.10 | 7.69 |
| 3 | 0.88 | 21.50 | 9.91 | 20.44 | 23.24 | 10.57 | 5.69 | 7.77 |
| 4 | 3.25 | 16.19 | 11.60 | 22.98 | 22.42 | 8.85 | 6.86 | 7.85 |
| 5 | 3.25 | 20.47 | 10.17 | 19.62 | 25.30 | 10.04 | 5.56 | 5.59 |
| 6 | 2.17 | 20.66 | 10.56 | 19.30 | 22.18 | 10.36 | 6.76 | 8.01 |
| 7 | 4.99 | 20.95 | 6.51 | 21.00 | 25.21 | 9.21 | 5.90 | 6.23 |
| 8 | 2.29 | 21.40 | 8.77 | 19.46 | 25.08 | 10.23 | 6.17 | 6.60 |
| 9 | 1.98 | 21.76 | 10.58 | 18.80 | 23.50 | 8.89 | 6.67 | 7.82 |
| 10 | 3.28 | 14.73 | 11.78 | 20.16 | 24.14 | 10.06 | 7.20 | 8.65 |
| 11 | 2.13 | 27.91 | 9.13 | 17.94 | 21.75 | 8.67 | 6.07 | 6.40 |
| 12 | 1.93 | 24.74 | 10.61 | 19.65 | 20.77 | 8.85 | 6.20 | 7.25 |
| 13 | 1.61 | 25.50 | 10.90 | 20.10 | 18.56 | 7.62 | 6.77 | 8.94 |
| Mean: | 2.67 | 21.61 | 10.32 | 19.78 | 22.60 | 9.27 | 6.32 | 7.37 |
| Standard error of the mean: | ±0.31 | ±0.97 | ±0.43 | ±0.36 | ±0.57 | ±0.25 | ±0.14 | ±0.26 |

* Expressed as percentage of total.

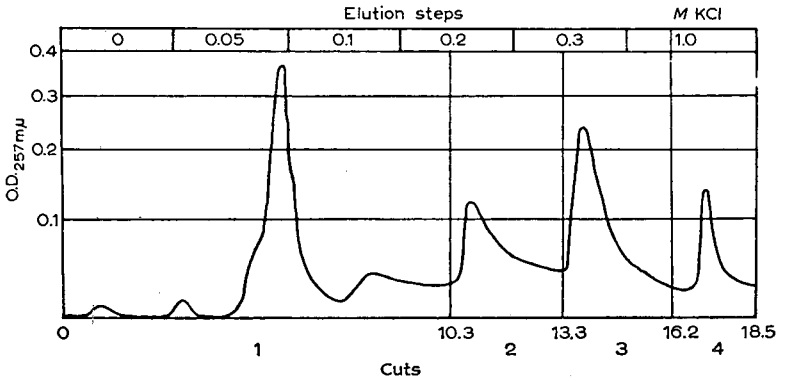



Fig. 6. Typical baleen kerateine chromatogram used for comparative analysis of mineralised preparations. Chromatogram "cuts" shown as described in text.

TABLE IV

MEAN FIGURES FOR THE CHROMATOGRAPHIC ANALYSIS OF SEI WHALE BALEEN TOTAL S-CARBOXYMETHYL-KERATEINE PREPARATIONS*

| *Chromatogram "Cut"* | *% Ash in baleen fraction* | | |
|---|---|---|---|
| | *2.8%* | *14.2%* | *27.0%* |
| 1 | 41.01 ± 0.07 | 39.13 ± 1.05 | 35.47 ± 0.59 |
| 2 | 21.31 ± 0.22 | 24.40 ± 0.35 | 20.90 ± 1.90 |
| 3 | 26.10 ± 0.20 | 25.16 ± 0.69 | 31.20 ± 1.70 |
| 4 | 11.70 ± 0.28 | 11.30 ± 0.21 | 13.57 ± 0.69 |

* Data are shown as percentage of the total "cut" weight.

shown in Fig. 6. The "cuts" for the quantitative analysis of the chromatograms were made in a manner such that each of the principal peaks was isolated in a single "cut", namely at 10.3, 13.3, 16.2 and 18.5 cm from the origin of the chromatogram. The data from multiple analyses of these preparations are shown in Table IV.

## DISCUSSION

The complexity of the baleen kerateine preparations is illustrated by the chromatographic analyses and is in accord with the findings of THOMPSON AND O'DONNELL[23]. However, it appears that the method of analytical chromatography described is capable of resolving these preparations into discrete fractions in a quantitatively reproducible manner. Such methods may be of value for the detailed quantitative analysis of protein preparations from keratinised tissues or other sources.

The comparative analyses of the mineralised baleen preparations suggest that there is a reduction in the proportion of the "initial fraction" (*e.g.* Cut 1, Fig. 6) of the total kerateine preparation with the degree of mineralisation, which is of interest in view of the finding of BURGESS AND MACLAREN[3] for tooth enamel proteins, and the suggested decrease in the α-keratose component of milled, density fractionated, baleen plate[24].

## ACKNOWLEDGEMENTS

I am indebted to Mr. A. H. ANSLOW and Mr. J. R. SCOTT of the Department of Biophysics Workshops for the detailed design and construction of the elution mechanism described. Financial assistance from the U.S. Institute of Health (Grant No. DE 01346-03) and the Agricultural Research Council is gratefully acknowledged.

## SUMMARY

A detailed study of the chromatographic fractionation of baleen S-carboxymethyl-kerateines has been made, using a system of automated step-wise elution. A comparison of the total kerateine preparations from baleen cells of widely differing inorganic content suggests a variation in protein proportions with the degree of mineralisation.

The type of chromatographic system used is described and its reliable reproducibility suggests it to be suitable for quantitative analyses of complex protein mixtures.

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# THE SEQUENCE OF ELUTION OF PLANT ORGANIC ACIDS FROM SILICA GEL CHROMATOGRAPHIC COLUMNS

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## INTRODUCTION

In a programme of work on the effect of potassium nutrition on the organic acids of leaves, a knowledge of the sequence of elution of the common plant acids proved valuable in identification of acids separated by partition chromatography. The sequence of elution of these and related acids (total 39), by gradient elution chromatography on silica gel, is presented together with a discussion on the effect of structural relationships on the sequence.

Work on the partition chromatography of acids has been comprehensively reviewed to 1956 by LEDERER AND LEDERER[1]. Based on paper chromatographic data, HOWE[2] demonstrated relationships between the $R_M$ values of about 100 acids and certain features of their molecular structure, *e.g.*, the number of carboxyl and other substituent groups, and indicated that this information should prove useful in identification of unknown compounds.

## EXPERIMENTAL

Silica gel chromatography of the acids was carried out by the gradient elution method of WAGER AND ISHERWOOD[3], essentially as modified by BLUNDSTONE AND DICKINSON[4]. In the present work, reservoir 1 contained initially *n*-butanol–chloroform, 50:50 (v/v) (400 ml) and reservoir 2 contained chloroform (340 ml). The eluate was initially pure chloroform and its *n*-butanol content was progressively increased to 35 % (v/v). The acids (0.25–1 mequiv. each) were chromatographed in groups of 2–8 components, chosen to minimise overlapping of peaks. The volume of the fraction collector (syphon device) was 2.6 ml. The volume of chloroform eluted from the column during addition of the sample was 10 ml (≡ 4 fractions) and this was combined with the first fraction for titration. The rate of transfer of mixed solvent from reservoir 1 to reservoir 2 was about 30 drops per min from a capillary tube of 2 mm I.D., 7 mm O.D., but maximum resolution in the early stages of a separation was obtained when this rate was halved. Two hundred fractions were collected during 8 h.

The column was packed with Mallinckrodt silicic acid (100 mesh powder, A.R.). As preliminary treatment, it was washed several times by decantation with water to remove the finer particles, washed once with 6 *N* hydrochloric acid and several times with distilled water to remove excess acid and finally washed with ethanol and with

acetone and dried overnight in an oven at 100°*. Chloroform (Hopkin & Williams Ltd., G.P.R. "A" grade) was treated with excess of anhydrous sodium carbonate to remove any traces of acid and filtered. Ethanol, 2 % (v/v), present as a preservative was not removed before use. *n*-Butanol (Hopkin & Williams Ltd., G.P.R. grade) was the other component of the solvent mixture. The acids were the purest samples available commercially in the U.K.

Non-volatile acids were identified by thin-layer chromatography on Silica Gel G (Merck) with *n*-butyl formate–90 % formic acid–water (7:2:1, v/v) as mobile phase[5] and the observations were confirmed by spot tests[6].

TABLE I

SEQUENCE OF ELUTION OF ACIDS FROM SILICA GEL COLUMN

The acids were eluted with chloroform containing a progressively increasing proportion of *n*-butanol. The stationary phase was 0.5 *N* sulphuric acid. Fraction volume: 2.6 ml.

| *Acid* | *Elution range (fraction numbers)* | *Peak maximum (fraction number)* | *Acid* | *Elution range (fraction numbers)* | *Peak maximum (fraction number)* |
|---|---|---|---|---|---|
| *n*-Butyric | 1–10 | 2 | 5-Pyrrolidone-2-carboxylic | 65– 80 | 69 |
| *n*-Valeric | 1– 9 | 2 | Glyoxylic | 53– 81 | 70 |
| Isobutyric | 1–12 | 3 | Diglycollic | 64– 76 | 70 |
| Propionic | 5–19 | 8 | Oxalacetic | 67– 79 | 73 |
| Acetic | 22–29 | 25 | Oxalic | 76– 95 | 81 |
| Mesaconic | 24–30 | 27 | Tricarballylic | 80– 93 | 88 |
| Pyruvic | 28–38 | 32 | Glycollic | 84– 98 | 92 |
| Adipic | 28–38 | 32 | Nitric | 87–147 | 93 |
| Formic | 31–38 | 34 | *cis*-Aconitic | 97–108 | 103 |
| Glutaric | 32–37 | 34 | DL-Malic | 103–120 | 111 |
| Citraconic | 34–45 | 39 | Citric | 134–153 | 141 |
| Itaconic | 39–47 | 42 | DL-Glyceric | 152–170 | 160 |
| Maleic | 38–50 | 45 | DL-Isocitric | 176–195 | 183 |
| Fumaric | 38–52 | 45 | Sulphuric | 178–192 | 181 |
| Thymol blue indicator | 47–53 | 50 | | 193–230 | 198 |
| | | | Shikimic | 175–206 | 198 |
| Succinic | 57–63 | 60 | D(+)-Tartaric | 200–233 | 211 |
| Lactic | 57–63 | 60[a] | Phosphoric | > 239 | — |
| α-Ketoglutaric | 54–67 | 62 | L-Glutamic | | |
| *trans*-Aconitic | 66–72 | 69 | Quinic | > 252 | — |
| Malonic | 64–75 | 69 | L-Aspartic | | |

[a] From ref. 4.

RESULTS

The sequence of elution of 39 acids is given in Table I in terms of the first and last fraction numbers in which the acids appeared and the fraction number at peak maximum for each. These fraction numbers were used for the sake of simplicity but may be converted to the more fundamental effluent volumes (ml) by multiplying by the factor 2.6 and addition of the column "hold-up" volume (10 ml). In the gradient elution method, the rate of transfer of the mixed solvent from reservoir 1 to reservoir 2

* A commercial product of controlled particle size is now available as Mallinckrodt silicic acid, SilicAR CC-4, 100–200 mesh.

is a variable which cannot be precisely controlled; this leads to a lack of reproducibility of the order of $\pm$ 5 fractions from the stage of elution of the volatile acids onwards. The emergence of the indicator (thymol blue) about 10 fractions earlier than the succinic acid peak maximum (prominent in the chromatograms of many plant extracts) provided a convenient marker. Recovery of acids was normally better than 95 %.

## DISCUSSION

The following generalisations were made on the effect of the structural relationships of the acids on their sequence of elution as described above (Table I).

### *Fatty acids, $H(CH_2)_nCOOH$*

The sequence was inversely related to $n$ in fatty acids of the type $H(CH_2)_n$-COOH, where $n$ = 0–4. There was a smooth relationship between $n$ and the fraction number ($F$) at peak maximum (graph not reproduced here). Mean $\Delta F$ per $CH_2$ group in the series formic to $n$-butyric acids was —11.

### *Monocarboxylic acids with zero to four hydrophilic groups*

The relationship between the number of polar groups (other than the carboxyl group) ($a$) in the molecule and the fraction number at peak maximum ($F$) is summarised in Table II. There was a linear relationship between ($a$) and ($F$). From the graph (not reproduced here), $\Delta F$ for first OH group was 55 and $\Delta F$ for second OH group was 80.

TABLE II

MONOCARBOXYLIC ACIDS—RELATIONSHIP BETWEEN NUMBER OF POLAR GROUPS AND ELUTION

| *Number of polar groups* ($a$) | *Number of carbon atoms* | *Acid* | *Peak maximum (fraction number)* ($F$) | |
|---|---|---|---|---|
| 0 | 2 | Acetic | 25 | |
| 1 | 3 | Lactic | 60 | |
| 1 | 5 | Pyrrolidonecarboxylic | 69 | Mean 66 |
| 1 | 2 | Glyoxylic | 70 | |
| 1 | 2 | Glycollic | 92 | |
| 2 | 3 | DL-Glyceric | 160 | |
| 3 | 7 | Shikimic | 198 | |
| 4 | 7 | Quinic | > 252 | |

### *Saturated dicarboxylic acids*

The effect of chain length ($n$) in dicarboxylic acids of the type $HOOC(CH_2)_n$-COOH, in the range $n$ = 0–4 is shown in Table III. Mean $\Delta F$ per $CH_2$ group in this series was —12, substantially the same as $\Delta F$ in the fatty acids. The introduction of an ether linkage, as in diglycollic acid, increased the hydrophilic character of the substance ($F$ = 70) as compared with succinic acid ($F$ = 60).

### *Cis- and trans-isomers*

It proved impossible to separate maleic and fumaric acids on the silica gel

column under the conditions described above but their methyl homologues (citraconic and mesaconic acids) were readily separated, particularly when the *n*-butanol content of the mixed solvent was slowly increased ($F$ mesaconic = 37; $F$ citraconic = 57). *cis*- and *trans*-aconitic acids were widely separated. In each of these cases the *trans*-acid was eluted before the corresponding *cis*-isomer.

TABLE III

SATURATED DICARBOXYLIC ACIDS—RELATIONSHIP BETWEEN CHAIN LENGTH ($n$) IN $HOOC \cdot (CH_2)_n \cdot COOH$ AND ELUTION

| *Chain length* (*n*) | *Acid* | *Peak maximum* (*fraction number*) ($F$) |
|---|---|---|
| 0 | Oxalic | 81 |
| 1 | Malonic | 69 |
| 2 | Succinic | 60 |
| 3 | Glutaric | 34 |
| 4 | Adipic | 32 |

*Tricarboxylic acids*

In citric and isocitric acids, the pronounced effect of the shift of the hydroxyl group, from the $\beta$- to the $\alpha$-position, in increasing the hydrophilic property is noteworthy. $\Delta F$ for the hydroxyl group in the above acids is 53 ($\beta$) and 95 ($\alpha$) as compared with 51 (first OH) and 100 (second OH) in the dicarboxylic acids and 55 (first OH) and 80 (second OH) in the monocarboxylic series, (see above). In the dicarboxylic acids, introduction of a double bond in the main chain increased hydrophobicity but *cis*-aconitic acid behaved in the contrary manner and was eluted later than tricarballylic acid.

*Oxalacetic and α-ketoglutaric acids*

Introduction of a methylene group into the main chain increased the hydrophobicity of oxalacetic acid and $\Delta F$ was —11. As compared with the parent acid, introduction of an $\alpha$-keto-group increased hydrophilic properties but the extent of the change varied considerably in the three examples studied. The variation may be due, at least in part, to the presence of the corresponding enol-forms of the acids.

Propionic ⟶ pyruvic acid, $\Delta F$ (CO) was 24

Glutaric ⟶ α-ketoglutaric acid, $\Delta F$ (CO) was 28

Succinic ⟶ oxalacetic acid, $\Delta F$ (CO) was 13

*Number of carboxyl groups*

In the unsubstituted polycarboxylic acid series (acetic, succinic, tricarballylic) and the corresponding monohydroxyl compounds (glycollic, malic and isocitric acids) the addition of a $-CH_2COOH$ group led to a marked increase of hydrophilic properties in the acids but the differences in the $\Delta F$ values are not regular. Addition of a carboxyl group in the keto-acid series (pyruvic and oxalacetic) had a similar effect.

Nitrate is present in relatively large amounts in many plants, particularly when

grown in sand culture. The acid was eluted from silica gel columns as an asymmetrical peak with a steep leading edge and a marked tailing edge and the peak therefore overlapped several others including the important malic and citric acid peaks. This occurred independently of column loading in the range 0.25–1 mequiv. of nitrate. The quenching acid, sulphuric, gave rather indefinite twin peaks, one of which may have been due to the acid sodium salt. Phosphoric (*cf.* ref. 7) and quinic acids and the amino acids, L-aspartic and L-glutamic were not eluted before fraction 239. As observed previously under similar though not identical conditions[7,8] the acids eluted early in the sequence gave sharp, symmetrical peaks (*aiguilles*) whereas those eluted late were less steep and tended to tail slightly. Thus, in a separation of α-ketoglutaric and D(+)tartaric acids (1 mequiv. each), the former gave a symmetrical peak in which the height/base ratio was 15.5 whereas the corresponding ratio for the latter was 1.8. With certain exceptions, the sequence of elution described above was similar to the order of increase of partition coefficients of organic acids between water and ethyl ether as reported by DERMER AND DERMER[9]. It is of interest that whereas the partition coefficients of L- and DL-malic and D- and *meso*-tartaric acids, respectively, were different, there are no reports of separation of these optical isomers on silica gel columns. BULEN *et al.*[8] drew attention to the effect of development solvent composition on the sequence of elution of acetic, fumaric, pyruvic and glutaric acids. Other publications relevant to the present work are as follows. Automatic determination of acids on a microscale (0.05–3 μequiv. per acid) was described by KESNER AND MUNTWYLER[10]. Thirteen acids were eluted from silica gel with chloroform–*tert.*-amyl alcohol mixtures and the sequence was, in general, similar to that found in the present work and by others with chloroform–butanol mixtures as the development solvent. An equation for calculating the concentration of solvent delivered by a mixing device similar to that used in this work has been reported[11].

ACKNOWLEDGEMENTS

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SUMMARY

The sequence of emergence of 39 plant and related acids from silica gel chromatographic columns by gradient elution with chloroform–*n*-butanol mixtures is described. The interference of nitrate in the determination of several acids and, most importantly, malic and citric is reported. The effect of structural relationships such as chain length, number of carboxyl, hydroxyl or keto groups and *cis* and *trans* isomerisation on the volume of solvent required for elution is discussed.

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# QUANTITATIVE CHROMATOGRAPHIC DETERMINATION OF FUCOSE AS TRITIATED FUCITOL*

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The reduction of the carbonyl group of reducing sugars to the corresponding sugar alcohols with sodium borohydride has been advantageously used in carbohydrate chemistry. It seemed possible that this reaction might be adapted to the determination of reducing sugar in ultramicro quantity by employing tritium-labeled sodium borohydride to introduce tritium into the corresponding alcohol. We have applied this principle to the estimation of L-fucose, a sugar of biochemical and immunological interest for which present analytical methods are relatively insensitive and unprecise. Fucose and hexoses, with ribose added as an internal standard, were reduced with tritium-labeled sodium borohydride, and the labeled reduction products were separated by thin-layer chromatography. The amount of radioactivity fixed in the sugar alcohols was proportional to the molar amounts of the sugars used. The method has been applied to the estimation of fucose content of orosomucoid. The procedure as described requires 0.3–1.6 $\mu$g of the sugar, but much greater sensitivities are possible.

## MATERIALS AND METHODS

Sugar preparations (L-fucose, D-ribose, D-galactose, D-mannose, L-fucitol, D-ribitol, D-galactitol and D-mannitol) were analyzed reagents obtained from Mann Research Laboratories.

To 10 mC of tritium-labeled sodium borohydride (purchased from New England Nuclear Corporation) was added enough recrystallized non-radioactive sodium borohydride to bring the total to 1 mmole. This was dissolved in 10 ml of 0.01 *N* NaOH. One ml aliquots containing 1 mC of tritium, 0.1 mmole of sodium borohydride and 0.01 mmole of NaOH were lyophilized in test tubes, and stored until used.

Shortly before use, 1 ml of water was added to a test tube containing the radioactive sodium borohydride. 10 $\mu$l of this solution was added to a mixture of fucose (0.02–0.1 $\mu$mole) and ribose (0.02–0.1 $\mu$mole) in 20 $\mu$l of water. After incubation overnight at room temperature, one drop of glacial acetic acid was added to destroy the excess borohydride, and the mixture was lyophilized.

The sugar alcohols were separated by thin layer chromatography on Kieselguhr. Twenty grams of Kieselguhr containing $CaSO_4$ as a binder was mixed with 40 ml of

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0.1 *M* phosphate buffer (pH 5.7) in a mortar. A layer 0.25 mm thick was applied with an applicator to 20 × 20 cm plates. The plates were dried at room temperature for 15 min and were then kept in an oven at 105° for 2 h and stored in a desiccator until used.

The solvent system utilized for separation of the sugar alcohols was butanol–pyridine–water (6:4:3 by volume)[1].

The lyophilized samples were dissolved in 10 $\mu$l of water, and 1 $\mu$l was spotted on the plate. When the solvent had ascended to the top edge of the plate (about 3.5 h), the plates were dried overnight at room temperature. For the qualitative localization of sugars and sugar alcohols, sodium meta-periodate and benzidine[2] were used as detection reagents.

The location of radioactivity in the chromatograms was determined by cutting the dried, unstained Kieselguhr into forty, 5 mm sections. Each section was scraped off the plate and transferred into liquid scintillation counting vials. One-half ml of water was added, followed by 10 ml of the scintillation medium[3]. The samples were counted for 10 min on a model 7000B Nuclear-Chicago liquid scintillation counter.

*Estimation of the fucose content of orosomucoid*

Human orosomucoid (1–2 mg) prepared as described by BEZKOROVAINY AND WINZLER[4], was hydrolyzed with Dowex 50W-X8 resin (200–400 mesh, $H^+$ form) at 100° for 24 h[5]. The resin and hydrolyzate were separated by filtration and washing through a sintered glass filter. The filtrates were transferred to a 50 ml Erlenmeyer flask, and the hydrolyzate neutralized with Dowex 2-X8 (200–400 mesh, bicarbonate form)[6]. The resin was removed by filtration and rinsed with three 1 ml aliquots of water. The combined filtrate and washings were lyophilized. The residue was transferred quantitatively to a small test tube (1 × 7.5 cm) using 0.5 ml of water, relyophilized, and the lyophilized residue dissolved in 10 $\mu$l of water. Ten $\mu$l of ribose (0.1 $\mu$mole) was added as an internal standard followed by 10 $\mu$l of the tritium-labeled borohydride (1 $\mu$mole, 10 $\mu$C). The remainder of the procedure was as already described.

## RESULTS

Fig. 1 shows the chromatographic separation of ribose, galactose, mannose and fucose and for these same compounds reduced with sodium borohydride as described in the text; *i.e.*, ribitol, galactitol, mannitol and fucitol. Fucitol and ribitol are well separated from each other and from the hexitols, but galactitol is not separated from mannitol in this chromatographic system. The same results were found when the quantitative distribution of radioactivity was studied following reduction of the sugars with tritium-labeled sodium borohydride. As is shown in Fig. 2, three radioactive peaks are found when fucose and ribose are reduced with tritium-labeled borohydride and chromatographed. Two have the $R_F$ values of ribitol and fucitol, while another unidentified component moves with the solvent front.

Various amounts of ribose (0.02–0.1 $\mu$mole) were reduced with tritium-labeled sodium borohydride in separate tubes, and one-tenth of the reduced sugar alcohol in each tube was applied to the chromatographic plates. The sum of radioactivity in the ribitol peak plotted against the amount of ribose added gives the linear relationship

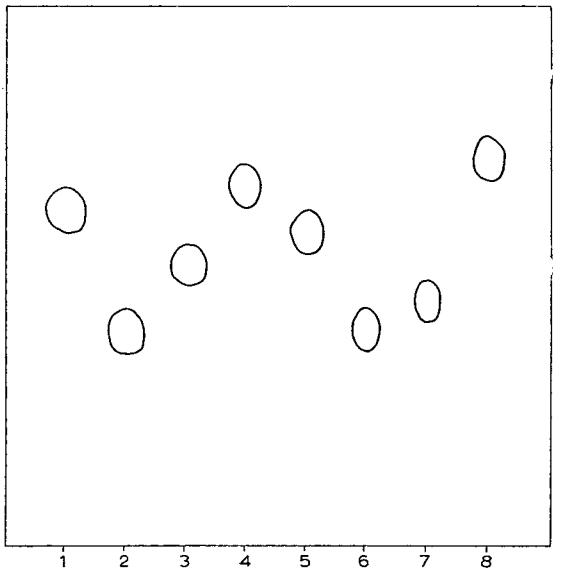


Fig. 1. Thin-layer chromatogram of the sugars and sugar alcohols. The circled spots correspond to: 1 = ribose ($R_F$ 0.57); 2 = galactose ($R_F$ 0.32); 3 = mannose ($R_F$ 0.46); 4 = fucose ($R_F$ 0.62); 5 = ribitol ($R_F$ 0.53); 6 = galactitol ($R_F$ 0.32); 7 = mannitol ($R_F$ 0.38); 8 = fucitol ( $R_F$ 0.68).

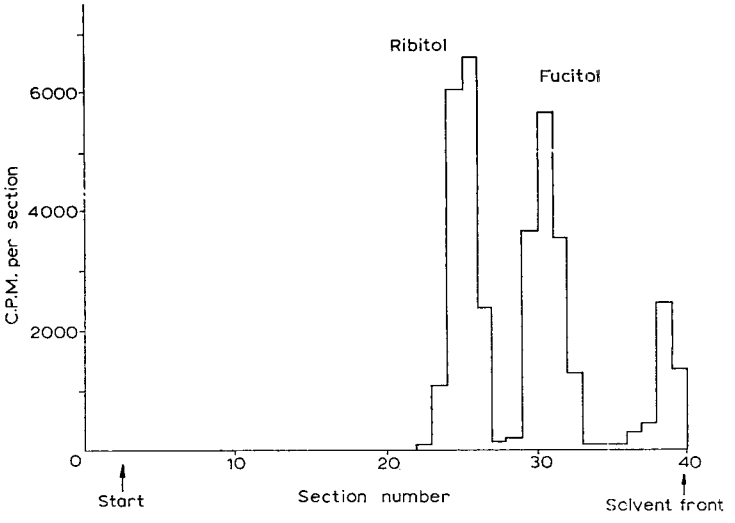


Fig. 2. Separation of radioactive fucitol and ribitol by thin-layer chromatography. In this experiment a mixture of fucose and ribose (0.1 $\mu$mole each) was reduced by the tritium-labeled sodium borohydride (1 $\mu$mole, 10 $\mu$C) and one-tenth of the amount was applied to the chromatographic plate. The plate was cut into 0.5 cm sections and the radioactivity in each section determined.

shown in Fig. 3. Similar linearity was found with fucose. For practical utilization of this procedure for fucose determination, it was felt that addition of a fixed quantity of ribose to a sample prior to reduction would serve as an internal standard. Fig. 4 shows the ratio of counts fixed in the fucitol component to those in ribitol at various amounts of fucose (0.002–0.01 $\mu$mole) and a fixed amount of ribose (0.01 $\mu$mole).

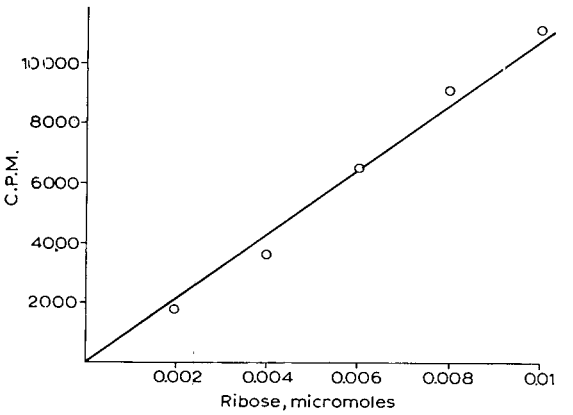


Fig. 3. Linear relationship between increasing concentrations of ribose and the counts per minute fixed in ribitol.

As is shown in Fig. 4 the counts fixed in fucitol are slightly lower than those of ribitol reduced from an equimolar amount of ribose. In 25 experiments the counts fixed in fucitol were 88.7 $\pm$ 4.2 % of those fixed in an equimolar amount of ribose. This difference persisted with samples of fucose and ribose recrystallized several times and dried to constant weight. The reason for this difference is not apparent.

Routinely a period of 16 h has been used for the reduction with borohydride. That this is an adequate time is evident from Fig. 5 which shows that the reduction of fucose and ribose is completed within 1 h under the conditions employed. It is evident that fucose is reduced somewhat more slowly than ribose but that both are fully reduced in 1 h.

The procedure outlined has been applied to the determination of the fucose content of human orosomucoid. Results given in Fig. 6 show that the radioactivity in the fucitol and ribitol peaks are separated from the large unresolved radioactive peak, consisting of galactitol and mannitol. The fucose content of the glycoprotein was calculated from the relationship:

$$\text{fucose } (\mu\text{mole}) = \text{ribose } (\mu\text{mole}) \times \frac{\text{c.p.m. in fucitol}}{\text{c.p.m. in ribitol}} \times \frac{100}{88.7}$$

$$\text{fucose } (\%) = \frac{\text{fucose } (\mu\text{mole}) \times 164 \times 100}{\text{orosomucoid } (\mu\text{g})}$$

The average fucose content of human orosomucoid in 5 determinations by the present method was 0.66 % $\pm$ 0.06 S.D. Previous data on the fucose content of

orosomucoid determined by the method of DISCHE AND SHETTLES[7] have varied from 0.7 to 1.5 %[4,8–13].

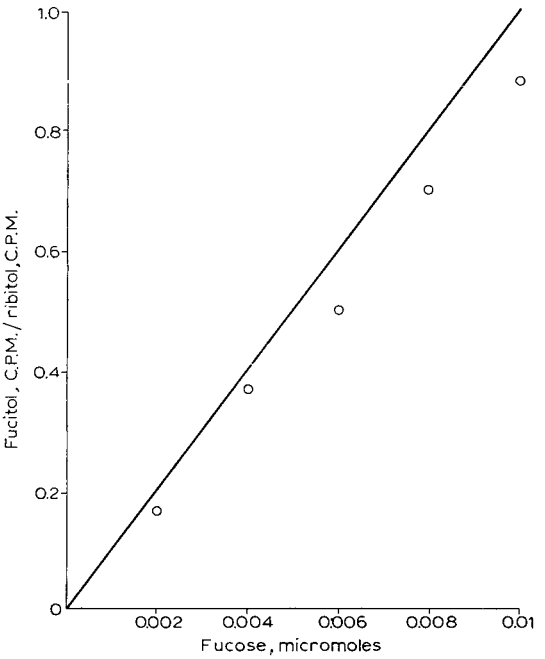


Fig. 4. Ribose as an "internal standard" for fucose determination. A constant amount of ribose (0.1 μmole) was mixed with varying amounts of fucose (0.02–0.1 μmole) and the mixture reduced. One-tenth of the amount was chromatographed and counted as described in the text. The solid line results from the assumption that fucose and ribose fix the same number of counts per mole. The points are experimental.

## DISCUSSION

The procedure described here is potentially adaptable to give much greater sensitivities by utilizing borohydride of higher specific activity, and the chromatography of a larger proportion of the sample. The procedure is also adaptable to any sugar which can be chromatographically separated after reduction to an alcohol. Another application of the procedure is its adaptation as an exceedingly sensitive method for determination of reducing end groups in oligosaccharides and polysaccharides.

It would be expected that the same amount of tritium should be fixed into the sugar alcohols prepared from equimolar amounts of sugars. The present results, however, showed that counts in fucitol were always lower than those in equimolar amounts of ribitol. Possible causes of this discrepancy include weighing errors, presence of impurities or moisture, incomplete reduction of fucose by the tritiated sodium borohydride, a non-reductive exchange between tritium of the sodium borohydride and hydrogen atoms of the sugar alcohol which was greater with ribose, and differences

between quenching rates associated with the liquid scintillation counting of the radioactive sugar alcohols. Each of these possibilities was investigated and was eliminated. The ribose and fucose samples used in the present experiments were prepared several times in separate weighings with similar results.

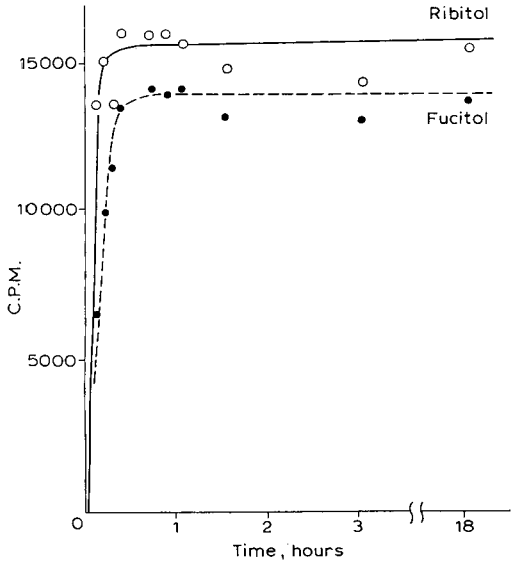


Fig. 5. Effect of time on reduction of fucose and ribose (0.01 $\mu$mole of each).

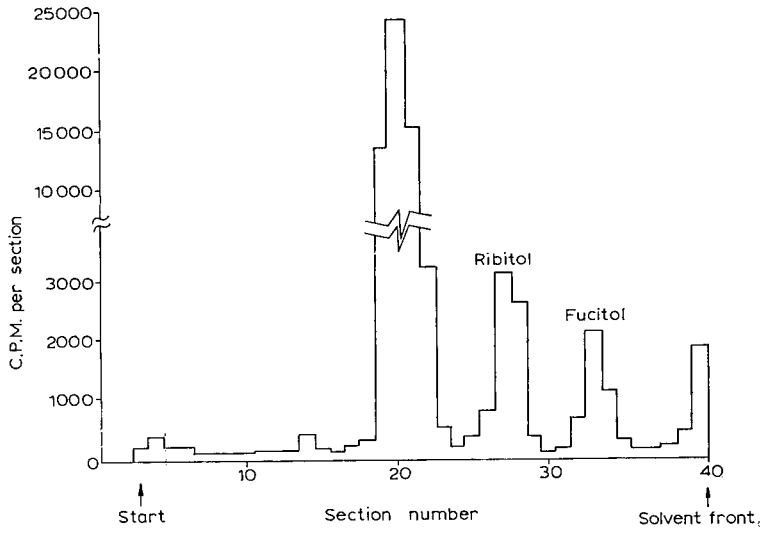


Fig. 6. Separation of radioactive neutral sugar alcohols in reduced hydrolysate of 1.6 mg of orosomucoid. Ribose (0.1 $\mu$mole) was added as an internal standard prior to reduction. One-twentieth of the reduction product was chromatographed and counted.

Ribose and fucose were recrystallized from hot ethanol and the crystallized sugars were dried *in vacuo* over calcium chloride at room temperature to a constant weight. The experiments using the recrystallized preparations gave the same results as those obtained by using the commercial preparations of the highest quality. Using gas–liquid chromatography of the trimethyl silyl derivatives no carbohydrate impurity could be detected in the recrystallized fucose and ribose.

Ribose and fucose were reduced with non-radioactive sodium borohydride under precisely the conditions described. Tritium-labeled sodium borohydride was then added to the reduced sugar mixture. No radioactivity was fixed in the sugar alcohols. It thus can be concluded that no exchange reaction occurred between tritium of the sodium borohydride and hydrogen of the sugar alcohols. It should be emphasized that reduction of the sugars with sodium borohydride was quantitative, since no radioactivity was found in the sugar alcohols previously reduced by the addition of non-radioactive sodium borohydride. Counting efficiencies of the samples determined by the channel ratio method[14,15] showed no differences between ribitol and fucitol.

The cause of the lower incorporation of tritium into fucitol is thus not clear. It is possible that there are steric differences which favor fucose reduction by the non-radioactive borohydride. Another possibility is that fucose is less stable than ribose leading to some destruction during reduction with borohydride.

The relationships are sufficiently constant, however, that the procedure can be applied to the determination of very small amounts of fucose.

SUMMARY

A procedure has been described which provides a highly sensitive method for fucose estimation. Fucose with ribose added as an internal standard, was reduced with tritium-labeled sodium borohydride, and the resulting radioactive sugar alcohols, fucitol and ribitol, were separated by thin-layer chromatography on Kieselguhr. Tritium was fixed into the sugar alcohols in proportion to amounts of sugars added. This method was applied to estimation of fucose content of human orosomucoid. The procedure as described requires about 0.3–1.6 $\mu$g of fucose, and is capable of much greater sensitivity.

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# ZUR CHROMATOGRAPHIE EINIGER 1-DIMETHYLAMINO-NAPHTHALIN-5-SULFONYL-DERIVATE AUF KIESELGEL G-SCHICHTEN

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(Eingegangen den 7. Oktober 1966)

In früheren Arbeiten haben wir Laufmittelgemische mitgeteilt, welche einerseits die nahezu vollständige Trennung der in einem Proteinhydrolysat vorkommenden Aminosäuren auf einem zweidimensionalen Dünnschichtchromatogramm[1], andererseits die Auftrennung einer grossen Reihe biologisch bedeutsamer Amine in Form ihrer 1-Dimethylamino-naphthalin-5-sulfonyl- (DANS-)Derivate***,[4] gestatten. Für die Trennung von DANS-Aminosäuren auf Dünnschichtchromatogrammen wurden inzwischen auch von anderen Autoren Lösungsmittelgemische mitgeteilt[5,6].

Die Beobachtung von NEADLE UND POLLIT[7] zeigte, dass DANS-Aminosäuren durch überschüssiges DANS-Cl in DANS-$NH_2$, CO und den entsprechenden Aldehyd gespalten werden. Nach unseren Versuchen lassen sich nahezu beliebige DANS-Aminosäuren durch einen genügenden Überschuss an DANS-Cl-Reagens in dieser Weise vollständig fragmentieren. Somit ist die quantitative Bestimmung von freien Aminosäuren in Form ihrer DANS-Derivate problematisch. Die Umsetzung von Aminen mit DANS-Cl verläuft hingegen nach unseren bisherigen Erfahrungen im allgemeinen ohne störende Nebenreaktionen. Unter günstigen Bedingungen ist daher die Bestimmung eines primären oder sekundären Amins noch in einer Menge von $5 \cdot 10^{-12}$ Mol Amin/Fleck durch direkte Auswertung der Dünnschichtchromatogramme möglich[8].

Die grosse Anzahl der Amine, die in biologischem Material nach Umsetzung mit DANS-Cl häufig nachzuweisen ist, gestattet es nur in Ausnahmefällen eine quantitative Bestimmung der Amine nach ihrer Auftrennung mit den früher von uns mitgeteilten Lösungsmittelgemischen auf den Chromatogrammen auszuführen. Müssen infolge sehr geringer Aminkonzentrationen grössere Gewebemengen aufgearbeitet werden, so ist häufig eine Vortrennung erforderlich. Vorallem die hohen Konzentrationen an Ammoniak sowie an Spermidin und Spermin in fast allen Geweben, machen sich recht störend bemerkbar.

Um das Auffinden und die Identifizierung biogener Amine zu erleichtern, waren wir einerseits bestrebt die früher erarbeitete Fleckenkarte[4] zu ergänzen und anderer-

* Unter Mitarbeit von Frau J. WIECHMANN.
** Leiter: Priv.-Doz. Dr. G. WERNER
*** DANS steht im folgenden für 1-Dimethylamino-naphthalin-5-sulfonyl-. Um Missverständnisse zu vermeiden möchten wir diese Abkürzung, die ursprünglich auch von HARTLEY benutzt wurde, vor einigen Jahren jedoch durch die Kurzbezeichnung DNS ersetzt wurde (vgl. z.B. Lit. 2), auch weiterhin beibehalten, denn im deutschen Schrifttum ist DNS für die abkürzende Bezeichnung von Desoxyribonucleinsäuren eingeführt. Leider ist die HARTLEY'sche Abkürzung bereits auch in der deutschsprachigen Literatur verwendet worden[3].

seits Trennsysteme zu finden, welche die Lösung auch spezieller Probleme im Zusammenhang mit der Biochemie der Aminosäuren und der Amine gestatten.

### DIE TRENNUNG VON DANS-VALIN UND -LEUCIN BZW. DER DISUBSTITUIERTEN DANS-DERIVATE VON TYROSIN, LYSIN UND ORNITHIN

In dem einen von uns früher angegebenen Laufmittelsystem trennten sich die DANS-Derivate der disubstituierten Aminosäuren Tyrosin, Lysin und Ornithin einerseits, sowie Valin und Leucin andererseits, nicht. Im anderen System waren DANS-Leucin, -Isoleucin, -Phenylalanin sowie Di-DANS-Tyrosin, -Lysin und -Ornithin auf sehr nahe benachbarten Punkten der Chromatogramme angeordnet[1].

TABELLE I

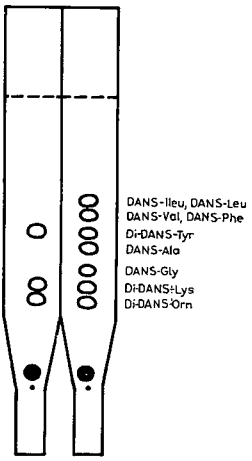
ZUSAMMENSETZUNG DER LAUFMITTELGEMISCHE, DEREN ANWENDUNG IN DER VORLIEGENDEN ARBEIT BESCHRIEBEN WIRD

Die Zahlenangaben sind Volumeneinheiten.

| *Laufmittel Nr.* | *Zusammensetzung* |
|---|---|
| I | Chloroform–Essigsäure–Wasser (50 : 45 : 5) |
| II | Methylacetat–Isopropanol–25%ig. Ammoniaklösung (45 : 35 : 20) |
| III | Äthylacetat–Chloroform–Methanol–Essigsäure (50 : 30 : 20 : 1) |
| IV | Chloroform–Methanol–Essigsäure (75 : 20 : 5) |
| V | Äthylacetat–Chloroform–Äthanol–Essigsäure (70 : 45 : 4 : 4) |
| VI | Äthylacetat–Cyclohexan (75 : 50) |
| VII | Benzol–Cyclohexan–Methanol (85 : 10 : 5) |
| VIII | Benzol–Triäthylamin (100 : 20) |
| IX | Cyclohexan–Butylacetat (80 : 30) |
| X | Tetrachlormethan–Triäthylamin (100 : 20) |
| XI | Chloroform–Butylacetat (100 : 20) |
| XII | Diisopropyläther |
| XIII | Diisopropyläther–Triäthylamin (100 : 20) |
| XIV | Chloroform |
| XV | Butylacetat–Cyclohexan–Äthylacetat–Triäthylamin (55 : 50 : 20 : 20) |
| XVI | Butylacetat–Triäthylamin (100 : 20) |
| XVII | Triäthylamin–Diisopropyläther (100 : 20) |
| XVIII | Äthylacetat–Butylacetat (100 : 20) |
| XIX | Benzol–Methanol (90 : 10) |
| XX | Chloroform–Triäthylamin (100 : 20) |
| XXI | Tetrachlormethan–Methanol (90 : 6) |
| XXII | Chloroform–Triäthylamin (100 : 10) |
| XXIII | Trichloräthylen–Methanol (95 : 5) |
| XXIV | Benzol–Methanol (95 : 5) |

Zur Trennung von DANS-Val und -Leu, sowie von Di-DANS-Tyr, -Lys und -Orn auf 250 $\mu$ Kieselgel G-Schichten erwies sich das Lösungsmittelgemisch I (Tabelle I) als geeignet, wie aus Fig. 1 hervorgeht. Kratzt man also aus dem zweidimensionalen Chromatogramm eines DANS-Aminosäurengemisches, welches mit den Lösungsmitteln II in der ersten und mit dem Gemisch III in der zweiten Laufrichtung entwickelt wurde (Einzelheiten der chromatographischen Bedingungen vgl.[1]), den Fleck mit DANS-Leu und -Val einerseits und den Fleck mit den disubstituierten Derivaten andererseits aus, und eluiert man die DANS-Aminosäuren aus dem Kieselgel mit

Methanol, so erhält man durch eindimensionale Trennung dieser Extrakte mit dem Trenngemisch I eine vollständige Aufklärung über die Aminosäurenzusammensetzung z.B. eines Proteinhydrolysates. Wählte man zur Trennung des Aminosäurengemisches in zweiter Phase das Laufmittel IV, so kann man das im Chromatogramm mit grösstem $R_F$-Wert laufende Aminosäurengemisch, welches DANS-Leu, -Ileu, -Val, -Phe und Di-DANS-Tyr, -Lys und -Orn enthält durch Kombination des Laufmittels III (in



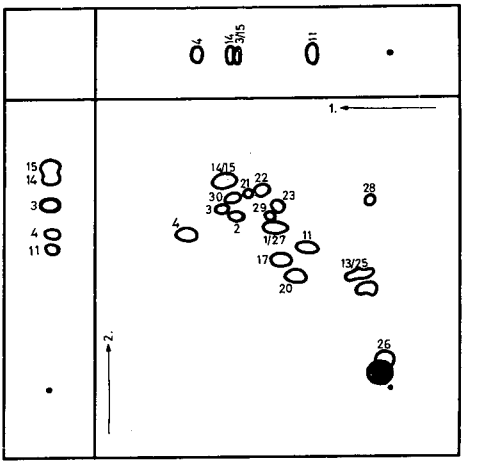




Fig. 1. Eindimensionale Dünnschichtchromatographie einiger DANS-Aminosäuren. Trennung von Di-DANS-Tyrosin, Di-DANS-Lysin und Di-DANS-Ornithin. Der schwarze Fleck entspricht DANS-OH. Laufmittel: Chloroform–Essigsäure–Wasser (50:45:5, V/V). 250 $\mu$ Kieselgel G-Schicht.

Fig. 2. Zweidimensionales Dünnschichtchromatogramm. Abtrennung von Di-DANS-Tyrsosin, Di-DANS-Lysin und Di-DANS-Ornithin von den übrigen DANS-Aminosäuren. 1. Laufrichtung: Äthylacetat–Chloroform–Äthanol–Essigsäure (70:45:4:4, V/V); 2. Laufrichtung: Chloroform–Essigsäure–Wasser (50:45:5, V/V). 1 = Ala; 3 = GABA; 4 = Ammoniak; 11 = Gly; 13 = HO-Pro; 14 = Ileu; 15 = Leu; 17 = Di-Lys; 20 = Di-Orn; 21 = Phe; 22 = Pro; 23 = Sar; 25 = Tau; 26 = Thre; 27 = Try; 28 = Mono-O-Tyr; 29 = Di-Tyr; 30 = Val. Die übrigen Aminosäuren laufen zusammen mit DANS-OH (schwarzer Fleck).

erster Phase) und des Gemisches I (in zweiter Phase) bis auf -Leu und -Ileu, welche nur unvollständig getrennt werden, in seiner Zusammensetzung aufklären. Als noch günstiger erwies sich die Anwendung des Gemisches V in der ersten Laufrichtung in Kombination mit dem Gemisch I in der zweiten Laufrichtung (Fig. 2). In diesem Falle gelingt es sogar in einem vollständigen Aminosäurengemisch Di-DANS-Lys und -Orn eindeutig, Di-DANS-Tyr allerdings nur unter günstigen Umständen sicher nachzuweisen, da letzteres zwischen Sarkosin, Tryptophan und Alanin läuft (vgl. Fig. 2). DANS-Phe und -Pro sind nicht immer sauber getrennt, -Leu und -Ileu laufen in diesem System gemeinsam.

Verwendet man die Laufmittelkombination V/I, so ist eine Aktivierung der Kieselgel-Schicht zwischen den einzelnen Läufen unnötig, denn die Trennung durch Gemisch I erfolgt, wie in der Papierchromatographie, in erster Linie durch Verteilung der Substanzen zwischen einer stationären und einer mobilen Phase, während in der Chromatographie mit den von uns sonst benutzten Lösungsmitteln die Adsorption an das Kieselgel für die Trennung wesentlich ist.

TRENNUNG DER DANS-DERIVATE BIOLOGISCH BEDEUTSAMER AMINE MIT HILFE DER LÖSUNGSMITTELKOMBINATION VI/VIII

Als recht allgemein anwendbar erwies sich die Kombination der Lösungsmittelgemische VI und VIII zur Trennung einer Reihe von DANS-Derivaten biologisch bedeutsamer Amine. Wir waren daher bestrebt die früher publizierte Fleckenkarte so weit als möglich zu ergänzen.

TABELLE II

SUBSTANZEN, DIE IN FORM IHRER DANS-DERIVATE AUF IHR CHROMATOGRAPHISCHES VERHALTEN GEPRÜFT WURDEN

Die Numerierung in dieser Tabelle entspricht der Numerierung der Substanzflecke in den Fig. 3–10.

| *Nr.* | *Amin bzw. Phenol* | *Nr.* | *Amin bzw. Phenol* |
|---|---|---|---|
| 1 | Ammoniak | 36 | 3,4-Dimethoxy-β-phenäthylamin |
| 2 | Methylamin | 37 | α-Methyl-dopamin |
| 3 | Dimethylamin | 38 | Noradrenalin |
| 4 | Äthylamin | 39 | 3-O-Methyl-noradrenalin (Normetanephrin) |
| 5 | Diäthylamin | | |
| 6 | Äthanolamin | 40 | α-Methyl-noradrenalin |
| 7 | N-Methyl-äthanolamin | 41 | Adrenalin |
| 8 | 2-Mercapto-äthylamin (Cysteamin) | 42 | 3-O-Methyl-adrenalin (Metanephrin) |
| 9 | 2,2'-Dithio-bis-(äthylamin) (Cystamin) | 43 | N-Methyl-adrenalin |
| | | 44 | N-Methyl-metanephrin |
| 10 | *n*-Propylamin | 45 | α-Methyl-adrenalin |
| 11 | *n*-Butylamin | 46 | Isoproterenol |
| 12 | Isobutylamin | 47 | 2,4-Dihydroxy-5-methoxy-β-phenäthylamin |
| 13 | Isoamylamin | | |
| 14 | Pyrrolidin | 48 | 2,3,4-Trimethoxy-β-phenäthylamin |
| 15 | *n*-Hexylamin | 49 | 3,4,5-Trimethoxy-β-phenäthylamin (Mezcalin) |
| 16 | Piperidin | | |
| 17 | 3-Hydroxy-piperidin | 50 | 2,4,5-Trimethoxy-β-phenäthylamin |
| 18 | Äthylendiamin | 51 | Amphetamin |
| 19 | Trimethylendiamin | 52 | Pervitin |
| 20 | Tetramethylendiamin | 53 | Ephedrin |
| 21 | Pentamethylendiamin | 54 | Pseudoephedrin |
| 22 | Hexamethylendiamin | 55 | Nor-pseudoephedrin (Catin) |
| 23 | Spermidin | 56 | 3,4-Dihydroxy-phenyl-äthanol |
| 24 | Spermin | 57 | 3,4-Dihydroxy-phenylglykol |
| 25 | β-Phenäthylamin | 58 | 3-Methoxy-4-hydroxy-phenylglykol |
| 26 | β-Hydroxy-β-phenyläthylamin | 59 | Adrenalon |
| 27 | 4-Hydroxy-β-phenäthylamin (Tyramin) | 60 | Tryptamin |
| 28 | N-Acetyl-tyramin | 61 | N-Methyl-tryptamin |
| 29 | Hordenin | 62 | 5-Hydroxy-tryptamin (Serotonin) |
| 30 | Octopamin | 63 | N-Acetyl serotonin |
| 31 | 4-Methoxy-β-phenäthylamin | 64 | Bufotenin |
| 32 | 3-Methoxy-β-phenäthylamin | 65 | 5-Methoxy-tryptamin |
| 33 | 3,4-Dihydroxy-β-phenäthylamin (Dopamin) | 66 | N-Methyl-5-methoxy-tryptamin |
| | | 67 | Imidazol |
| 34 | N-Acetyl-dopamin | 68 | Histamin |
| 35 | 3-Methoxy-4-hydroxy-β-phenäthylamin | 69 | N-Acetyl-histamin [4-(β-Acetaminoäthyl)-imidazol] |
| | | 70 | 1-Methyl-4-histamin |
| | | 71 | Pyridoxamin |

Die Amine wurden nach unseren früheren Angaben[8] umgesetzt und chromatographiert[4]. Die Fig. 3 gibt ein Chromatogramm aus einem Gemisch der DANS-Amide der Tabelle II wieder. (Die Fleckennumerierung in dieser und allen folgenden Abbil-

dungen bezieht sich auf die Kennziffern der Substanzen in Tabelle II.) In die Tabelle II sind nicht nur physiologischerweise vorkommende Substanzen aufgenommen worden, sondern in geringem Umfang auch solche, die den natürlichen β-Phenäthylaminen nahe verwandt sind und von pharmakologischem oder theoretischem Interesse sind.

Es ist selbstverständlich, dass ein so komplexes Gemisch z.T. nahe verwandter Verbindungen nicht in einem einzigen zweidimensionalen Chromatogramm vollständig aufgetrennt werden kann. Welche analytischen Probleme mit Hilfe der Dünnschichtchromatographie der DANS-Amide zu lösen sind, lehrt aber ohne weiteres ein Blick auf die Fig. 3.

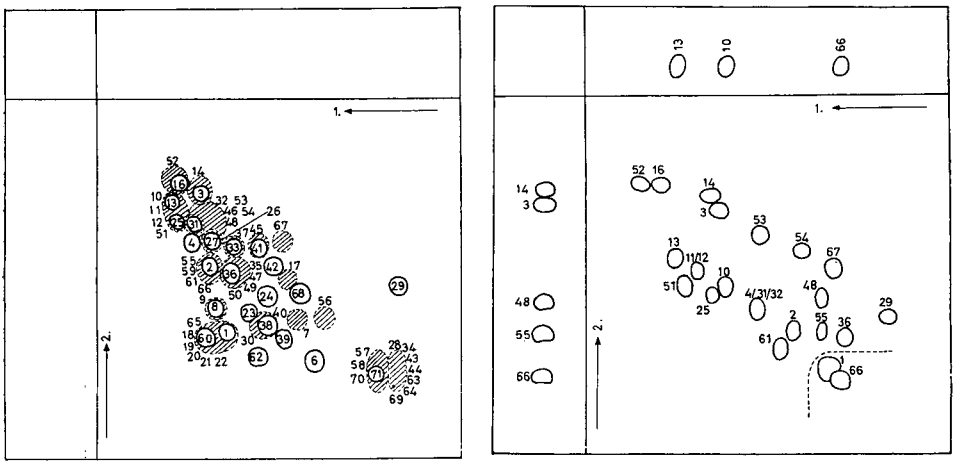



Fig. 3. Zweidimensionales Dünnschichtchromatogramm der DANS-Derivate der Substanzen in Tabelle II. Die dick umrandeten Zonen geben die Lage der in der früheren Mitteilung[4] chromatographierten DANS-Amide wieder. Die schraffierten Flächen entsprechen der Lage der Flecken der übrigen in Tabelle II aufgeführten Verbindungen. 1. Laufrichtung: Äthylacetat–Cyclohexan (75:50, V/V); 2. Laufrichtung: Benzol–Triäthylamin (100:20, V/V).

Fig. 4. Zweidimensionales Dünnschichtchromatogramm. Trennung der DANS-Derivate aliphatischer Amine und im Benzolkern nicht substituierter β-Phenäthylamine. 1. Laufrichtung: Cyclohexan–Butylacetat (80 + 30, V/V); 2. Laufrichtung: Tetrachlormethan–Triäthylamin (100 + 20, V/V) (zweimalige Chromatographie in jeder Richtung). Die in dem Chromatogramm nicht aufgeführten Substanzen der Tabelle II laufen innerhalb der umgrenzten Zone in Startnähe.

## TRENNUNG DER DANS-DERIVATIVE ALIPHATISCHER AMINE UND EINIGER IN WENIG POLAREN LÖSUNGSMITTELN CHROMATOGRAPHIERBARER DANS-AMIDE

Die DANS-Derivate der aliphatischen Amine sowie eine Reihe zumeist im Benzolkern nicht substituierte Abkömmlinge des β-Phenäthylamins laufen in den früher beschriebenen Laufmitteln VI–VIII (Tabelle I) mit wenig Ausnahmen ziemlich rasch[4]. Es ergibt sich daraus, dass diese Gruppe von Aminen auf Kieselgel G durch relativ unpolare Lösungsmittelgemische zum Wandern gebracht werden kann, was ihre Abtrennung von den übrigen Aminen und damit ihre bessere Identifizierung und Bestimmung neben einer Vielzahl anderer Amine, Aminosäuren, Phenolcarbonsäuren usw. ermöglicht.

Für die Trennung der aliphatischen Amine in Form ihrer DANS-Derivate fanden wir die Laufmittel IX und X am vorteilhaftesten. In der Fig. 4 ist das zwei-

dimensionale Chromatogramm dargestellt, das man nach Trennung der Substanzen der Tabelle II durch kombinierte Anwendung der beiden Laufmittel erhält. Man chromatographiert zweimal in der gleichen Richtung über eine Strecke von 13 cm zunächst mit dem Laufmittelgemisch IX und dann senkrecht dazu ebenfalls zweimal mit dem frisch angesetzten Gemisch X. Die Verwendung von aktivierten Platten ist zu empfehlen, zwischen den einzelnen Läufen ist jedoch eine Reaktivierung der Schicht nicht erforderlich. Die in der Fig. 4 umrandete Zone um den Startfleck wird von dem Grossteil der in der Tabelle II aufgeführten DANS-Amiden eingenommen. In Startnähe laufende Substanzen können daher in diesem Lösungsmittelsystem im allgemeinen nicht identifiziert werden. Einen zweiten Weg für die Trennung der im System VI/VII und VI/VIII rasch laufenden DANS-Amide fanden wir in der aufeinanderfolgenden Anwendung der Lösungsmittel XI, XII und XIII. Man chromatographiert zunächst über eine Strecke von 15 cm mit dem Gemisch XI, dann senkrecht zur ersten Laufrichtung je zweimal mit Diisopropyläther (XII) und noch weiter zweimal in der gleichen Richtung mit dem Gemisch XIII. Die Reaktivierung der Platten ist auch in diesem Falle nicht nötig. Der Zeitbedarf für diese Trennung beträgt etwa 3.5 Stdn. Ausser für die Auftrennung der aliphatischen Amine ist diese Lösungsmittelkombination insbesondere auch für den Nachweis einiger seltener Tryptaminderivate geeignet, wie aus der Fig. 5 hervorgeht. In Startnähe laufende Substanzen sind naturgemäss auf diesem Chromatogramm nicht zu identifizieren. Auf eine weitere einfache Möglichkeit zur Trennung der DANS-Derivate einiger aliphatischer Amine soll noch kurz hingewiesen werden, da sie zur Identifizierung unbekannter Flecke in manchen Fällen gute Dienste leisten kann: Die Dünnschichtelektrophorese. Wir verwendeten eine Desaga-Dünnschichtelektrophoresekammer. Die Platten wurden in der

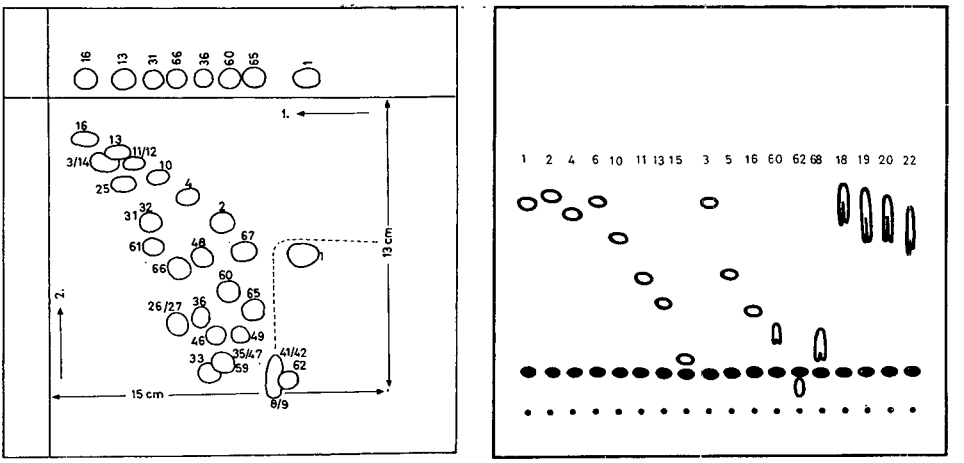



Fig. 5. Zweidimensionales Dünnschichtchromatogramm. Trennung der im System VI/VIII rasch laufenden DANS-Amide. 1. Laufrichtung: Chloroform–Butylacetat (100:20, V/V); 2. Laufrichtung: (a) Diisopropyläther (zweimalige Chromatographie); (b) Diisopropyläther–Triäthylamin (100 + 20, V/V) (zweimalige Chromatographie).

Fig. 6. Dünnschichtelektrophoretische Trennung einiger DANS-Amide. 250 $\mu$ Cellulose-Schicht (MN-Cellulosepulver 300; Macherey, Nagel & Co). Puffer: 120 g Essigsäure + 23.7 g Ameisensäure im Liter; pH 2.1; 480 V, 1.5 Stdn. Desaga Dünnschichtelektrophoresekammer. Schwarze Flecke: DANS-OH.

üblichen Weise[9] mit einer Celluloseschicht (MN-Cellulose 300; Macherey, Nagel & Co., Düren) versehen. Als Puffer diente ein Essigsäure–Ameisensäure–Wasser-Gemisch (120 g Essigsäure + 23.7 g Ameisensäure im Liter) vom pH 2.1. Ebenso geeignet ist 1.5 *M* Propionsäure als Puffer. Nach Anlegen einer Spannung von 480 V (24 V/cm) erhält man nach 1.5 Stdn. eine Trennung der DANS-Amide, wie sie aus der Fig. 6 ersichtlich ist. Infolge der geringen Löslichkeit im Puffer und der Adsorption der DANS-Amide an der Celluloseschicht laufen unter den geschilderten Bedingungen die meisten der in der Tabelle II aufgeführten Substanzen nicht oder nur in geringem Masse unter Schwanzbildung. Lediglich die DANS-Derivate der aliphatischen Amine geben runde Flecke.

Erhöht man die Löslichkeit der DANS-Amide im Puffer durch Zugabe von Alkohol, so bewegen sie sich mit nahezu gleicher Geschwindigkeit, so dass nur sehr unvollständige Trennungen erzielt werden. Auch die Anwendung verschiedener alkalischer Pufferlösungen führte im Falle der DANS-Aminderivate nicht zu befriedigenden Trennungen durch Elektrophorese. Auf Kieselgel G-Schichten sind die Laufstrecken der DANS-Amide für praktische Zwecke etwas zu gering, wenn man den Essigsäure–Ameisensäure-Puffer verwendet. Die erzielten Trennungen beruhen in erster Linie auf der Ausnutzung der geringen Löslichkeit der DANS-Derivate in wässrigem Milieu. Die Fleckenform kann zusätzliche Auskunft über die Art eines Amins geben.

## TRENNUNG EINIGER DANS-BRENZCATECHIN-DERIVATE

Brenzcatechinderivate können z.B. durch Adsorption an Aluminiumoxid von anderen Phenolen und von Aminen abgetrennt werden[10], so dass man sie als einheitliche analytische Gruppe betrachten darf. Ihre Trennung in Form der DANS-Derivate suchten wir so auszuführen, dass gleichzeitig auch die Separierung von ihren zwar physiologischerweise nicht vorkommenden, aber sowohl vom pharmakologischen als auch vom theoretischen Standpunkt aus interessanten α-Methylderivaten möglich wurde.

Wir fanden eine Reihe von Lösungsmittelkombinationen als geeignet zur Lösung dieses Problems. Zwei chromatographische Systeme sollen hier besprochen werden. Eine grössere Auswahl an geeigneten Trenngemischen macht die Identifizierung unbekannter Flecke zuverlässiger und lässt hoffen, dass Substanzen, die wir noch nicht auf ihre chromatographischen Eigenschaften hin haben prüfen können, sich in dem einen oder anderen Trenngemisch von den in die Fleckenkarte bereits aufgenommenen Substanzen werden abtrennen lassen.

In dem einen System chromatographiert man in der ersten Richtung zweimal mit Chloroform (XIV) und in der zweiten Richtung ebenfalls zweimal mit dem Lösungsmittelgemisch XV. Das Ergebnis einer solchen Trennung zeigt die Fig. 7. Da die zweite Lösungsmittelkombination in beiden Gemischen Triäthylamin enthält, chromatographieren wir in diesem Fall in der ersten Richtung zunächst mit XII, um Spuren von nicht verseiftem Säurechlorid zu entfernen und daraufhin noch zweimal in der gleichen Richtung mit dem Trenngemisch XVI. Senkrecht dazu wird das Chromatogramm ebenfalls zweimal mit dem Gemisch XVII entwickelt. (Fig. 8). Zwischen den einzelnen Läufen werden die Chromatogramme jeweils nur 3–4 Min an der Luft getrocknet.

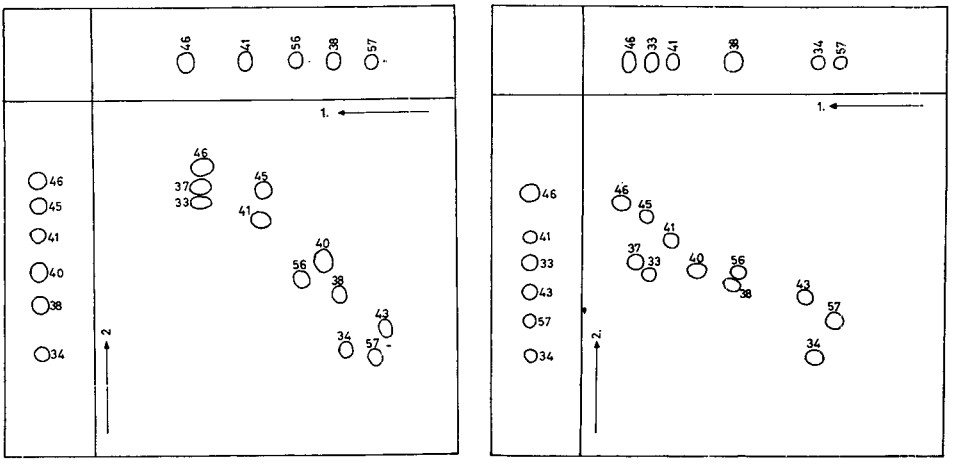



Fig. 7. Zweidimensionales Dünnschichtchromatogramm. Trennung von Brenzcatechinaminen und anderen Brenzcatechinderivaten in Form ihrer DANS-Derivate. 1. Laufrichtung: Chloroform; 2. Laufrichtung: Butylacetat–Cyclohexan–Äthylacetat–Triäthylamin (55:50:20:20, V/V) (zweimalige Chromatographie in jeder Richtung).

Fig. 8. Zweidimensionales Dünnschichtchromatogramm. Trennung von Brenzcatechinaminen und anderen Brenzcatechinderivaten in Form ihrer DANS-Derivate. 1. Laufrichtung: (a) Diisopropyläther; (b) Butylacetat–Triäthylamin (100:20, V/V) (zweimalige Chromatographie); 2. Laufrichtung: Triäthylamin–Diisopropyläther (100 + 20, V/V) (zweimalige Chromatographie).

## TRENNUNG VON N-ACETYLDERIVATEN BIOGENER AMINE SOWIE VON AMINOPHENOLEN MIT TERTIÄREM STICKSTOFF

In allen bisher beschriebenen Laufmitteln wanderten die an der primären Aminogruppe acetylierten Amine, wie N-Acetyl-dopamin, -Tyramin, -Histamin und -Serotonin, sowie die Aminophenole mit einer tertiären Aminogruppe (N-Methyl-adrenalin, Hordenin, Bufotenin usw.) und auch 1-Methyl-histamin nur sehr wenig. Es erschien lohnend, diese langsam wandernde Gruppe von Substanzen von den übrigen Aminen und Phenolen abzutrennen, da ihre Identifizierung in biologischem Material von Bedeutung ist, handelt es sich doch z.T. um sehr bedeutsame Stoffwechselprodukte biogener Amine.

Chromatographiert man das Gemisch der DANS-Derivate der Substanzen, die in der Tabelle II aufgeführt sind, zunächst in einer Richtung bis zum Plattenrand (Laufstrecke 17 cm) mit dem Gemisch XVIII und dann noch zweimal in der gleichen Richtung mit Gemisch XIX, so hat man die Abtrennung sämtlicher Amine von der Gruppe der N-Acetylderivate und der Dimethylamino-phenole erreicht. Nunmehr kann man hinter dem DANS-Äthanolamin-Fleck eine Begrenzungslinie ziehen und nach kurzem Trocknen der Platte bei Raumtemperatur in der zweiten Richtung mit dem Trenngemisch XX entwickeln. Man erhält ein Chromatogramm, wie es in der Fig. 9 dargestellt ist. (In die Fig. 9 sind lediglich die Fleckenlagen der hier interessierenden Amine sowie zur Orientierung die von Ammoniak und Äthanolamin eingezeichnet. Alle übrigen DANS-Amide der Tabelle II befinden sich vor der Begrenzungslinie. Die DANS-Aminosäuren laufen, wie in allen beschriebenen chromatographischen Systemen, unmittelbar in Startnähe, so dass sie die Chromatographie der Amine nicht stören).

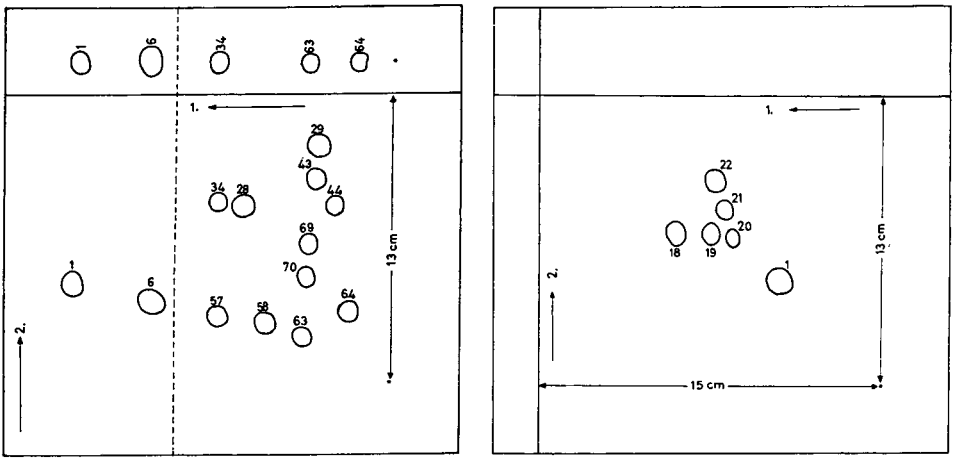



Fig. 9. Zweidimensionales Dünnschichtchromatogramm. Trennung der N-Acetylderivate biogener Amine sowie von Aminophenolen mit tertiärer Aminogruppe. 1. Laufrichtung: (a) Äthylacetat–Butylacetat (100 + 20, V/V); (b) Benzol–Methanol (90 + 10, V/V) (zweimalige Chromatographie); 2. Laufrichtung: Chloroform–Triäthylamin (100 + 20, V/V).

Fig. 10. Zweidimensionales Dünnschichtchromatogramm. Trennung der Di-DANS-Derivate aliphatischer Diamine. 1. Laufrichtung: Tetrachlormethan–Methanol (90 + 6, V/V) (fünfmalige Chromatographie); 2. Laufrichtung: Chloroform–Triäthylamin (100:10, V/V).

## DIE TRENNUNG DER DI-DANS-DERIVATE EINIGER ALIPHATISCHER DIAMINE

In der homologen Reihe der aliphatischen Diamine haben vorallem Putrescin (Tetramethylendiamin) und Cadaverin (Pentamethylendiamin), sowie als struktureller Bestandteil des Spermins und des Spermidins auch Trimethylendiamin, biologische Bedeutung. Diese Amine, Äthylendiamin und Hexamethylendiamin laufen auf einem zweidimensionalen Dünnschichtchromatogramm nach dem Entwickeln mit der Lösungsmittelkombination VI/VII gemeinsam mit Ammoniak; nach der Chromatographie mit den Trenngemischen VI/VIII sind auch noch Tryptamin und 5-Methoxytryptamin auf dem gleichen Fleck (vgl. Fig. 3).

Bisher gelang es nicht Laufmittelgemische zu finden, welche die Abtrennung der DANS-Derivate der Diamine von den übrigen DANS-Amiden der Tabelle II und gleichzeitig die Separierung der Diamine gestatten würden. Trennt man jedoch die DANS-Amide mit Hilfe der Trenngemische VI/VII vor und gewinnt man durch Extraktion des Kieselgels mit Methanol oder einem anderen geeigneten Lösungsmittel[8] das Gemisch der DANS-Derivate des Ammoniaks und der Diamine, so können auch in Gegenwart einer sehr grossen Menge DANS-Amid die Diamine (vom Äthylendiamin bis zum Hexamethylendiamin) auf die folgende Weise vollständig getrennt werden: Man chromatographiert in der ersten Laufrichtung fünfmal mit dem Laufmittelgemisch XXI in der üblichen Weise mit Zwischentrocknung bei Raumtemperatur und dann in der zweiten Richtung einmal mit dem Gemisch XXII. Trotz dieser häufigen Chromatographie erhält man kleine, scharf umgrenzte Flecken. Die Fig. 10 gibt das Ergebnis einer Trennung nach diesem Verfahren wieder. Die beiden erwähnten Tryptamine laufen gemeinsam mit Tetramethylendiamin, so dass ihre Abwesenheit gesichert sein muss. Im Hinblick auf die Bedeutung des Problems scheint uns der nicht

geringe Arbeitsaufwand gerechtfertigt, insbesondere, wenn man auf eine quantitative Bestimmung dieser Amine hinzielt.

Eine für qualitative Zwecke ausreichende Trennung der Diamine (auch in Gegenwart der beiden Tryptamine anwendbar) erzielt man durch jeweils einmalige Chromatographie mit den Trenngemischen XXIII und XXIV in der ersten und mit Gemisch XXII in der zweiten Laufrichtung. Die Güte der Trennung von Äthylendiamin, Trimethylendiamin und Tetramethylendiamin, und insbesondere die Abtrennung von Tryptamin und 5-Methoxy-tryptamin von den Diaminen, hängt in hohem Masse von den chromatographischen Bedingungen, vorallem von der Kammersättigung ab. Chromatographiert man z.B. nur eine Platte in einem Chromatographietank üblicher Grösse, so wird der Fleck mit den beiden Tryptaminen in unmittelbare Nähe des Äthylendiaminflecks versetzt. Es ist daher empfehlenswert in diesem Falle immer zwei Platten gleichzeitig zu entwickeln. Wenig beeinflusst ist die Trennung von Tetra-, Penta- und Hexamethylendiamin. Sie entspricht etwa der in der Fig. 10 dargestellten Fleckenverteilung.

## ALLGEMEINE BEMERKUNGEN ZUR CHROMATOGRAPHIE DER DANS-DERIVATE

Geringfügige Verunreinigungen bestimmter Art in den für die Umsetzung der Amine mit DANS-Cl benutzten Lösungsmitteln und Chemikalien können die Chromatographie u.U. erheblich stören. So reagieren z.B. Methanol, Äthanol und weniger leicht auch höhere Alkohole in Gegenwart von Soda mit DANS-Cl. Der DANS-Methylester läuft in System VI/VIII ähnlich wie DANS-Piperidid. Sollen aus grösseren Gewebemengen Amine durch Extraktion angereichert werden, so muss auf grosse Reinheit der Lösungsmittel geachtet werden. Aus dem Reaktionsansatz verschleppte Alkalispuren können das Ergebnis der Trennung, je nach Trenngemisch, u.U. erheblich beeinflussen. Sie können auch zu Zersetzungsreaktionen der DANS-Derivate Anlass geben. Peroxide (im Äther) sind ebenfalls eine mögliche Ursache für Zersetzungen. Die Trenngemische sollten daher, wie in der Dünnschichtchromatographie meist üblich, im allgemeinen unmittelbar vor dem Gebrauch hergestellt werden. Die in der vorliegenden Arbeit aufgeführten Chromatogramme erhielten wir ausschliesslich durch Chromatographie auf selbstbeschichteten Platten (Kieselgel G der Firma Merck A.G., Darmstadt), wobei auf sorgfältige Beschichtung grosser Wert gelegt wurde, um eine gute Reproduzierbarkeit der Fleckenmuster zu gewährleisten. Diese ist bei genauer Einhaltung der beschriebenen chromatographischen Bedingungen auch ohne weiteres gegeben. Wichtig ist, dass die Platten vor der Chromatographie durch 2 Stdn. langes Erhitzen auf 100° aktiviert werden. Sie sind nach dem Abkühlen auf Raumtemperatur sofort verwendbar. Die Trocknungszeiten von 3–4 Min (bei Raumtemperatur) zwischen den einzelnen Läufen sind möglichst einzuhalten.

Von den von uns bisher geprüften handelsüblichen, fertig beschichteten Dünnschichtplatten, ermöglichte lediglich die Selekta-Platte Nr. 1500 (Kieselgel mit Stärkebinder) der Firma C. Schleicher & Schüll, Dassel, sowie die DC-Fertigplatte "Merck", Kieselgel $F_{254}$ Trennungen mit den beschriebenen Laufmittelgemischen, die mit den Trennungen auf selbstbeschichteten Platten vergleichbar sind. Die leicht erhöhte Laufzeit dieser Platten i. Vgl. zu den selbstbeschichteten Dünnschichtplatten fällt nicht sehr ins Gewicht. Die Fertigplatte der Firma Merck A.G., Darmstadt enthält jedoch den für die Chromatographie der DANS-Derivate entbehrlichen Fluores-

zenzindikator, der die vermutliche Ursache geringer, mit einigen Lösungsmitteln eluierbarer Verunreinigungen ist, welche die quantitative Auswertung der Chromatogramme stören können.

Die Güte der Trennungen wird erheblich durch die Grösse des Startflecks beeinflusst. Es zeigte sich als günstig eine Lösung der DANS-Amide in Benzol auf die Chromatogramme aufzutragen. Auf diese Weise erreicht man eine Adsorption der Substanzen auf geringster Fläche.

Auf den photochemischen Zerfall der DANS-Derivate wurde wiederholt hingewiesen[1,8]. Um Zersetzungen durch Lichteinfluss zu vermeiden ist es ratsam, sämtliche Arbeitsgänge in einem leicht abgedunkelten Raum auszuführen. Im Verlaufe von qualitativen Untersuchungen kann die Güte der Trennung z.B. nach dem Entwickeln der Platte in der ersten Laufrichtung jedoch ohne Schaden durch kurzes Betrachten der Chromatogramme unter der U.V.-Lampe beurteilt werden, längere Belichtungszeiten sind aber zu vermeiden.

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Dr. J. AXELROD, Bethesda, Dr. J. DALY, Bethesda, Dr. L. R. GJESSING, Asker, Dr. H. GROBECKER, Frankfurt/M, Prof. P. KARLSON, Marburg, Dr. G. LEGLER, Bonn, Dr. C. E. SEKERIS, Marburg, Dr. N. MATUSSEK, München, Prof. K. H. SLOTTA, Miami und Prof. R. TSCHESCHE, Bonn, möchten wir herzlich für die uns grosszügig überlassenen, z.T. sehr wertvollen Vergleichssubstanzen, danken.

ZUSAMMENFASSUNG

Es werden Lösungsmittelsysteme für die ein- und zweidimensionale Trennung einer grossen Anzahl 1-Dimethylamino-naphthalin-5-sulfonyl-Derivate biogener Amine auf 250 Kieselgel G-Schichten beschrieben. Spezielle Lösungsmittelsysteme für die Trennung von aliphatischen Aminen, Brenzcatechinderivaten, N-Acetylaminophenolen und Dimethyl-aminophenolen (N-Methyl-adrenalin, Hordenin, Bufotenin usw.) werden mitgeteilt. Einige DANS-Amide konnten auch durch Dünnschichtelektrophorese getrennt werden. Die früher publizierten Trennungen von DANS-Aminosäuren wurden durch neue Lösungsmittelgemische vervollständigt.

SUMMARY

Solvent systems are described for the one- and two-dimensional separation of a large number of 1-Dimethylaminonaphthalene-5-sulphonyl (DANS)-derivatives of biogenic amines on thin layers of Silica Gel G. Special solvent systems are presented for the separation of a number of aliphatic amines, catechols, N-acetylaminophenols and dimethylaminophenols (N-methylepinephrine, Hordenin, Bufotenin etc.). DANS-amides were separated also by thin-layer electrophoresis. The formerly published DANS-amino acid separations have now been completed.

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# DETERMINATION OF LIPIDS IN BIOLOGICAL MATERIALS BY PAPER CHROMATOGRAPHY

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## INTRODUCTION

Although column[1,2] and thin-layer[3] chromatography have been used for the analysis of plant and animal products for lipid and provitamin components, the direct application of paper chromatography to untreated extracts does not appear to have been used extensively. In techniques reported, preliminary removal of interfering substances incurs the risk of losses, and involves time-consuming procedures unsuitable for routine examination of large numbers of samples.

Coloured pigments in marine algae have been separated by paper chromatography[4] and a two-dimensional paper chromatographic method has been employed for the analysis of tocopherols in foods and oils[5] and also in liver and faeces[6].

More recently, the separation of plant leaf pigments by paper chromatography has been described by BOOTH[7], and LICHTENTHALER[8] has developed systems for more rapid examination of plant materials.

Included in the current programme of research in this laboratory are studies on muscular dystrophy in grazing animals, and the definition of vitamin A requirements under drought conditions. For these purposes, more accurate and reliable techniques have been sought for the routine determination of vitamin A, $\beta$-carotene and the tocopherols in plant and animal products.

A technique employing two-dimensional paper chromatography, similar in principle to an earlier method for plants[7], but including minor modifications, has now been applied directly to extracts of sheep feeds and faeces without any preliminary treatment.

This technique has been extended to the quantitative measurement of $\alpha$-tocopherol and $\beta$-carotene in these materials, and also in fresh pastures used by experimental animals.

Rates of decay of $\beta$-carotene and $\alpha$-tocopherol in samples of pasture have also been determined by this method.

## MATERIALS AND METHODS

### *Extraction of samples*

Duplicate samples of freshly-collected feed or faeces, of about 0.3 g, were accurately weighed without previous drying and mixed with 1 g silica gel, 50–100 mesh chromatographic grade.

The prepared samples were then thoroughly stirred with several 5 ml portions of acetone–light petroleum (40–60°), 1:1, until no more colour was extracted. Usually a total of 50 ml of solvent was sufficient.

Each washing was decanted through a small pledget of cotton wool into a suitable flask. The solvent was evaporated under reduced pressure on a warm water bath, thereby eliminating the recommended drip-wash procedure for removing the acetone[7].

*Paper chromatography*

Chromatography papers, Whatman No. 4, were impregnated with zinc carbonate and sodium fluorescein, as recommended by the Analytical Methods Committee[9], and then cut to form a square, of side 28 cm.

The papers were drawn up to contain a starting area, 7 cm × 3 cm in the lower left-hand corner, 3 cm from the edge and 2 cm from the bottom.

The dried extract of feed or faeces was redissolved in the minimum of acetone, three 1 ml portions usually being sufficient, and applied to the starting area with the aid of a current of warm air.

Edges of the paper were stapled or pinned to form a cylinder, which was allowed to stand in a jar containing light petroleum (40–60°)–acetone (98.5:1.5) for development in the first dimension (75–90 min).

After impregnating the unused portion of the paper with medicinal paraffin (3 % in light petroleum) it was developed in a similar manner in the second dimension, using 90 % aqueous methanol (2½ h).

Chromatographic equipment consisted of rectangular specimen jars, 30 cm high and having a base 15 cm by 7.5 cm or 20 cm by 7.5 cm. These allowed rapid development and required only small quantities of developing solvents. Chromatography was complete in 4 h without the need to equilibrate papers or to line the developing tanks with papers saturated with solvent.

Papers impregnated with zinc carbonate were conveniently stored vertically in a sealed cylindrical jar containing silica gel desiccant.

*Detection and estimation of lipids*

Coloured pigments, such as the chlorophylls and carotenoids, were detected in the feed and faecal extracts by their characteristic appearance and their location on the chromatogram. Colourless substances, such as α-tocopherol, α-tocopherylquinone and other fluorescing materials, were detected by viewing the chromatogram under ultraviolet light. The more prominent constituents were identified by removing them from the chromatogram, eluting with appropriate solvents (Table I) and determining the absorption curve on a Beckman spectrophotometer, model DU.

Quantitative estimation of β-carotene was made from its absorbance at 452 mμ, in light petroleum, using appropriate conversion factors[10].

Quantitative estimation of α-tocopherol was carried out by the recommended colorimetric method[9]. Spots of α-tocopherol were cut out and eluted with 4 ml of ethanol to which was added 0.5 ml each of α,α'-dipyridyl (0.5 % w/v in ethanol) and ferric chloride (0.2 % w/v in ethanol). Optical density was read at 520 mμ after two minutes and concentrations were derived from a standard graph based on pure α-tocopherol.

TABLE I

ABSORPTION MAXIMA OF CONSTITUENTS SEPARATED FROM SHEEP FEED (KIKUYU GRASS) AND SHEEP FAECES BY PAPER CHROMATOGRAPHY

| *Constituent* | *Solvent* | *Absorption maxima (mμ)* | |
|---|---|---|---|
| | | *Green feed* | *Sheep faeces* |
| Chlorophyll 1 | Acetone | 410, 420, 520, 570, 610, 660 | 410, 425, 530, 570, 610, 655 |
| Chlorophyll 2 | Acetone | 420, 520, 570, 610, 660 | 410, 425, 530, 570, 610, 660 |
| Chlorophyll 3 | Acetone | 410, 425, 500, 520, 570, 610, 660 | 410, 425, 480, 510, 530, 570, 610, 655 |
| Chlorophyll 4 | Acetone | 430, 455, 600, 645 | 430, 455, 595, 645 |
| Chlorophyll 5 | Chloroform | 420, 520, 540, 610, 660 | 420, 520, 560, 610, 655 |
| Chlorophyll 6 | Acetone | 410, 425, 510, 530, 570, 610, 660 | 410, 425, 510, 530, 570, 610, 660 |
| Xanthophyll | Ethanol | 425, 445, 470 | 420, 440, 470 |
| β-Carotene | Light petroleum | 420, 452, 480 | 420, 452, 480 |
| α-Tocopherol | Ethanol | 290–292 | 290–292 |
| α-Tocopheryl-quinone* | Ethanol | 261, 268 | 262, 268 |

* Detected in green feed several hours after collection.

*Materials examined*

The basic analytical procedures described above were established using feed and faeces collected from sheep grazing Kikuyu grass (*Pennisetum clandestinum* Hochst.).

Subsequently, these methods were applied to feed and faecal samples collected from two merino lambs, maintained since birth on a dry ration based on lucerne hay (*Medicago sativa* L.) and also when the diet was later changed to fresh green Kikuyu grass.

Pastures grown specifically for the maintenance of experimental animal colonies were examined for their tocopherol and carotene contents.

The rates of decomposition of α-tocopherol and β-carotene were determined simultaneously in Kikuyu grass, stored in the laboratory at room temperature, sampled and analysed at weekly intervals.

## RESULTS

*Qualitative studies*

*Separation and detection of lipids.* Chromatographic patterns obtained for extracts of Kikuyu grass and for extracts of sheep faeces from this diet were similar, with differences only in the concentrations of some constituents. A chromatogram, illustrating the separation of lipid constituents in extracts of sheep faeces from animals on green feed, is shown in Fig. 1. This chromatogram is also typical of that obtained for extracts of green feed and other samples of fresh pasture (see Table III).

Six green or yellow-green components were clearly separated on the chromatograms, and their absorption maxima corresponded with spectral data for various chlorophylls and closely related substances published elsewhere[4].

A pronounced orange-yellow band of β-carotene, isolated from feed and faeces, was located at the solvent front in the first dimension. The absorption spectrum in

light petroleum showed two principal maxima at 452 mμ and 480 mμ, in agreement with published data[4,10].

A yellow, xanthophyll-like spot and many other coloured and fluorescing spots were also noted in the feed and faecal extracts. Some of these appeared to correspond with carotenoid and quinone-like substances described in plants[7,8].

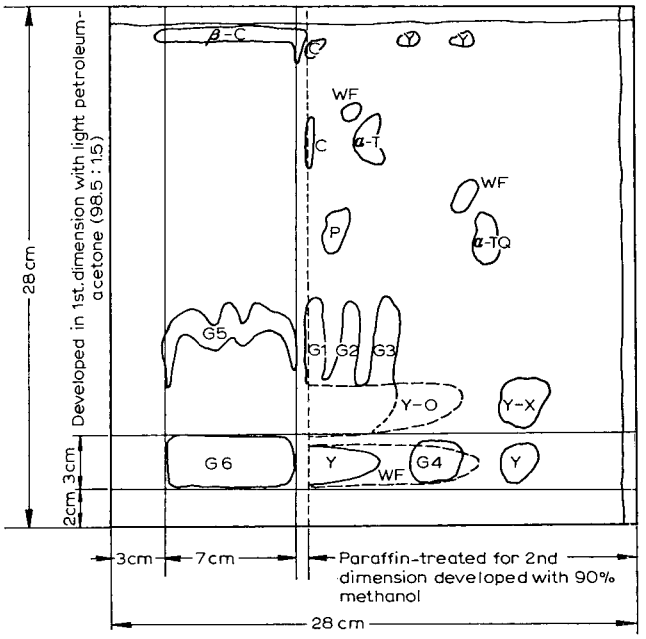


Fig. 1. Two-dimensional paper chromatogram showing separation of lipid components in extracts of sheep faeces (green feed diet). β-C = β-Carotene; α-T = α-tocopherol; α-TQ = α-tocopherylquinone; G1–G6 = green components (chlorophylls and closely related substances); Y-X = yellow xanthophyll; Y-O = yellow-orange pigments; Y = yellow pigments; P = pink pigments; W-F = white fluorescent substances; C = colourless, unknown, ultraviolet-absorbing substances.

α-Tocopherol was detected as a mauve spot under ultraviolet light, about midway between the chlorophyll front and β-carotene, and just inside the paraffin-treated area of the paper. The absorption spectrum in ethanol showed the expected maximum at 290–292 mμ[11].

A colourless spot with an intense absorption in the ultraviolet region ($\lambda_{max}$ 261 mμ, 268 mμ) was detected between the chlorophylls and α-tocopherol. On the basis of its spectrum[12,13], and by comparison with the spectral and chromatographic properties of the quinone prepared from pure α-tocopherol, it was considered to be α-tocopherylquinone.

Absorption maxima for some of the constituents, isolated from green feed and faeces by paper chromatography, are shown in Table I.

Absorption curves for β-carotene, α-tocopherol and α-tocopherylquinone, isolated from sheep faeces, and typical of those obtained from green feed, fresh pastures and reference substances, are shown in Fig. 2.

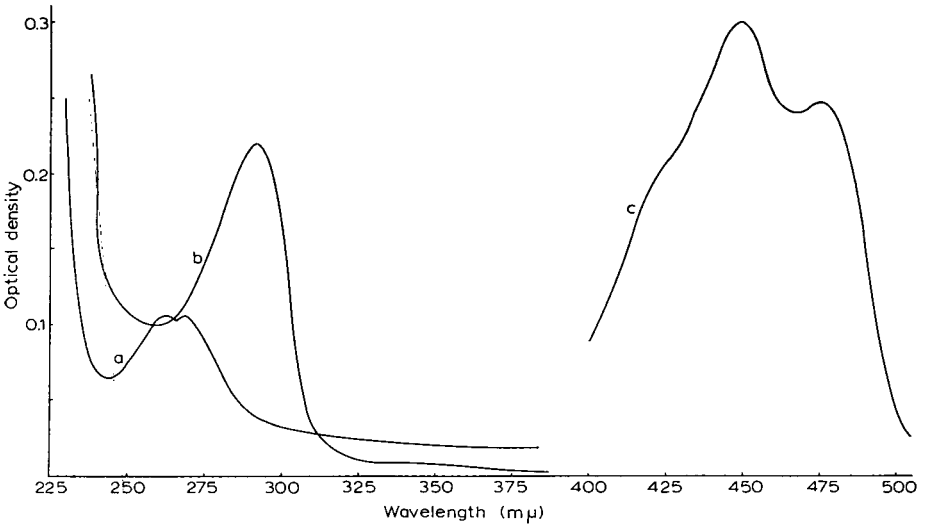


Fig. 2. Absorption curves of lipids, separated by paper chromatography, from extracts of sheep faeces. a = α-Tocopherylquinone; b = α-tocopherol; c = β-carotene.

*Quantitative analysis of biological materials*

*Sheep feeds and faeces.* Application of the method to feeds and faeces from lambs maintained under controlled experimental conditions showed differences in lipid concentrations.

On the dry ration, the concentration of β-carotene in the faeces was from 10 to 30 times that of the feed, on a dry weight basis. This feed contained no detectable α-tocopherol, but the faeces contained 1 to 2.3 mg per 100 g.

On the green feed, the concentration of β-carotene in the faeces was about 3 times that in the feed, and α-tocopherol levels were 4 to 5 times greater.

Concentrations obtained are shown in Table II and are expressed on a dry weight basis, as the means of duplicate determinations.

*Fresh pastures.* β-Carotene, and α-tocopherol contents of a range of pasture materials showed a wide variation and are listed in Table III.

TABLE II

CONCENTRATIONS OF β-CAROTENE AND α-TOCOPHEROL IN SHEEP FEEDS AND FAECES

All values are means of duplicate analyses and are expressed on a dry weight basis.

| *Component* | *Dry feed* | | *Green feed* | | *Sheep No.* |
|---|---|---|---|---|---|
| | *Feed (mg/100 g)* | *Faeces (mg/100 g)* | *Feed (mg/100 g)* | *Faeces (mg/100 g)* | |
| β-Carotene | 0.13 | 0.93 | 27.85 | 99.1 | 226 |
| | 0.13 | 3.73 | 27.85 | 89.2 | 232 |
| α-Tocopherol | 0.0 | 1.39 | 20.93 | 89.3 | 226 |
| | 0.0 | 2.31 | 20.93 | 89.8 | 232 |

TABLE III

β-CAROTENE AND α-TOCOPHEROL CONTENTS OF FRESH PASTURE

Values expressed on a wet weight basis.

| *Pasture* | *β-Carotene* | | *α-Tocopherol* | |
|---|---|---|---|---|
| | *mg/g* | *mg/lb* | *mg/g* | *mg/lb* |
| Kikuyu grass 1 | 0.064 | 29.1 | 0.085 | 38.5 |
| Kikuyu grass 2 | 0.081 | 36.8 | 0.083 | 37.7 |
| White clover leaf (*Trifolium repens* L.) | 0.125 | 57.0 | 0.024 | 10.8 |
| Berseem clover leaf (*Trifolium alexandrinum* L.) | 0.170 | 77.2 | 0.052 | 23.7 |
| Corn leaf | 0.077 | 35.0 | 0.067 | 30.3 |
| Oats leaf (mature) (*Avena sativa* L.) | 0.081 | 36.8 | 0.010 | 4.5 |
| Oats leaf (young) | 0.046 | 20.9 | 0.015 | 6.8 |

*Decomposition in drying pastures.* Results for the determination of β-carotene and α-tocopherol in air-dried Kikuyu grass, reported in Table IV and illustrated in Fig. 3, showed that during the first two days deterioration was negligible, but beyond one week the decomposition was more rapid. α-Tocopherol deteriorated more readily than β-carotene.

TABLE IV

RATE OF DECOMPOSITION OF β-CAROTENE AND α-TOCOPHEROL IN KIKUYU GRASS

Values are means of duplicate determinations and are calculated on an oven-dry basis.

| *Time interval (days)* | *% loss* | |
|---|---|---|
| | *α-Tocopherol* | *β-Carotene* |
| 2 | Negligible | Negligible |
| 8 | 50% | 30% |
| 13 | 70% | 50% |
| 21 | 90% | 70% |
| 30 | 100% | 80% |

## DISCUSSION

The use of column chromatography, employing a variety of adsorbents has been described for carotene[2] and the tocopherols[9,14], but frequently difficulties are encountered, such as destruction or retention of tocopherols on the column. This process can lead to spurious recoveries[15] and care has to be exercised in choice of adsorbent[16].

Direct application of the two-dimensional paper chromatographic method to feed and faecal extracts, though more time-consuming than unidimensional methods for plants[8], gives an added degree of separation of β-carotene and α-tocopherol from interfering substances. The accurate quantitative determination of α-tocopherol with

the iron–dipyridyl reagents[9] requires rigid freedom from reducing contaminants, as provided by the two-dimensional method.

The performance of labile constituents on the chromatograms was influenced by the amount of extract applied to the paper. In agreement with findings for plant extracts[7], it was observed for sheep faeces from green feed that, when the chlorophylls travelled about one-third the distance of the solvent front, α-tocopherol was well separated about midway between the chlorophylls and β-carotene.

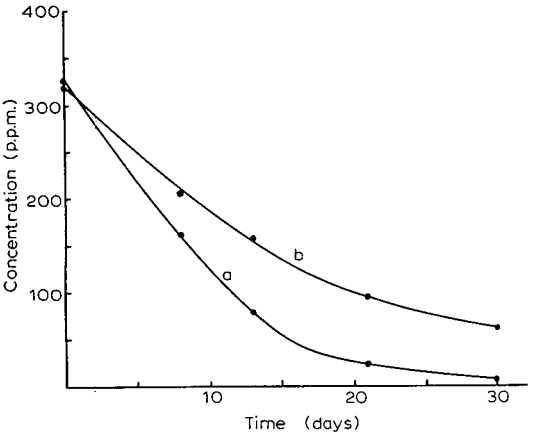


Fig. 3. Rate of decomposition of α-tocopherol (a) and β-carotene (b) in Kikuyu grass stored at room temperature.

Because of the high concentration of tocopherol in the faecal extracts, no difficulty was experienced in determining the absorption spectrum. However, for the green feed, several spots had to be combined before a satisfactory absorption curve could be obtained.

Location of the spots of α-tocopherol from both feed and faeces was always very similar and compared well with the position obtained for pure α-tocopherol.

Detection of α-tocopherylquinone in feeds and faeces, showing a typical bicuspid peak (261, 268 mμ), was of interest because of its position in metabolic pathways involving α-tocopherol.

Although α-tocopherylquinone was found as a prominent spot in chromatograms of fresh corn leaves (*Zea mays* L.) and has been reported in other plants[13], it was not detected in the fresh Kikuyu grass. However, as the drying process progressed and decomposition of lipids, including tocopherol, continued, the quinone appeared as a clearly detectable spot. α-Tocopherylquinone was also found in Kikuyu grass examined several hours after harvesting.

Studies using vitamin E as an antioxidant for β-carotene frequently measure the decay of the carotene only[17]. In contrast, the present method allows the convenient determination of both lipids simultaneously, after chromatographic separation. This technique is now being used in studies of digestibility in sheep.

SUMMARY

Two-dimensional paper chromatography was used, without preliminary removal of interfering substances, for examination of pastures, feeds and sheep faeces. α-Tocopherol, β-carotene and other lipids were adequately separated in a total chromatography time of four hours. α-Tocopherol and β-carotene were measured quantitatively in the diets and faeces. Rates of decay were also determined in samples of pasture.

In general, chromatographic patterns obtained from the faeces and the relevant diet were similar, but there were notable differences in the concentrations of both tocopherol and carotene.

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# CHROMATOGRAPHIE DES ACIDES PHÉNOLIQUES URINAIRES SUR PAPIER IMPRÉGNÉ D'ACÉTATE DE SODIUM*

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(Reçu le 31 octobre 1966)

A la suite des travaux de ARMSTRONG et coll.[1], SMITH[2], DALGLIESH[3] et d'autres auteurs, la chromatographie sur papier est largement utilisée pour préciser l'identité et étudier les modalités d'excrétion des acides phénoliques urinaires, et même pour l'évaluation quantitative de certains d'entre eux par comparaison visuelle[4] ou mesure photométrique après élution[5,6].

En raison du nombre élevé des composés présents sur les chromatogrammes, les techniques proposées font habituellement appel à une séparation bi-dimensionnelle. Cependant, quels que soient les solvants choisis, les similitudes de $R_F$ rendent certaines identifications difficiles ou impossibles. C'est, en particulier, le cas lorsque le prélèvement biologique dont on est parti provient d'un malade (phénylcétonurie, phéochromocytome) ou d'un individu qui n'a pas été soumis au préalable à une restriction médicamenteuse ou alimentaire appropriée.

On sait que certains auteurs ont eu recours avec succès—pour la séparation chromatographique de composés phénoliques[7–9] ou de substances de faible polarité comme les alcaloïdes[10]—à l'emploi de papiers imprégnés de sels ou de systèmes tampons particuliers.

En nous référant aux travaux de ces auteurs nous nous sommes proposés de rechercher si, dans le cas des acides phénoliques urinaires, une telle imprégnation saline du papier jointe à l'emploi de solvants appropriés, ne serait pas susceptible de conduire à une meilleure distinction des taches, en modifiant les solubilités et les équilibres ioniques des substances présentes.

De nombreux essais ont été effectués sur des papiers imprégnés de sels ou de systèmes tampons divers. Ce sont les résultats obtenus sur papier Whatman No. 20 imprégné d'acétate de sodium 0.1 *M* que nous nous proposons de rapporter ici.

## MATÉRIEL ET MÉTHODES

Tous les produits de référence cités sont d'origine commerciale**.

L'extraction des acides phénoliques de l'urine est effectuée selon ARMSTRONG, SHAW ET WALL[1], par l'acétate d'éthyle. Le volume final de l'extrait est amené à une valeur telle que 1 ml corresponde au volume d'urine qui renferme 1 mg de créatinine. On dépose habituellement sur un chromatogramme 0.5 ou 1 ml d'extrait.

* Travail effectué avec l'aide de la Délégation Générale à la Recherche Scientifique et Technique, Convention 61-Fr-202, avec la collaboration scientifique de Mlle R. BELLIMAZ et la collaboration technique de Mlle G. GIRARD.

** Produits Fluka, Buchs, Suisse.

*Préparation du papier*

Après en avoir découpé le bord inferieur en dents de scie, les feuilles de papier Whatman No. 20 (44 × 56 cm) sont suspendues à des baguettes de verre (fibre du papier dans le sens vertical) puis arrosées régulièrement*, en allant du haut vers le bas et sur les deux faces, avec une solution d'acétate de sodium 0.1 *M*. On laisse ensuite secher spontanément à l'air.

Les caractères du papier ainsi traité se révèlent, après plus d'une centaine d'essais, parfaitement constants. La vitesse de migration du solvant devient plus grande sur le papier traité que sur le papier normal, ce qui est sans doute lié à une modification de la texture des fibres résultant du gonflement: le papier imprégné d'acétate de sodium prend un aspect plus irrégulier et plus rugueux que le papier normal. Dans le solvant *n*-butanol–acide acétique–eau (4:1:5) on obtient un déplacement du front de 35 cm environ en 14–16 heures, contre 36 heures (HAIS ET MACEK[11]) sur le papier Whatman No. 20 normal.

Le papier Whatman No. 1 peut être utilisé, mais il semble plus fragile pendant le traitement et donne des spots moins beaux que le papier Whatman No. 20.

*Solvants*

Voir Tableau II. L'emploi du solvant isopropanol–ammoniaque–eau (8:1:1), déjà préconisé par ARMSTRONG[1], demande certaines précautions: cuves à chromatographie placées dans un local thermostatisé et sans courants d'air, nécessité d'en charger le couvercle pour assurer son étanchéité.

Nous avons toujours utilisé la technique descendante, à une temperature du Laboratoire comprise entre 20° et 25°, et en équilibrant les chromatogrammes dans l'atmosphère de la cuve pendant 1 heure avant le début de la migration. Le solvant est renouvelé à chaque essai.

*Révélation des taches d'acides phénoliques*

Les chromatogrammes sont d'abord examinés sous lumière U.V. à 360 mμ et 254 mμ puis, après avoir été privés de toute trace de solvant acide soit par un séchage sous courant d'air de 1 heure au moins, soit par un séjour prolongé à l'air (24 heures), ils sont révélés par l'acide sulfanilique ou la *p*-nitraniline diazotés. Ces réactifs sont particulièrement sensibles et ils donnent des colorations spécifiques avec de nombreux composés phénoliques. En vue de l'utilisation sur papier imprégné d'acétate de sodium, nous avons adopté les formules suivantes:

*Réactif à la diazo-p-nitraniline*

(a) *p*-Nitraniline pure en solution à 0.1 % dans HCl 0.1 *M*;

(b) Nitrite de sodium en solution à 1 % dans l'eau distillée, cette solution est diluée au 1/10 e au moment de l'emploi;

(c) $Na_2CO_3$ 0.6 *M*: ce titre doit être controlé.

Au moment de l'emploi, à 10 ml de (a) on ajoute, dans l'ordre, 10 ml de (b) dilué au 1/10 e, puis 10 ml de (c). Réactif à pulvériser aussitôt après sa préparation.

*Réactif à l'acide sulfanilique diazoté*

Même formule, mais en substituant à (a):

(a') Acide sulfanilique pur en solution à 1.25 % dans HCl 0.1 *M*.

* À l'aide d'une "pissette" de laboratoire.

Ces formules donnent également de bonnes colorations sur le papier chromatographique normal. Dans tous les cas, l'apparition des taches colorées n'est pas instantanée, elle demande quelques heures. Le fond du chromatogramme reste incolore.

## RÉSULTATS

Le Tableau I donne les caractères d'identification des acides phénoliques de référence, sur papier imprégné d'acétate de sodium, en lumière U.V. (254 m$\mu$) et après révélation par les deux réactifs décrits.

Le Tableau II indique les $R_F$ de ces mêmes acides phénoliques sur papier Whatman No. 20 normal et imprégné d'acétate de sodium 0.1 $M$, dans quatre solvants différents.

La Fig. 1 illustre l'intérêt de l'emploi du papier imprégné d'acétate de sodium. On remarque, en particulier, la position privilégiée qu'occupe, sur le chromatogramme de droite, l'acide 4-hydroxy-3-méthoxymandélique (AVM), nettement séparé des autres acides phénoliques. Cette distinction de l'acide vanillomandélique en chromatographie mono-dimensionnelle est encore plus nette avec le solvant isopropanol–acide acétique–eau (8:1:1) (voir Tableau II) dans lequel on peut l'isoler totalement, à $R_F$ 0.25, des autres composés phénoliques normaux de l'urine qui réagissent avec les diazo-réactifs. Cette proprieté peut être mise à profit pour une évaluation quantitative de l'AVM (visuelle ou photométrique) par comparaison avec des quantités connues de substance pure chromatographiées parallèlement.

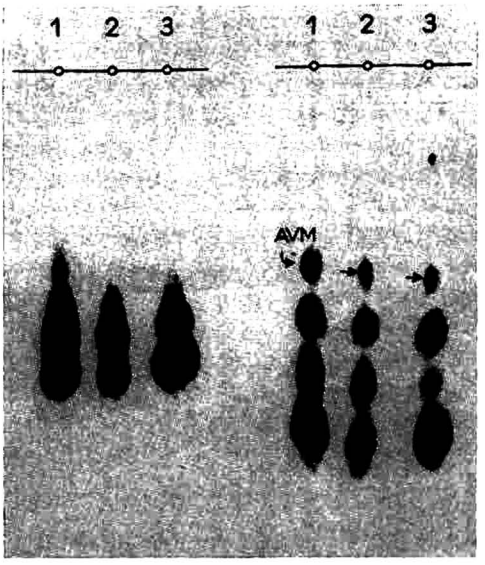


Fig. 1. Chromatogrammes de trois extraits urinaires (*n*-butanol–acide acétique–eau (4:1:5) sur papier Whatman No. 20 normal (à gauche) et imprégné d'acétate de sodium (à droite). Les quantités d'extrait déposées à gauche et à droite sont identiques. On voit que les taches d'acides phénoliques sont beaucoup mieux séparées les unes des autres, et plus nettes, sur le papier imprégné d'acétate de sodium. Il faut noter, en particulier, la position privilégiée de l'acide 4-hydroxy-3-méthoxymandélique (AVM), nettement distinct, après une migration monodimensionnelle, des autres composés présents.

TABLEAU I

IDENTIFICATION DES TACHES D'ACIDES PHÉNOLIQUES

| *Acides* | *U.V. 254 mμ* | | *Dz PN* | *Sulfa Dz* |
|---|---|---|---|---|
| | *A* | *B* | | |
| *o*-Hydroxybenzoïque (salicylique) | bleu clair | — | rose pâle | jaune pâle |
| *m*-Hydroxybenzoïque | bleu | — | rose | jaune vif |
| *p*-Hydroxybenzoïque | bleu sombre | bleu sombre | rose | jaune vif |
| *o*-Hydroxyphénylacétique | bleu sombre | bleu sombre | rouge violet | jaune orangé |
| *m*-Hydroxyphénylacétique | — | — | rose foncé | jaune |
| *p*-Hydroxyphénylacétique | — | — | violet clair | rose saumon |
| *m*-Hydroxymandélique | — | — | rose vif | jaune |
| *p*-Hydroxymandélique | — | — | rose | jaune |
| *p*-Hydroxyphénylpropionique | — | — | violet clair | rose saumon |
| *p*-Hydroxyphénylpyruvique | bleu pale | — | violet pâle | orangé pâle |
| *p*-Hydroxyphényllactique | — | — | violet | rose saumon |
| 3,4-Diméthoxybenzoïque (vératrique) | bleu sombre | bleu sombre | violet t. pâle | — |
| 3,4-Diméthoxyphénylacétique (homovératrique) | bleu sombre | bleu sombre | — | — |
| 4-Hydroxy-3-méthoxybenzoïque (vanillique) | bleu sombre | — | violet | orange |
| 4-Hydroxy-3-méthoxyphénylacétique (homovanillique) | bleu | — | bleu gris | rose |
| 4-Hydroxy-3-méthoxymandélique (vanillomandélique) | — | — | violet | orange |
| 3-Hydroxy-4-méthoxymandélique | — | — | violet | orange |
| 4-Hydroxy-3-méthoxycinnamique (férulique) | turquoise vif | turquoise vif | gris bleu | rose violacé |
| 3,4-Dihydroxybenzoïque (protocatéchique) | bleu | bleu | violet pâle | jaune pâle |
| 3,4-Dihydroxyphénylacétique (homoprotocatéchique) | bleu sombre | bleu sombre | violet pâle | gris |
| 2,5-Dihydroxybenzoïque (gentisique) | turquoise vif | turquoise vif | jaune pâle | jaune pâle |
| 2,5-Dihydroxyphénylacétique (homogentisique) | violet sombre | violet sombre | brun pâle | gris brun |
| 3,5-Dihydroxybenzoïque (α-résorcylique) | bleu | bleu | jaune d'or | jaune citron |
| 2,4-Dihydroxybenzoïque (β-résorcylique) | bleu vif | bleu | jaune orangé | jaune citron |
| 2,6-Dihydroxybenzoïque (γ-résorcylique) | bleu sombre | bleu sombre | jaune | jaune citron |
| 3,4-Dihydroxycinnamique (caféïque) | jaune vert | jaune vert | gris pâle | gris vert |
| 3,4,5-Trihydroxybenzoïque (gallique) | bleu | bleu | gris pâle | gris |
| 4-Hydroxy-3,5-diméthoxybenzoïque (syringique) | bleu sombre | bleu sombre | bleu vif | rose vif |
| Cynurénique | jaune vert | jaune vert | — | — |
| Xanthurénique | jaune orangé | jaune clair | violet | rose vif |
| 3-Hydroxyanthranilique | bleu | bleu | violet pâle | rose pâle |
| 5-Hydroxyindoleacétique | rose | rose | rose violet | rouge saumon |
| Dihydroxyphénylalanine (Dopa) | jaune orangé | rose orangé | rose pâle | orangé pâle |
| Tyrosine | — | — | violet clair | rose saumon |

Abbréviations: A = sur papier Whatman No. 20 imprégné d'acétate de sodium; B = sur papier Whatman No. 20 normal; Dz PN = *p*-nitraniline diazotée, sur papier Whatman No. 20 imprégné d'acétate de sodium; Sulfa Dz = acide sulfanilique diazoté sur papier Whatman No. 20 imprégné d'acétate de sodium.

TABLEAU II

$R_F$ DES ACIDES PHÉNOLIQUES DANS DIFFERENTS SOLVANTS

(1) Trainée; (2) décomposition; (3) une tache secondaire jaune (Dz PN et Sulfa Dz) de $R_F$ 0.07; (4) une tache secondaire jaune (Dz PN et Sulfa Dz) de $R_F$ 0.11; (5) deux taches secondaires visibles sous lumière U.V. de $R_F$ 0.22 (jaune clair) et 0.46 (bleu clair); (6) une tache secondaire visible sous lumière U.V. de $R_F$ 0.57 (bleu clair); (7) deux taches secondaires visibles sous lumière U.V. de $R_F$ 0.09 (jaune clair) et 0.26 (bleu clair); (8) une tache secondaire visible sous lumière U.V. de $R_F$ 0.35 (bleu clair).

| *Acides* | *Bu–Ac (4:1:5)* | | *Bu–Ac (12:3:5)* | | *Isoprop–Ac* | | *Isoprop–$NH_3$* | |
|---|---|---|---|---|---|---|---|---|
| | *A* | *B* | *A* | *B* | *A* | *B* | *A* | *B* |
| *o*-Hydroxybenzoïque (salicylique) | 0.68 | 0.85 | 0.75 | 0.85 | 0.56 | 0.81 | 0.55 | 0.56 |
| *m*-Hydroxybenzoïque | 0.74 | 0.82 | 0.83 | 0.84 | 0.67 | 0.81 | 0.25 | 0.24 |
| *p*-Hydroxybenzoïque | 0.78 | 0.83 | 0.88 | 0.85 | 0.78 | 0.82 | 0.14 | 0.13 |
| *o*-Hydroxyphénylacétique | 0.72 | 0.82 | 0.81 | 0.84 | 0.69 | 0.80 | 0.57 | 0.55 |
| *m*-Hydroxyphénylacétique | 0.72 | 0.82 | 0.80 | 0.84 | 0.71 | 0.81 | 0.34 | 0.28 |
| *p*-Hydroxyphénylacétique | 0.73 | 0.80 | 0.81 | 0.84 | 0.72 | 0.79 | 0.27 | 0.25 |
| *m*-Hydroxymandélique | 0.34 | 0.67 | 0.41 | 0.69 | 0.31 | 0.63 | 0.22 | 0.23 |
| *p*-Hydroxymandélique | 0.33 | 0.66 | 0.40 | 0.66 | 0.32 | 0.63 | 0.17 | 0.18 |
| *p*-Hydroxyphénylpropionique | 0.81 | 0.84 | 0.88 | 0.85 | 0.75 | 0.81 | 0.29 | 0.32 |
| *p*-Hydroxyphénylpyruvique | 0.36 | 0.72 | 0.48 | 0.69 | 0.38 | 0.69 | (1) | 0.21 |
| *p*-Hydroxyphényllactique | 0.53 | 0.71 | 0.54 | 0.69 | 0.35 | 0.67 | 0.25 | 0.22 |
| 3,4-Diméthoxybenzoïque (vératrique) | 0.80 | 0.80 | 0.85 | 0.84 | 0.75 | 0.78 | 0.29 | 0.29 |
| 3,4-Diméthoxyphénylacétique (homovératrique) | 0.80 | 0.80 | 0.85 | 0.85 | 0.73 | 0.78 | 0.33 | 0.32 |
| 4-Hydroxy-3-méthoxybenzoïque (vanillique) | 0.76 | 0.81 | 0.86 | 0.82 | 0.65 | 0.77 | 0.07 | 0.09 |
| 4-Hydroxy-3-méthoxyphénylacétique (homovanillique) | 0.74 | 0.79 | 0.80 | 0.80 | 0.70 | 0.74 | 0.22 | 0.21 |
| 4-Hydroxy-3-méthoxymandélique (vanillomandélique) | 0.31 | 0.62 | 0.36 | 0.64 | 0.25 | 0.57 | 0.13 | 0.16 |
| 3-Hydroxy-4-méthoxymandélique | 0.31 | 0.60 | 0.36 | 0.62 | 0.23 | 0.55 | 0.14 | 0.17 |
| 4-Hydroxy-3-méthoxycinnamique (férulique) | 0.79 | 0.80 | 0.88 | 0.82 | 0.70 | 0.75 | 0.08 | 0.15 |
| 3,4-Dihydroxybenzoïque (protocatéchique) | 0.69 | 0.73 | 0.77 | 0.75 | 0.64 | 0.69 | 0.02 | 0.05 |
| 3,4-Dihydroxyphénylacétique (homoprotocatéchique) | 0.62 | 0.71 | 0.62 | 0.72 | 0.57 | 0.65 | (2) | 0.08 (1) |
| 2,5-Dihydroxybenzoïque (gentisique) | 0.43 | 0.80 | 0.53 | 0.81 | 0.37 | 0.73 | 0.37 | 0.39 |
| 2,5-Dihydroxyphénylacétique (homogentisique) | 0.57 | 0.69 | 0.59 | 0.70 | 0.52 | 0.72 | (2) | (1) |
| 3,5-Dihydroxybenzoïque (α-résorcylique) | 0.62 (3) | 0.75 | 0.72 (4) | 0.78 | 0.64 | 0.73 | 0.06 | 0.11 (1) |
| 2,4-Dihydroxybenzoïque (β-résorcylique) | 0.60 | 0.82 | 0.70 | 0.85 | 0.52 | 0.78 | 0.13 | 0.19 |
| 2,6-Dihydroxybenzoïque (γ-résorcylique) | 0.49 | 0.45 | 0.75 | 0.53 | 0.46 | 0.40 | 0.64 | 0.68 |
| 3,4-Dihydroxycinnamique (caféïque) | 0.69 | 0.71 | 0.78 | 0.76 | 0.60 | 0.66 | (1) | 0.05 |
| 3,4,5-Trihydroxybenzoïque (gallique) | 0.45 | 0.50 | 0.52 | 0.55 | 0.54 | 0.52 | (1) | (1) |
| 4-Hydroxy-3,5-diméthoxybenzoïque (syringique) | 0.73 | 0.78 | 0.82 | 0.79 | 0.67 | 0.71 | 0.05 | 0.08 |
| Cynurénique | 0.35 | 0.46 | 0.50 | 0.51 | 0.24 | 0.29 | 0.29 | 0.32 |
| Xanthurénique | 0.33 (5) | 0.42 | 0.46 (6) | 0.49 | 0.19 (7) | 0.30 | 0.02 (8) | 0.05 |
| 3-Hydroxyanthranilique | 0.75 | 0.78 | 0.85 | 0.77 | 0.67 | 0.40 | (1) | 0.05 |
| 5-Hydroxyindoleacétique | 0.62 | 0.68 | 0.70 | 0.69 | 0.59 | 0.63 | 0.14 | 0.12 |
| Dihydroxyphénylalanine | 0.13 | 0.08 | 0.20 (1) | 0.14 | 0.07 | 0.07 | (2) | 0.05 |
| Tyrosine | 0.28 | 0.22 | 0.35 | 0.28 | (1) | 0.16 | 0.15 | 0.16 |

Abréviations: Bu–Ac (4:1:5) = *n*-butanol–acide acétique–eau (4:1:5), phase organique; Bu–Ac (12:3:5) = *n*-butanol–acide acétique–eau (12:3:5); Isoprop–Ac = Isopropanol–acide acétique–eau (8:1:1); Isoprop–$NH_3$ = Isopropanol–ammoniaque–eau (8:1:1); A = sur papier Whatman No. 20 imprégné d'acétate de sodium 0.1 *M*; B = sur papier Whatman No. 20 normal.

Cependant le recours à une séparation bi-dimensionnelle reste indispensable pour l'identification de l'ensemble des composés présents dans les extraits d'urine. Les deux solvants qui nous ont donné les meilleurs résultats sont les suivants:

1. (Première dimension): isopropanol–ammoniaque–eau (8:1:1),
2. (Deuxième dimension): isopropanol–acide acétique–eau (8:1:1).

La Fig. 2 montre la localisation des taches d'acides phénoliques urinaires sur un chromatogramme bi-dimensionnel. On peut observer, pour certains composés,

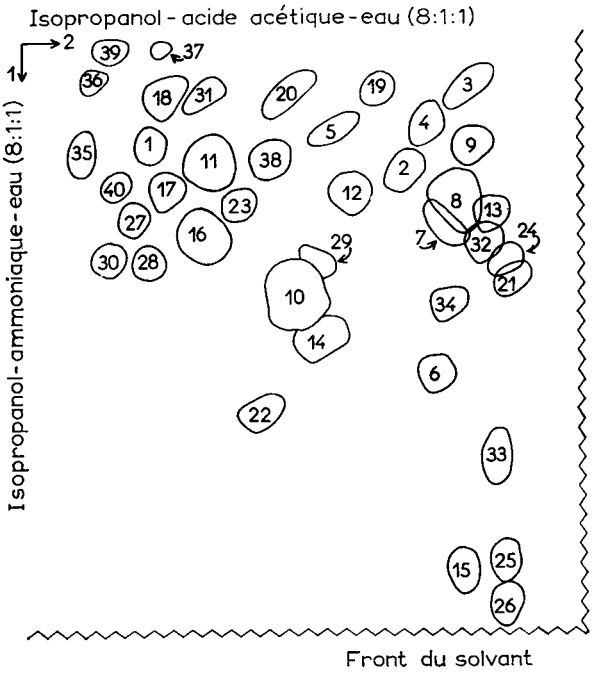


Fig. 2. Les acides phénoliques de l'urine humaine. Chromatographie bi-dimensionelle descendante sur papier Whatman No. 20 imprégné d'acétate de sodium 0.1 *M*. DzPN = *p*-Nitraniline diazotée; Sulfa Dz = acide sulfanilique diazoté; U.V. = examen sous lumière ultra-violette à 254 mμ.

(1) Ac. 4-hydroxy-3-méthoxymandélique (vanillomandélique); (2) ac. 4-hydroxy-3-méthoxyphénylacétique (homovanillique); (3) ac. vanillique; (4) ac. 4-hydroxy-3-méthoxycinnamique (férulique); (5) ac. 5-hydroxyindoleacétique; (6) ac. *o*-hydroxyphénylacétique; (7) ac. *m*-hydroxyphénylacétique; (8) ac. *p*-hydroxyphénylacétique; (9) ac. *p*-hydroxybenzoïque; (10) ac. hippurique (Dz PN + O, Sulfa Dz = O, U.V. = bleu sombre); (11) ac. *p*-hydroxyhippurique (Dz PN = rouge clair, Sulfa Dz = jaune orangé, U.V. = bleu); (12) Dz PN = rose, Sulfa Dz = jaune; (13) U.V. = bleu clair; (14) U.V. = bleu clair; (15) U.V. = bleu; (16) U.V. = bleu sombre; (17) U.V. = bleu sombre; (18) U.V. = bleu turquoise vif; (19) ac. 3,5-dihydroxybenzoïque (α-résorcylique); (20) ac. *o*-hydroxyhippurique (salicylurique) (Dz PN = rose, Sulfa Dz = jaune, U.V. = bleu vif); (21) ac. *p*-hydroxyphénylpropionique; (22) ac. *o*-hydroxybenzoïque (salicylique); (23) ac. *p*-hydroxyphényllactique; (24) Dz PN = rose, Sulfa Dz = jaune; (25) Dz PN = rose, Sulfa Dz = jaune; (26) Dz PN = rose violacé, Sulfa Dz = rose saumon; (27) U.V. = bleu clair; (28) U.V. = bleu; (29) U.V. = bleu clair; (30) U.V. = bleu clair; (31) ac. *m*-hydroxyhippurique (Dz PN = rose clair, Sulfa Dz = jaune clair, U.V. = bleu sombre); (32) Dz PN = bleu foncé, Sulfa Dz = rose; (33) U.V. = bleu clair; (34) U.V. bleu; (35) U.V. = jaune; (36) et (37) U.V. = bleu sombre; (39) ac. xanthurénique (?); (40) tyrosine (?). Les taches 1 à 18 ont été observées sur presque tous les chromatogrammes; en revanche la présence des taches 19 et suivantes est inconstante. Les noms de plusieurs acides phénoliques, reconnus comme constituents normaux de l'urine humaine, ne figurent pas sur cette liste qui comporte seulement les noms des substances que nous avons identifiées en nous référant aux seuls produits purs en notre possession.

des différences sensibles de $R_F$ selon qu'il s'agit du produit pur our de la même substance présente dans l'extrait urinaire.

Le solvant 1 présente l'inconvénient de décomposer ou d'altérer certains acides phénoliques fragiles. Il serait bon de pouvoir lui substituer un autre solvant qui, tout en permettant une résolution comparable des taches, n'aurait pas cet inconvénient. Nos recherches dans ce sens, à l'aide de solvants contenant plusieurs types de bases: propylamine, isopropylamine, *n*-butylamine, isobutylamine, $Na_2CO_3$, ont été jusqu'à ce jour infructueuses.

La comparaison entre les chromatogrammes obtenus avec ces solvants, sur papier Whatman No. 20 imprégné d'acétate de sodium 0.1 *M*, et ceux réalisés sur papier normal à l'aide de solvants préconisés par d'autres auteurs (isopropanol–ammoniaque–eau, 8:1:1, suivi de benzène–acide propionique–eau, 2:2:1 phase organique (1) ou anisol–acide acétique–eau, 70:29:1 (2)) montre que l'on obtient, sur le papier traité par imprégnation saline, une meilleure répartition des spots sur l'ensemble du chromatogramme. Ils se distinguent donc mieux les uns des autres.

Ces constatations corroborent celles de MUNIER ET MACHEBOEUF[10,12]. On sait que ces auteurs ont préconisé pour la chromatographie des alcaloïdes et des bases organiques—substances dont les constantes de dissociation varient dans de larges limites, comme c'est le cas pour les acides phénoliques—l'emploi de papier imprégné d'un sel dont l'anion forme un système tampon avec l'acide présent dans la phase solvante. Les résultants que nous avons obtenus montrent que ce principe peut être appliqué avec succès à la séparation des acides phénoliques: en phase solvante acide et sur papier normal, un grand nombre de ces composés ont des $R_F$ voisins, ce qui n'est plus le cas sur le papier imprégné d'acétate de sodium.

On peut observer (Tableau II) que les modifications de $R_F$ des acides phénoliques, sous l'effet de l'imprégnation saline du papier, varient de l'un à l'autre dans de grandes proportions. La comparaison, pour un certain nombre d'entre eux, entre la constante de dissociation $K$[13,14] et la différence entre le $R_F$ sur papier normal et le $R_F$ sur papier imprégné d'acétate de sodium ($R_{F(B)} - R_{F(A)}$) montre que ce sont ceux qui ont une constante de dissociation élevée ($K > 10^{-3}$) pour lesquels la valeur de $R_{F(B)} - R_{F(A)}$ est la plus élevée. Les acides phénoliques dont la constante de dissociation est moyenne ou faible ($K < 10^{-4}$) ne voient leur comportement chromatographique que peu ou pas modifié.

Quelques exemples (Tableau III) illustrent ces faits.

En phase solvante alcaline, comme il était prévisible, les $R_F$ et l'aspect des chromatogrammes sont sensiblement identiques pour le papier normal et le papier imprégné d'acétate de sodium.

RÉSUMÉ

La séparation chromatographique des acides phénoliques extraits de l'urine humaine peut être effectuée dans de bonnes conditions sur papier Whatman No. 20 imprégné d'acétate de sodium 0.1 *M*, et à l'aide des solvants suivants:

Première dimension: isopropanol–ammoniaque–eau (8:1:1),

Deuxième dimension: isopropanol–acide acétique–eau (8:1:1).

Par rapport aux techniques antérieurement décrites, la chromatographie sur papier imprégné d'acétate de sodium conduit à une meilleure répartition des taches

TABLEAU III

RELATION, POUR QUELQUES ACIDES PHÉNOLIQUES, ENTRE LA CONSTANTE DE DISSOCIATION $K$ ET LA MODIFICATION DE LEUR $R_F$ SOUS L'EFFET DE L'IMPRÉGNATION SALINE DU PAPIER.

Se reporter au texte.

| *Acides* | $K^*$ | $R_{F(B)} - R_{F(A)}{}^{**}$ |
|---|---|---|
| 2,5-Dihydroxybenzoïque | $1.08 \cdot 10^{-3}$ | 0.36 |
| 2,4-Dihydroxybenzoïque | $1.14 \cdot 10^{-3}$ | 0.26 |
| *o*-Hydroxybenzoïque | $1.25 \cdot 10^{-3}$ | 0.25 |
| *p*-Hydroxybenzoïque | $3.3 \cdot 10^{-5}$ | 0.04 |
| 3,4-Dihydroxybenzoïque | $3.3 \cdot 10^{-5}$ | 0.05 |
| 3,4,5-Trihydroxybenzoïque | $3.9 \cdot 10^{-5}$ | 0.02 |

* Première dissociation.
** Dans le solvant: isopropanol–acide acétique–eau (8:1:1).

d'acides phénoliques sur l'ensemble du chromatogramme. Elle améliore les possibilités d'identification et, dans certains cas, d'évaluation quantitative de ces composés.

SUMMARY

The chromatographic separation of phenolic acids extracted from human urine can be carried out under good conditions on Whatman No. 20 paper, impregnated with 0.1 *M* sodium acetate, using the following solvents:

1st dimension: isopropanol–ammonia–water (8:1:1),

2nd dimension: isopropanol–acetic acid–water (8:1:1).

In comparison with techniques previously described, chromatography on paper impregnated with sodium acetate leads to a better distribution of the spots of the phenolic acids over the whole chromatogram. It improves the possibility of identification and, in certain cases, the quantitative determination of these compounds.

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# DETERMINATION OF RADIOCHEMICAL PURITY OF SOME RADIOCHEMICALS AND PHARMACEUTICALS BY PAPER CHROMATOGRAPHY, THIN-LAYER CHROMATOGRAPHY AND HIGH-VOLTAGE ELECTROPHORESIS

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## INTRODUCTION

Much attention has been paid during the last few years to the radiochemical purity of radiochemicals, now generally defined as the portion of the radioisotope concerned that is in the stated chemical form. Chromatography and electromigration techniques have been utilized mostly for these determinations, and the subject has been dealt with by several authors[1–7]. In our laboratory the methods mentioned are used for control of the routine production of radioisotopes and imported materials. This paper gives a review of methods and some practical details which may be of value to other laboratories.

Prior to radiochemical analysis the samples under investigation are analyzed with regard to radioisotopic purity or the proportion of total radioactivity which is in the form of the main or stated radioisotope. This is usually done by gamma spectroscopy or beta absorption measurements. The chemical purity of radiochemicals and pharmaceuticals is examined by methods such as emission spectrography, infrared spectrometry, U.V.-spectrometry, melting point determinations, etc. Chemical impurities may also, of course, be identified by partition paper chromatography followed by specific colour reactions on the paper strips.

The various procedures for radiochemical purity determinations described in this paper have been tested experimentally, and found to give reasonably good reproducibilities, whereas the accuracy has been difficult to establish for some of the methods.

## EQUIPMENT

Paper chromatography (PC) is mainly carried out by the ascending technique on Whatman paper No. 1 and 3. Application of the samples to paper strips and thin layer plates is done by means of lambda pipettes connected to a micro syringe. Shandon equipment is used for thin-layer chromatography (TLC), ordinary electrophoresis and high-voltage electrophoresis (HVE).

Scanning of paper strips and plates for radioactivity is usually undertaken with a Packard Radiochromatogram Scanner Model 2701. The quantitative distribution of activity along the strips is determined by registering with a scaler the counts under each peak.

EXPERIMENTAL

The sample should have a reasonable counting rate in a suitable volume (say 0.005 ml), and to obtain this it is frequently necessary to dilute the sample with an appropriate solvent. In the case of so-called carrier-free radiochemicals, for instance sodium iodide—$^{131}I$ and sodium phosphate—$^{32}P$, it is advisable to add a carrier. If a certain radioactive impurity is sought or suspected in the sample under investigation, a carrier for the impurity should also be included. In some instances the solution in which the radiochemical is supplied commercially, may cause serious interference in the chromatographic procedure. In such cases the solvent has to be removed completely before starting the chromatogram.

*Iodide-$^{131}I$ ions*

This compound is conveniently analyzed by PC, over a period of 2–3 h, using a mixture of methanol and water in the ratio 70:30[8].

If rapid results are required, the sample may be analyzed by HVE using 0.1 *N* NaOH as electrolyte[9]. The spot is placed in the middle of a paper sheet (Whatman No. 1 paper, dimensions about 56 × 2.5 cm) and constant current is applied for 5 minutes (10 mA/cm width of paper). Under these conditions $I^-$ migrates approximately 130 mm against the anode, whereas the $IO_3^-$ moves around 40 mm. The presence of periodate is indicated by a tail between the point of application and the iodate spot. A recently published TLC method[10] provides a good and rapid separation of iodide and iodate ions.

*Phosphate-$^{32}P$ ions*

The chemical composition of phosphate-$^{32}P$ products has been thoroughly investigated previously[11,12]. It was found that the product may contain up to 2 % radiochemical impurities, comprising pyro-, tri-, tetra- and other long-chain polyphosphates. Routine analysis of this product is performed by ascending PC using the following solvent: isopropanol–trichloroacetic acid (20 % w/v)–5 *M* ammonia solution in water (75:25:1)[13]. The $R_F$ value for orthophosphate in this system is around 0.8. Small amounts of impurities in phosphate-$^{32}P$ can be determined with TLC, using a layer of cellulose and starch[14].

*Sulphate-$^{35}S$ ions*

This radioisotope is produced by pile irradiation of potassium chloride and contains $^{36}Cl$, $^{38}Cl$, $^{42}K$ and $^{32}P$ from secondary nuclear reactions. After chemical separation the product still contains minor amounts of $^{32}P$. By passing it through a column of aluminium shavings the content of $^{32}P$ in the product is brought down to 0.1 % or less. The radiochemical purity is easily determined by PC, using Whatman No. 1 paper. A suitable solvent mixture is isopropyl alcohol and 1.5 *N* ammonia in the ratio 50:50[15]. The $R_F$ value for sulphate under these conditions is approximately 0.7.

*Chlormerodrin-$^{203}Hg$*

The lack of commercial supplies of 3-chloromercuri-2-methoxy-propyl urea as reference material complicates the establishment of a convenient analytical procedure for control of the corresponding labelled compound. By using locally made reference

material, however, we found that TLC gives satisfactory reproducibility. Silica Gel G was used in 0.2 mm layers with *n*-butanol–acetic acid–water (60:15:25) as solvent. Under these conditions the labelled chlormerodrin has a $R_F$ value of approximately 0.65. A second small peak appeared at around $R_F$ 0.4 in all preparations, and it was found that it did not consist of $^{203}Hg$ ions. Running its reference material stained with iodine vapour gives a corresponding unidentified spot. Probably this effect is due to the same phenomenon as previously mentioned in the literature[16]. The spot usually amounts to 1–3 % of the total activity. Reference runs of mercuric chloride stained with 1 % diphenyl carbazide in alcohol followed by exposure to ammonia revealed that $Hg^{2+}$ ions move nearly to the solvent front.

A PC method, using *n*-butanol–methanol–water–ammonia (5:7:1:3)[17] as solvent has also been used. On Whatman No. 1 paper the following $R_F$ values were found: Chlormerodrin-$^{203}Hg$: 0.28–0.33, 3-chloromercuri-2-methoxy-propyl urea as reference: 0.28, mercuric ions: 0, with slight tail formation. The small separate peak appearing on the TLC plates mentioned above does not emerge on the paper. Otherwise the analytical results from the two methods coincide.

*Cyanocobalamin-$^{58}Co$ (Vitamin $B_{12}$)*

The radiochemical purity of this product has been examined by PC using a mixture of *sec.*-butanol–acetic acid–water–5 % KCN solution (100:1:50:0.25) or *sec.*-butanol–aqueous $NH_3$–water–5 % KCN solution (100:1:50:0.25)[18]. The $R_F$ values for cyanocobalamin are 0.25 and 0.30 for the two solvents respectively.

A more rapid TLC method for separation of mixed water soluble vitamins is published by GÄNSHIRT AND MALZACHER[19]. The conditions are: Silica Gel G layer and a solvent consisting of glacial acetic acid–acetone–methanol–benzene (5:5:20:70). The $R_F$ value for cyanocobalamin is 0 in this system. In addition if a Silica Gel G layer is used with water as developing solvent[20] the cyanocobalamin has a $R_F$ value of 0.22. The latter system has been found particularly useful for our purposes and is now used for the control of cyanocobalamin-$^{58}Co$ preparations in general.

*Gold-198 colloid*

Several methods for PC analysis of these solutions mentioned in the literature have been tried, but most of them seem to suffer from either tail formation or inadequate reproducibility. The best solvent was found to be a mixture of acetone, water and HCl (70:20:10)[21].

Recently we introduced paper electrophoresis (PE) in this connection[22] and later HVE and thin-layer electrophoresis (TLE). In these experiments 0.075 *M* sodium thiosulphate is used as electrolyte. The experimental conditions are: For HVE the spot is placed in the middle of a paper sheet (Whatman No. 1, 56 × 2.5 cm), and constant current is applied for 10 minutes at 6000 volts. Auric ions move around 160 mm against the anode, while colloidal gold is kept primarily at the zero point. Negligible tail formation is observed after this procedure. For TLE, the plates (20 × 5 cm) are prepared from Silica Gel G, layer thickness 2 mm. After air-drying for some minutes the plates are activated in an oven for one hour at 100°. The sample is applied in the form of a streak in the middle of the plate. Using constant current (2.5 mA/cm width of plate), the auric ions move approximately 60 mm against the anode. Colloidal gold moves a few millimeters toward the anode.

Comparing the analytical results from these three techniques, it has been found that PC nearly always gives the highest percentage of auric ions. The following is typical: PC—1.2 % auric ions, HVE—0.8 % auric ions and TLE—0.6 % auric ions.

*Human serum albumin-$^{131}I$*

$^{131}$I-labelled human serum albumin for blood volume measurements, etc. requires a reliable knowledge of the percentage of free iodide in the product. The conventional PC method (methanol–water, 75:25) does not always give reproducible results. We therefore found it necessary to verify the results by other methods. The PE method described in the British Pharmacopoeia[23] has been adopted for our HVE equipment, and has given excellent results. The experimental conditions are: Carrier (0.5 mg normal serum albumin, iodide and iodate) plus the sample under investigation is placed on a sheet of Whatman No. 1 paper (56 × 3 cm) and submitted to electrophoresis for 10 min using constant current (about 3.4 mA/cm width of paper). Under these circumstances the albumin moves around 10 mm towards the anode, the iodate around 70 mm and the iodide around 150 mm towards the anode.

A technique based upon precipitation of the albumin by trichloro-acetic acid is also used for checking purposes, and this method probably gives even more precise results. 0.1 ml 0.1 % KI solution in water is added to 0.4 ml of the solution of labelled albumin in a centrifuge tube. 0.1 ml of the mixture is calibrated in a scintillation counter. 0.4 ml of 10 % trichloroacetic acid in water is added to the tube, followed by shaking. The tube is then immersed for a moment in boiling water and afterwards in ice. After approximately three minutes' centrifugation at 3000 r.p.m. or filtration using blue band filter, 0.1 ml of the supernatant containing possible free ions is counted and the percentage of free ions calculated.

Typical results for a sample analyzed by the three methods are as follows: PC—3.3 % iodide, HVE—2.0 % iodide, precipitation techniques—2.9 %free ions. In the PC and HVE analysis the free ions have been identified as iodide only.

*Insulin-$^{131}I$*

The radiochemical purity of insulin—$^{131}$I for immunoassay work is principally determined by PC in this laboratory. The procedure in use has been described previously[24]. Due to the high purity requirements for this product and also the uncertainty concerning the nature of the impurities appearing we found it desirable to verify the analytical results, mainly by using various solvent systems. At present the best solvents appear to be veronal buffer (pH 8.6)[25] and a mixture of *n*-butanol, acetic acid and water (30:10:40)[26]. The first solvent keeps the insulin at the point of application, while the second gives an $R_F$ value of approximately 0.4 for insulin. This work is still in hand.

*Iodohippurate-$^{131}I$*

Radiochemical analysis of iodohippurate—$^{131}$I is described in detail by MAGNUSSON[27], and the PC method (*n*-butyl alcohol–acetic acid–water) was found convenient for our purpose. However, when commercial sodium iodohippurate is applied on a reference strip for identification, the spot appears at an entirely different place, $R_F$ around 0.3 when stained with bromophenol blue as compared with $R_F$ around 0.9 for the $^{131}$I-labelled sodium iodohippurate. Some twenty arbitrarily chosen solvents

were tried in order to test the discrepancy, out of which six solvents gave acceptable separation of iodohippurate—$^{131}$I from iodide. The $R_F$ values for commercial sodium iodohippurate and $^{131}$I-labelled sodium iodohippurate differed, however, for all the six solvents, the labelled material running faster on the strips than commercial iodohippurate. Products from different suppliers were analyzed during these tests, all showing the same behaviour. The identity of the iodohippurate used was verified by infrared spectrometry.

Thin-layer chromatography with silica gel layer and acetic acid–water (3:97) as solvent was found to give corresponding $R_F$ values for labelled and unlabelled sodium iodohippurate, $R_F$ around 0.75, while iodide ions move with the solvent front. Spraying techniques for iodohippurate do not give a distinct band in this case. Better results are obtained by colouring the material before application on the glass plates. The following procedure may be useful: 10 % sodium iodohippurate (w/v) in water is mixed in the ratio 1:1 with a solution of 40 mg bromophenol blue in 100 ml ethyl alcohol, to which has been added 0.5 ml 3 % (w/v) sodium hydroxide solution. Around 5 $\mu$l is applied on the plate. A reference spot of bromophenol blue on the same plate leaves a yellow tail behind and has a $R_F$ value of about 0.9.

*Rose Bengal-$^{131}$I*

Several methods for examination of the radiochemical purity of this labelled compound have been published[28–31]. It seems to be generally accepted that Rose Bengal—$^{131}$I preparations contain an unknown colourless component, which is separated from the main component when subjected to paper chromatography. This is in agreement with our experience, as all examinations so far performed have revealed a colourless peak, which is not "free" iodide. Using TLC (Silica Gel G layer) in the system water–acetic acid (97:3) the colourless component moves with the solvent front, and the main component is kept at the point of application, whereas in the system ethyl acetate–acetic acid (90:10) the two components travel in the opposite manner. As to the chemical composition of the unknown fraction it has been indicated that some less iodinated tetrachloro-(P)-fluoresceins—$^{131}$I may be found in these preparations[30]. It is also stated that their clinical behaviour is similar to that of the main component.

The TLC method published by MITTA *et al.*[31] has been found to give reproducible and reliable results. The thin-layer plates are prepared with silica gel G according to STAHL[32], and butyl acetate–acetic acid (90:10) is used as solvent. $R_F$ values are 0.9 for Rose Bengal and 0 for iodide. The colourless labelled component was found as a tail in our experiment. The tail formed a maximum at about $R_F$ 0.6.

SUMMARY

Procedures for determination of the radiochemical purity of some important radiochemicals are described. The methods have been verified and found to give reliable results with good reproducibility. Some practical details of experience gained during the study are given.

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# HOCHSPANNUNGSPAPIERIONOPHORETISCHE TRENNUNG UND IDENTIFIZIERUNG $^{35}S$ UND $^{75}Se$ MARKIERTER SELENOPOLYTHIONATE

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Zur Trennung der bei der Wackenroderschen Reaktion entstehenden Polythionate benutzten BLASIUS und Mitarbeiter[1,2] die Hochspannungspapierionophorese.

Die Instabilität der höheren Selenopolythionate erschwerte bisher eine genaue Analyse dieser Verbindungen[3], so dass die wenigen in der Literatur gemachten Angaben zum Teil stark veraltet sind und zum anderen sich oft widersprechen[4–8].

In der vorliegenden Arbeit werden die bei Reaktion von seleniger Säure mit schwefliger Säure entstehenden Selenopolythionate hochspannungspapierionophoretisch getrennt. Ein Verfahren zur Identifizierung der isolierten Produkte wird ausgearbeitet.

## TRENNUNG DER SUBSTANZEN

Hinsichtlich der Trennmethode wird auf frühere Veröffentlichungen verwiesen[1,2].

Da die Selenopolythionate in sauren Lösungen beständiger sind als in alkalischen[9], werden die in Tabelle I angeführten Puffer verwendet.

TABELLE I

| *pH-Wert* | *Zusammensetzung* |
|---|---|
| 1.5 | 0.1 *M* $H_2SO_4$ + 0.1 *M* Glykokoll |
| 3 | 0.1 *M* $H_2SO_4$ + 1 *M* Glykokoll |
| 4 | 0.4 *M* $CH_3COONa$ + 0.6 *M* $CH_3COOH$ |
| 7 | 0.6 *M* $CH_3COONa$ |

Die Reihenfolge der Selenopolythionate auf den Pherogrammen ist in allen Puffern die gleiche:

$$SeS_2O_6^{2-} > Se_2S_2O_6^{2-} > SeS_4O_6^{2-} > Se_3S_2O_6^{2-} > Se_4S_2O_6^{2-}$$

Die $R_B$-Werte aller in Frage kommender Ionen bezogen auf $^{36}Cl^-$ sind als Mittelwerte vieler Einzelbestimmungen in Tabelle II zusammengefasst.

TABELLE II

| | *pH 1.5* | *pH 3* | *pH 4* |
|---|---|---|---|
| $SO_4^{2-}$ | 0.75 | 0.81 | 0.75 |
| $S_2O_3^{2-}$ | — | 0.53 | — |
| $SeO_3^{2-}$ | 0.17 | 0.42 | 0.48 |
| $SeS_2O_6^{2-}$ | 0.81 | 0.78 | 0.73 |
| $Se_2S_2O_6^{2-}$ | 0.71 | 0.68 | 0.68 |
| $Se_3S_2O_6^{2-}$ | 0.62 | 0.59 | 0.62 |
| $Se_4S_2O_6^{2-}$ | 0.52 | 0.46 | 0.53 |
| $SeS_4O_6^{2-}$ | — | 0.65 | — |

Der $R_B$-Wert des $SeS_4O_6^{2-}$ wird aus der Reaktion von seleniger Säure mit Thiosulfat ermittelt.

Die Trennung der Selenopolythionate ist mit den angegebenen Puffern (mit Ausnahme von pH 7) ausgezeichnet. Bei pH 4, 3 und 1.5 kommt es zur Ausbildung scharfer Zonen.

Im Puffer pH 4 sind alle Selenopolythionate und $SO_4^{2-}$ voneinander getrennt. Jedoch ist die infolge der hohen Ionenstärke auftretende Joulesche Wärme schwierig abzuführen. Eine unvollständige Trennung zwischen $SeS_2O_6^{2-}$ und $SO_4^{2-}$ wird bei pH 3 beobachtet. Bei pH 1.5 ist eine schlechte Trennung zwischen $Se_2S_2O_6^{2-}$ und $SO_4^{2-}$ zu verzeichnen. Da die $SO_4^{2-}$-Zone innerhalb des Systems der Selenopolythionate liegt, wirkt sich diese bei den späteren Aktivitätsmessungen der Selenopolythionate nachteilig aus. Für die vorliegenden Versuche wird deshalb vorwiegend der Puffer pH 3 verwendet. Aus den geringen Wanderungsunterschieden der Selenopolythionate in den einzelnen Puffern ist zu schliessen, dass die Ladung der einzelnen Ionen identisch ist. Sie liegen vermutlich als $Se_xS_2O_6^{2-}$ vor.

Zur Einschränkung von Sekundärreaktionen[2] ist die Zugabe von Formaldehyd (1 %) zur Pufferlösung günstig. Formaldehyd bildet mit $SO_3^{2-}$, $HSO_3^-$ und $S_2O_5^{2-}$ stabile Anlagerungsprodukte und verhindert weitere Reaktionen mit diesen Ionen auf dem Papier. Die Sulfitgleichgewichte werden "eingefroren", was sich durch das Auftreten scharfer Doppelzonen anzeigt. Andernfalls ergibt Sulfit die zu erwartende steile Frontzone, die in einen langen Schwanz ausläuft.

## IDENTIFIZIERUNG DER SELENOPOLYTHIONATE

Der Nachweis der getrennten Selenopolythionate durch chemische Reaktionen auf dem Papier bereitet häufig Schwierigkeiten. Die Reduktion mittels $SnCl_2$-Lösung und Nachweis des ausgeschiedenen Selens ist bei geringen Mengen zu unempfindlich. Verläuft der Nachweis auf dem Papier positiv, so können die ermittelten $R_B$-Werte zur Identifizierung herangezogen werden. Eindeutige Aussagen lassen sich jedoch nicht machen, da die $R_B$-Werte der Selenopolythionate analog den Polythionaten Schwankungen unterworfen sind. Deshalb ist eine neue Methode zur Identifizierung der Selenopolythionate auf dem Papier ausgearbeitet worden.

Für die mit $^{35}S$ und $^{75}Se$ doppelmarkierten Selenopolythionate wird experimentell ein Quotient ermittelt, in dem im Zähler die $\varepsilon^-$-Aktivität des $^{35}S$ und im Nenner die $\gamma$-Aktivität des $^{75}Se$ erscheint. Der für ein bekanntes Selenopolythionat z.B. $SeS_2O_6^{2-}$ ermittelte Quotient, entspricht einem bestimmten Verhältnis von Schwefel- zu

Selenatomen im Molekül. Der Vergleich der ermittelten Quotienten unbekannter Selenopolythionate mit dem Quotienten des bekannten Selenopolythionats ergibt genaue Aussagen über deren Zusammensetzung.

Zur Messung der $e^-$- und $\gamma$-Aktivitäten muss die Lage der einzelnen Selenopolythionate auf dem Pherogramm bekannt sein. Hierzu wird die Fluoreszenslöschung im U.V. herangezogen. Die Löschflecken werden markiert und anschliessend an diesen Stellen 1 cm breite Streifen aus dem Pherogramm herausgeschnitten. Diese Streifen werden über das Loch eines Präparateschiebers geklebt und die $e^-$-Aktivitäten mit Hilfe eines Endfensterzählrohres (FHZ 15) ausgewertet. Die $\gamma$-Aktivität wird nach Überführung des Papierstreifens in ein Reagenzglas im Bohrloch eines Szintillationszählers (FH 421/Z6) gemessen.

Während die Ermittlung der $\gamma$-Aktivität keinerlei Schwierigkeiten bereitet, können bei der $e^-$-Messung erhebliche Fehler auftreten. Es sind deshalb hier identische Bedingungen zu wählen. U.a. ist darauf zu achten, dass die Pherogramme in gleicher Zeit und bei konstanter Temperatur getrocknet werden und eine Konzentrierung des Puffers durch Abdecken der Puffertröge weitgehend vermieden wird. Eine auf den Pherogrammen zurückbleibende, unterschiedlich starke Salzkruste führt zu einer mehr oder minder starken Negatronen-Absorption. Bei längeren Reaktionszeiten sind die unterschiedlichen Halbwertszeiten des $^{35}S$ (87 d) und des $^{75}Se$ (127 d) zu berücksichtigen. Deshalb sind die erhaltenen Quotienten jeweils auf den Zeitpunkt der ersten Messung umzurechnen.

Die Überprüfung der Ergebnisse wird mit Hilfe des $t$-Testes[10] durch Vergleich des Mittelwertes der Quotienten $\bar{F}$ mit dem Sollwert $u$ vorgenommen. Als statistische Prüfgrösse wird $\tau$ herangezogen:

$$\tau = \frac{(\bar{F} - u)}{s} \sqrt{n}$$

Hierbei bedeuten $S$ die Standardabweichung und $n$ die Anzahl gemessener Proben. Zwischen Soll- und Mittelwert bei den einzelnen Quotienten ist kein Unterschied feststellbar, zumindest ist das Ergebnis statistisch gesichert.

Liegt die Vergleichszone auf dem selben Streifen, so können auch Quotienten, die stärker vom Mittelwert abweichen, zur Auswertung herangezogen werden, da alle Abweichungen gleichsinnig sind.

## REAKTION SCHWEFLIGE SÄURE–SELENIGE SÄURE

Die Reaktion der schwefligen Säure mit seleniger Säure wird in wässriger Lösung bei einem Überschuss an schwefliger Säure bzw. einem solchen an seleniger Säure untersucht. Als erstes Selenopolythionat kann immer $SeS_2O_6^{2-}$ nachgewiesen werden, gemäss:

$$3\ HSO_3^- + HSeO_3^- \rightleftharpoons SeS_2O_6^{2-} + SO_4^{2-} + 2\ H_2O.$$

Als Beispiel für die Reaktionsfolge dient das Gemisch:

0.4 ml 0.2 $M$ $Na_2{}^{35}SO_3$ + 0.1 ml 0.2 $M$ $H_2{}^{75}SeO_3$

mit der spezifischen Aktivität:

$^{35}S$ 0.13 mCi/mg und $^{75}Se$ 0.01 mCi/ml.

Mit dem verwendeten Methandurchflusszähler FH 407 werden auf den folgenden Aktivitätsverteilungskurven nur die mit $^{35}S$ markierten Verbindungen erfasst, so dass $H_2{}^{75}SeO_3$ als $\gamma$-Strahler auf den Pherogrammen nicht in Erscheinung tritt.

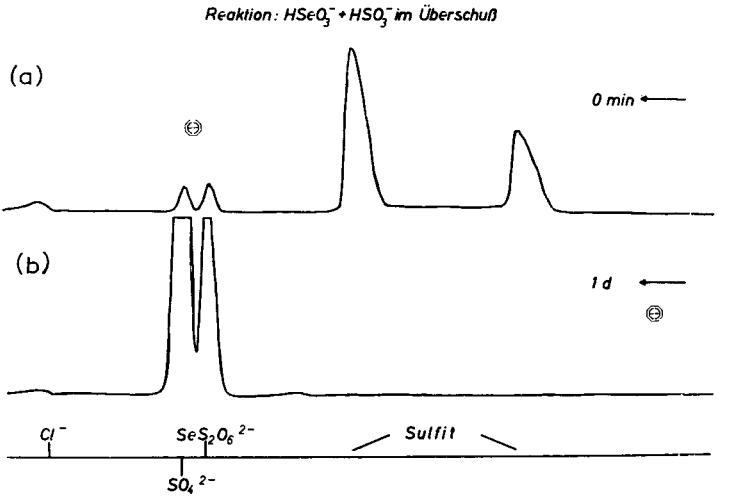


Fig. 1. Reaktion schweflige Säure–selenige Säure. Papierionophorese pH 3.

Fig. 1a gibt die Zusammensetzung zur Zeit "Null" wieder, d.h. bei Probenahme sofort nach dem Mischen der Lösungen. $SeS_2O_6^{2-}$, $SO_4^{2-}$ und die beiden Sulfitzonen sind deutlich zu erkennen. Nach 1 Tag (Fig. 1b) ist kein Sulfit mehr vorhanden. Das Reaktionsgemisch enthält jetzt nur noch $SeS_2O_6^{2-}$, $SO_4^{2-}$ und selenige Säure. Durch Reaktion des $SeS_2O_6^{2-}$ mit seleniger Säure entsteht $Se_2S_2O_6^{2-}$ (Fig. 2a). Nach 14 Tagen (Fig. 2b) wird $Se_3S_2O_6^{2-}$ gut sichtbar.

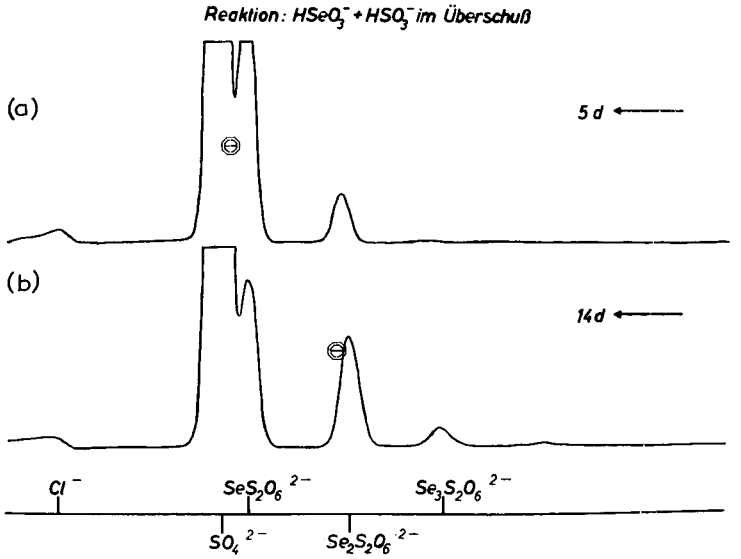


Fig. 2. Reaktion schweflige Säure–selenige Säure. Papierionophorese pH 3.

Nach 21 Tagen (Fig. 3) sind $Se_4S_2O_6^{2-}$ und $Se_5S_2O_6^{2-}$ zu erkennen, wobei letzteres nicht mit Sicherheit nachgewiesen werden konnte.

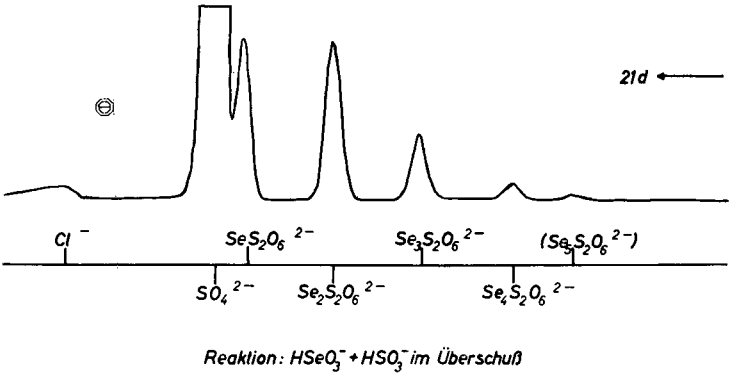


Fig. 3. Reaktion schweflige Säure–selenige Säure. Papierionophorese pH 3.

Somit wird der schrittweise Aufbau der Selenopolythionate bei der Reaktion der schwefligen Säure mit seleniger Säure deutlich.

Die Angabe von SCHULZE[4], dass sich bei einem Überschuss an seleniger Säure Diselenotrithionat bildet, wird nicht bestätigt.

Die Ergebnisse decken sich jedoch weitgehend mit denen von JANITSKII und Mitarbeitern[11]. Jedoch sind die hier verwendeten Trenn- und Identifizierungsmethoden den bisher angewandten Methoden hinsichtlich Aufwand, Zeit und Empfindlichkeit weit überlegen.

## AUSBLICK

Die ausgearbeiteten Trenn- und Bestimmungsmethoden gestatten die Untersuchung von Bildungsreaktionen und Reaktionen der Selenopolythionate untereinander. Somit ergibt sich die Möglichkeit, das bisher wenig bekannte Gebiet der Schwefel-Selen-Verbindungen systematisch zu ergründen. Zudem lassen sich mit den gleichen Methoden die noch weniger bearbeiteten Schwefel-Tellur-Verbindungen mit in die Untersuchung einbeziehen. Hierbei bieten sich $^{132}$Te-markierte Verbindungen an.

## ZUSAMMENFASSUNG

Mit Hilfe der Hochspannungspapierionophorese unter Verwendung saurer Puffer werden die Selenopolythionate erfolgreich getrennt. Für die Bestimmung der getrennten Selenopolythionate wird eine neue Methode ausgearbeitet, die auf der Doppelmarkierung mit $^{35}$S und $^{75}$Se beruht.

Bei Verfolgung der Reaktion von schwefliger mit seleniger Säure können auf den Pherogrammen zeitlich nacheinander $SeS_2O_6^{2-}$, $Se_2S_2O_6^{2-}$, $Se_3S_2O_6^{2-}$ und $Se_4S_2O_6^{2-}$ nachgewiesen werden.

Für die angegebenen Selenopolythionate und $SeS_4O_6^{2-}$ werden die $R_B$-Werte bezogen auf $^{36}Cl^-$ für Puffer von pH 4, 3 und 1.5 ermittelt.

SUMMARY

Selenopolythionates were successfully separated, by means of high-voltage paper ionophoresis, in an acid buffer medium. A new method, based on a double labelling technique with $^{35}S$ and $^{75}Se$, was worked out for the estimation of the separated selenopolythionates.

If the reaction between sulphurous and selenious acid is followed by running pherograms at various time intervals, $SeS_2O_6^{2-}$, $Se_2S_2O_6^{2-}$, $Se_3S_2O_6^{2-}$ and $Se_4S_2O_6^{2-}$ can be detected successively.

The $R_B$ values, with respect to $^{36}Cl^-$, of the above mentioned selenopolythionates and $SeS_4O_6^{2-}$ were determined in buffers of pH 4, 3, and 1.5.

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## Notes

### Evaluation of hydrogen-bond energies by means of gas–liquid chromatography

In the paper by IOGANSEN, KURKCHI AND LEVINA[1], presented under the above title at the 6th International Symposium on Gas Chromatography in Rome, a simple approach to the evaluation of hydrogen-bond energies has been suggested. The controversy between the consistency claimed for the results obtained and the apparent inconsistency in the theoretical approach used deserve closer inspection.

The attempt at the determination of the H-bond energy from chromatographic data seems promising; but owing to the complexity of molecular interactions, especially in polar systems, and to the fact, that chromatographic data reflect all types of interaction along with statistical effects, it is unlikely that such a simple procedure as has been presented will elucidate the contribution of individual types of interaction. It would be more appropriate to speak of rough estimation or of correlation of the heats of mixing rather than of the evaluation of H-bond energies. Thus, it is questionable whether the so-called nonspecific interactions, involving, for the systems discussed, the contribution of inductive and dispersion forces, may be assumed the same both in polar electron donating and in nonpolar media. $\Delta H_{ib}$, defined as $\Delta H_v$ (liquid $b$) $-\Delta H_v$ (liquid $i$), will in any case involve the contribution of inductive forces, which are playing a role only in liquid $b$. Moreover, the Raoult's law activity coefficient involves a geometric term, a distribution term and an interaction term[2]. On the one hand, the first two terms have been found[3] to be significant contributors to the total excess Gibbs free energy, and on the other hand, they obviously cannot be declared nonspecific.

The procedure for calculating $\Delta H_v$ seems to be ambiguous. The relation $V_g = \bar{v}t'/m$ corresponds to the well known equation:

$$V_g = RT/f^0 M_L \gamma = RT/M_L H\gamma^* \qquad (1)$$

where $f^\circ$ is fugacity of pure solute, $\gamma$ and $\gamma^*$ are, respectively, the Raoult's law and the Henry's law activity coefficients of the solute in the given solvent at temperature $T$, $M_L$ is the molecular weight of the solvent and $H$ is the Henry's law constant. Differentiation of $\ln V_g$, expressed from eqn. (1), with respect to $1/T$, gives:

$$\mathrm{d} \ln V_g/\mathrm{d}(1/T) = (\Delta H_v/R) - T. \qquad (2)$$

Hence for the determination of $\Delta H_v$, as used in the earlier paper[1], it is necessary to use $V_g$ $(273/T)$, *i.e.*, $V_g^\circ$, for:

$$V_g^0 = R\,273/f^0 M_L \gamma = R\,273x/M_L P y \qquad (3)$$

where $x$ and $\gamma$ are the mole fractions of solute in the gas and liquid phases and $P$ is the total pressure of the sorption system.

and therefore:

$$\mathrm{d} \ln V_g^0/\mathrm{d}(1/T) = \Delta H_v/R. \quad (4)$$

The calculation of $\Delta S_v$ is not clear either. It is well known that:

$$\Delta G^0 = -RT \ln K \quad (5)$$

where $K$ is the thermodynamic distribution coefficient defined as the ratio of solute activity in the gaseous and liquid phases, respectively, and $\Delta G^\circ$ is the change of the standard Gibbs free energy per mole of solute. When choosing as standard states the 1 atm perfect gas state for the solute in the gas phase and an extremely dilute solute in the solvent for the solute in the liquid phase, it may be written as[4]:

$$K = Py/x. \quad (6)$$

Combining eqns. (3), (5) and (6) we obtain for the standard molar entropy change:

$$\Delta S_v^0 = \Delta H_v^0/T + R \ln (R\,273/V_g^0 M_L),$$

which relation can hardly be correlated with:

$$4.57 \times \log V_g = \Delta S_v - (\Delta H_v/T).$$

Concerning the relation between reaction enthalpy and equilibrium constant $K_a$, it is necessary to keep in mind that the relation $-\Delta H_a = R\,\mathrm{d}\ln K_a/\mathrm{d}(1/T)$ becomes valid only when $\Delta H_a$ is a change of standard enthalpy, which makes it necessary to define the standard states. $K_a$ should also be defined by activities rather than by concentrations, which will both differ for the two systems discussed.

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## Comments on the standard addition method used in quantitative gas chromatographic analysis

Quantitative analysis by gas chromatography, using the standard addition method, is based on the relation between the chromatogram of the initial sample under analysis and the chromatogram of a blend of this sample and a defined added

quantity of the component to be determined. There are three versions of this well-known technique: (1) The areas (or heights) of chromatographic peaks of the component to be determined and the corresponding injected quantities of sample are measured; (2) peak areas (or heights) of the compound to be determined and the peak areas (or heights) of an appropriate reference compound, already present in the initial sample, are measured; (3) the same procedure is used as under (2), but the auxiliary reference compound is added to the initial sample.

In the last mentioned two cases, the auxiliary reference substance serves as a measure of the quantity of sample injected. General relations between the amount of substance analysed, present in the chromatographic zone and the area of the corresponding chromatographic peak[1], and mass balance, lead to the following equations:

*Method (1)*

$$g_i = \frac{W_s}{W_{(i)}} \frac{100}{\frac{A_i^* \cdot w_{(i)}}{A_i \cdot w_{(i)}^*} 1 + \frac{W_{(s)}}{W_{(i)}} - 1} \tag{1}$$

where $g_i$ is the weight per cent of component $i$ to be determined, $W_{(i)}$ and $W_s$ are the weight of the initial sample and that of the standard substance added (as pure component $i$), $A_i^*$ and $A_i$ are the respective peak areas (heights) of the component under analysis corresponding to the injection of a sample–standard blend and of the initial sample, $w_{(i)}^*$ and $w_{(i)}$ are the weight of the sample–standard mixture and the weight of the initial sample introduced.

*Method (2)*

$$g_i = \frac{W_s}{W_{(i)}} \frac{100}{\frac{A_i^* \cdot A_p}{A_i \cdot A_p^*} - 1} \tag{2}$$

where, in addition to the symbols already defined, $A_p^*$ and $A_p$ are, respectively, the peak area (height) of the auxiliary reference compound corresponding to the injection of the sample–standard mixture and that pertaining to the injection of the initial sample.

*Method (3)*

The relation for calculation of $g_i$ is identical with eqn. (2); the values of $A_i^*$, $A_p^*$ and $A_i$, $A_p$ correspond, however, to the injection of the sample–auxiliary substance–standard mixture, and to the injection of a mixture of sample and auxiliary substance only.

It is worth mentioning that the corresponding equations quoted in the literature, are mostly erroneous including those used several years ago by the second author[2] of this paper. The only exception to this general inconsistency is the relation given by DAL NOGARE AND JUVET[3], which is correct and equivalent to eqn. (1). Besides this relationship, one can find in the literature the eqn.[2]

$$g_i = \frac{100\, g_s A_i}{A_i^* - A_i} \qquad \text{(a)}$$

which has been declared equal to (1), provided that the injections $w_i$ and $w_i^*$ are of identical weight; $g_s$ in (a) represents the weight per cent of the standard added to the mixture of sample and standard.

The equation:

$$g_i = \frac{100\, g_s A_i/A_p}{(A_i^*/A_p^*) - (A_i/A_p)} \qquad \text{(b)}$$

has also been suggested[2] as equivalent to (2). Relations (a) and (b) have been adopted by other authors[4,5]. In another paper[6] the equation:

$$g_i = g_s A_i/(A_i^* + A_i) \qquad \text{(c)}$$

is quoted as equivalent to (a), and:

$$g_i = \frac{g_s A_i/A_p}{(A_i^*/A_p^*) - (A_i/A_p)} \qquad \text{(d)}$$

as equivalent to (2).

In addition to eqns. (a)–(d) one can find the following equations[7,8]:

$$g_i = \frac{100\, g_s A_i}{(A_i^* A_p/A_p^*)\,(100 - g_s)} \qquad \text{(e)}$$

and

$$g_i = \frac{W_s}{W_i}\,\frac{100\, A_i}{A_i^* - A_i(A_p^*/A_p)} \qquad \text{(f)}$$

The errors introduced by using relations (a)–(f) can be illustrated by the following simple model.

Let us analyse a mixture containing by weight 25 % benzene, 25 % toluene and 50 % xylene. Let benzene be the component to be determined and toluene the auxiliary reference substance. When mixing the sample to be analysed with the standard (pure benzene) in the ratio of 1 : 1 by weight and injecting the same quantities (on a weight basis) of the initial sample and its blend with the standard substance, then, under these conditions, the products of peak area and appropriate correction factors are proportional to the weight percentages of the corresponding compounds. Let us, therefore, introduce directly weight percentages. A simple calculation using the above relations furnishes data, summarized in Table I.

$f_b$, $f_t$ and $f_x$ are conversion factors enabling one to express the peak areas of benzene, toluene and xylene in terms of quantitative data on a weight basis; $g_i^*$ stands for the weight percentages of the corresponding components in the mixture of initial sample with added standard. In Table II, the results are listed for the $g_i$ of benzene, as calculated according to the above equations.

In cases (a) and (b), multiplication of the numerator by 100 is obviously superfluous and the relations are, nevertheless, incorrect. Correct results (agreeing

TABLE I

NUMERICAL DATA SUBSTITUTED FOR THE RESPECTIVE QUANTITIES INTO EQUATIONS (1), (2), (a)–(f)

| | $g_i$ | $g_i^*$ | $A_i$ | $A_i^*$ | $A_p$ | $A_p^*$ |
|---|---|---|---|---|---|---|
| Benzene | 25 | 62.5 | $25f_b$ | $62.5f_b$ | | |
| Toluene | 25 | 12.5 | $25f_t$ | $12.5f_t$ | $25f_t$ | $12.5f_t$ |
| Xylene | 50 | 25 | $50f_x$ | $25f_x$ | | |

$W_s/W_{(i)} = 1$; $g_s = 50$.

TABLE II

THE RESULTS OBTAINED BY SUBSTITUTING THE NUMERICAL DATA FROM TABLE I INTO THE RESPECTIVE EQUATIONS

| | *Equation* | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | (1) | (2) | (a) | (b) | (c) | (d) | (e) | (f) |
| $g_i$ | 25 | 25 | 3333 | 1250 | 14.3 | 12.5 | 20.0 | 50 |

with the above assumptions) are obtained only when using eqns. (1) or (2) for the calculation, and, of course, also when proceeding according to the equation given by DAL NOGARE AND JUVET[3].

It is of interest to note how long these same and relatively obvious errors can be referred to, and what diverse forms they may acquire. A similar problem has been already pointed out in polarography[9], where the standard addition method is used more often than in gas chromatography. The standard addition method is advantageous, mainly when chromatographing a very complex mixture where no free space remains available for the peak of the standard, or for eliminating efficiently the socalled "matrix effects" (*cf.* ref. 10).

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## Gas chromatographic analysis of some isomeric phenyl-substituted perfluoro-olefins

In the course of an investigation[1] involving the fluoride ion catalyzed isomerization of terminal polyfluorinated olefins to mixtures of *cis* and *trans* internally substituted olefins (eqn. 1), it became necessary to separate and analyze isomeric

$$CF_2{=}C(C_6H_5){-}CF_2CF_3 + F^{\ominus} \longrightarrow CF_3C(C_6H_5){=}CFCF_3 + F^{\ominus} \quad (1)$$

*cis–trans*

mixtures of the terminal, *cis*- and *trans*-perfluoro-olefins by gas–liquid chromatography (*cf.* Table I for physical properties of these olefins). The problem consisted primarily in the separation of the *cis* and *trans* isomers of 2-phenylheptafluoro-2-butene (compounds 1 and 3) and 2-phenylnonafluoro-2-pentene (compounds 4 and 5) respectively; and separation of a mixture consisting of the terminal 2-phenylheptafluoro-1-butene (compound 2) and the *cis* and *trans* isomers of 2-phenylheptafluoro-2-butene (1 and 3).

Although several columns gave some partial separation, silicone gum rubber, fluorosilicone rubber, Carbowax 20 M, and $\beta,\beta'$-oxydipropionitrile gave moderate to good separation of the *cis*- and *trans*-2-phenylheptafluoro-2-butene and 2-phenylnonafluoro-2-pentene isomers. However, only the use of $\beta,\beta'$-oxydipropionitrile (Fig. 1) and fluorosilicone rubber (Fig. 2) as liquid phases gave satisfactory separation of the three isomers of 2-phenylheptafluorobutenes (compounds 1, 2 and 3).

TABLE I

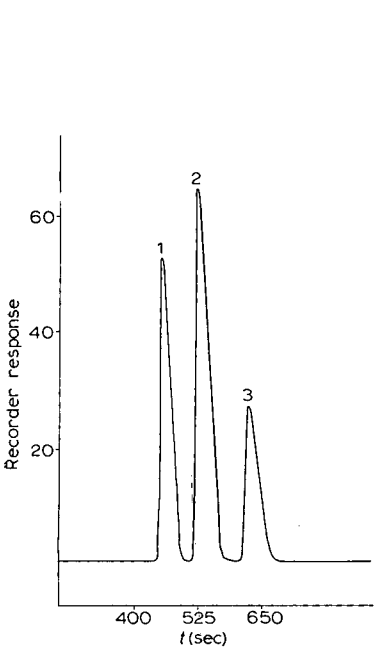
PHENYL SUBSTITUTED PERFLUORO-OLEFINS

$$H_5C_6(A)C{=}C(B)(C)$$

| *Compound No.* | *A* | *B* | *C* | *B.p. °C (mm)* | $n_D^{20}$ |
|---|---|---|---|---|---|
| 1 | $CF_3$ | F | $CF_3$ | 140–141 (740) | 1.4049 |
| 2 | $C_2F_5$ | F | F | 138–139 (737) | 1.4032 |
| 3 | $CF_3$ | $CF_3$ | F | 133–134 (740) | 1.4004 |
| 4 | $CF_3$ | $C_2F_5$ | F | 145–146 (730) | 1.3872 |
| 5 | $CF_3$ | F | $C_2F_5$ | 150–151 (739) | 1.3918 |

*Experimental and results*

Carbowax 20 M (F & M Scientific) and fluorosilicone rubber (Dow Corning) were used to prepare 10 % w/w packings on 100–120 mesh Gas Chrom P (Applied Science Lab.) and packed into a 10 ft. length of ¼ in. O.D. copper tube and coiled. $\beta,\beta'$-Oxydipropionitrile (F & M Scientific) was used to prepare a 10 % w/w packing on 80–100 mesh Chromosorb P (Applied Science Lab.) and packed into a 10 ft. length of $^1/_4$ in. O.D. copper tube and coiled. Silicone gum rubber (F & M Scientific) was used to prepare a 10 % w/w packing on 100–120 mesh Gas Chrom P and packed into a



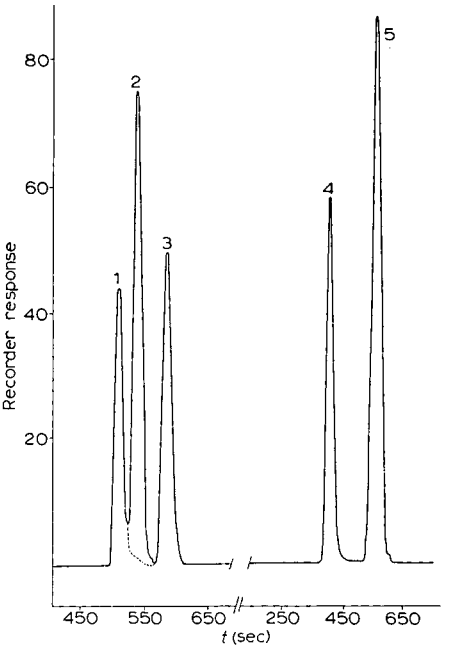




Fig. 1. Chromatogram of fluoro-olefins on $\beta,\beta'$-oxydipropionitrile. The numbers refer to those given in Table I.

Fig. 2. Chromatogram of fluoro-olefins on fluorosilicone rubber. The numbers refer to those given in Table I.

6 ft. length of $^1/_4$ in. O.D. copper tube and coiled. All chromatograms were obtained on an F & M Model 720 gas chromatograph with a helium flow rate of 60 ml/min with a pressure drop of 20 to 30 p.s.i. across the column. The sample size in all cases was 0.003 ml.

The results of the separations are given in terms of retention times (Table II) and the chromatograms of the fluoro-olefins are shown in Figs. 1 to 4. The dotted lines in Figs. 2, 3 and 4 indicate the separation of the *cis*- and *trans*-2-phenylhepta-

TABLE II

RETENTION TIMES OF FLUORO-OLEFINS

| *Packing* | *Temperature (°C)* | *Retention time (sec) of olefins* | | | | |
|---|---|---|---|---|---|---|
| | | *1* | *2* | *3* | *4* | *5* |
| Silicone gum rubber | 60 | 188 | 236 | 236 | — | — |
| Silicone gum rubber | 70 | — | — | — | 160 | 215 |
| Fluorosilicone rubber | 110 | 493 | 535 | 605 | — | — |
| Fluorosilicone rubber | 120 | — | — | — | 439 | 542 |
| Carbowax 20 M | 100 | 210 | 245 | 270 | — | — |
| Carbowax 20 M | 110 | — | — | — | 185 | 234 |
| $\beta,\beta'$-Oxydipropionitrile | 70 | 431 | 521 | 632 | — | — |

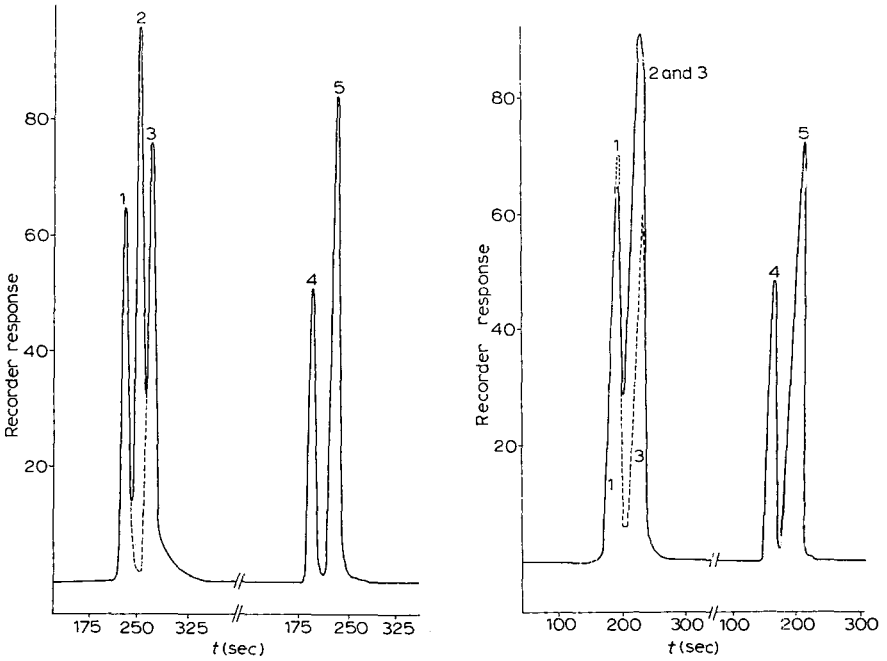



Fig. 3. Chromatogram of fluoro-olefins on Carbowax 20 M. The numbers refer to those given in Table I.

Fig. 4. Chromatogram of fluoro-olefins on silicone gum rubber. The numbers refer to those given in Table I.

fluoro-2-butene isomers on these columns, respectively, in the *absence* of the terminal olefin, 2-phenylheptafluoro-1-butene.

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## Gas chromatography of fluorinated fatty acids

## II. Separation and identification of the methyl esters of 2-fluorofatty acids to 18 carbons

A rationale for our interest in the 2-fluorofatty acids has previously been reported[1]. In that paper, a gas chromatographic method was presented for the separation and identification of those free acids to 6 carbons and in combination with a similar series of unfluorinated aliphatic fatty acids. Since that time, GERSHON AND PARMEGIANI[2] have reported on the preparation and antifungal properties of additional members of the 2-fluorofatty acid series to 20 carbon atoms. Of further interest are the results of PATTISON, BUCHANAN AND DEAN[3], who reported the comparatively low mammalian toxicity of 2-fluorofatty acids, and that this was due to their inability to undergo $\beta$-oxidation.

The present report is concerned with a gas chromatographic study of the methyl esters of these acids alone and together with a similar series of methyl esters of unfluorinated fatty acids.

*Experimental*

*Apparatus.* All separations were carried out in an Aerograph Model 204 gas chromatograph, fitted with a flame ionization detector. The column employed was 5 ft. $\times$ $\frac{1}{8}$ in. O.D. stainless steel tube packed with 5 % diethyleneglycol succinate (DEGS) on acid washed Chromosorb W (80/100 mesh) with a flow rate of nitrogen of 25 ml/min. Retention data were obtained under isothermal conditions at two different temperatures. For the lower fatty acid esters, the column temperature was maintained at 85°, and the detector and injector temperatures were 100° and 140°, respectively. The higher fatty acid esters were chromatographed at a column temperature of 180° with the detector temperature at 205° and the injector temperature at 250°.

The mixture of the methyl esters of the 2-fluorofatty acids was separated using linear temperature programming at 5°/min from 100° to 200°, after which, isothermal conditions were maintained. The complete mixture of the methyl esters of the fluorinated and unfluorinated fatty acids was separated as above, except that the initial temperature was 75°. The detector temperature was kept at 210°, and the injector temperature was 220° in both cases.

*Compounds.* The methyl esters of the unfluorinated fatty acids were commercially available and the preparation of the 2-fluorofatty acids was according to the method of GERSHON AND PARMEGIANI[2]. Esterification of the 2-fluorofatty acids was performed by means of methanolic boron trifluoride[4]. All of the fluorinated fatty acid esters were purified by preparative gas chromatography in an Aerograph Autoprep Model A-700, and acetone solutions of the compounds were employed for injection into the chromatographs.

*Results and discussion*

Table I contains the analytical data characterizing the methyl esters of the 2-fluorofatty acids. A chromatogram of the separation of the methyl esters of the 2-fluorofatty acids can be seen in Fig. 1, and the gas chromatographic separation of the combined mixture of methyl esters of fatty acids and 2-fluorofatty acids is shown

TABLE I

ANALYTICAL DATA FOR METHYL ESTERS OF 2-FLUOROFATTY ACIDS

| *Methyl ester of* | $n_D^{25}$ *or m.p.* | $\nu_{max}^{C=O}$ | *Formula* | *Calculated* (%) | | | *Found* (%) | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | *C* | *H* | *F* | *C* | *H* | *F* |
| Fluoroacetic acid[a] | 1.3634[c] | 1778, 1758 | | | | | | | |
| 2-Fluoropropionic acid[a,b] | 1.3708[d] | 1772, 1758 | | | | | | | |
| 2-Fluorobutyric acid[b] | 1.3795[e] | 1772, 1758 | $C_5H_9FO_2$ | 49.93 | 7.55 | 15.82 | 49.83 | 7.45 | 15.98 |
| 2-Fluorovaleric acid[b] | 1.3888[f] | 1772, 1756 | $C_6H_{11}FO_2$ | 53.72 | 8.27 | 14.16 | 53.91 | 8.44 | 14.01 |
| 2-Fluorohexanoic acid | 1.3961 | 1778, 1754 | $C_7H_{13}FO_2$ | 56.74 | 8.84 | 12.82 | 56.21 | 8.72 | 12.39 |
| 2-Fluoroheptanoic acid | 1.4027 | 1775, 1754 | $C_8H_{15}FO_2$ | 59.24 | 9.32 | 11.71 | 59.40 | 9.28 | 11.96 |
| 2-Fluorooctanoic acid | 1.4091 | 1772, 1752 | $C_9H_{17}FO_2$ | 61.29 | 9.72 | 10.78 | 61.44 | 9.73 | 10.54 |
| 2-Fluorononanoic acid | 1.4131 | 1778, 1758 | $C_{10}H_{19}FO_2$ | 63.13 | 10.07 | 9.99 | 63.16 | 10.04 | 9.81 |
| 2-Fluorodecanoic acid | 1.4175 | 1778, 1755 | $C_{11}H_{21}FO_2$ | 64.67 | 10.36 | 9.30 | 63.77 | 10.23 | 8.93 |
| 2-Fluoroundecanoic acid | 1.4212 | 1778, 1752 | $C_{12}H_{23}FO_2$ | 66.02 | 10.62 | 8.70 | 66.03 | 10.24 | 8.73 |
| 2-Fluorododecanoic acid | 1.4240 | 1774, 1750 | $C_{13}H_{25}FO_2$ | 67.20 | 10.85 | 8.18 | 67.56 | 10.54 | 7.93 |
| 2-Fluorotetradecanoic acid | 1.4298 | 1772, 1758 | $C_{15}H_{29}FO_2$ | 69.19 | 11.23 | 7.30 | 69.02 | 10.88 | 6.95 |
| 2-Fluorohexadecanoic acid | 35–36° | 1756 | $C_{17}H_{33}FO_2$ | 70.79 | 11 53 | 6.59 | 71.23 | 11.35 | 6 46 |
| 2-Fluorooctadecanoic acid | 37–38° | 1755 | $C_{19}H_{37}FO_2$ | 72.10 | 11.78 | 6.00 | 72.08 | 11.48 | 6.00 |

[a] Previously prepared, lit. refs. 5 and 6.
[b] Previously prepared, lit. ref. 7.
[c] Lit. ref. 6, $n_D^{20}$ 1.3679.
[d] Lit. ref. 7, $n_D^{20}$ 1.3707.
[e] Lit. ref. 7, $n_D^{20}$ 1.3809.
[f] Lit. ref. 7, $n_D^{20}$ 1.3920.

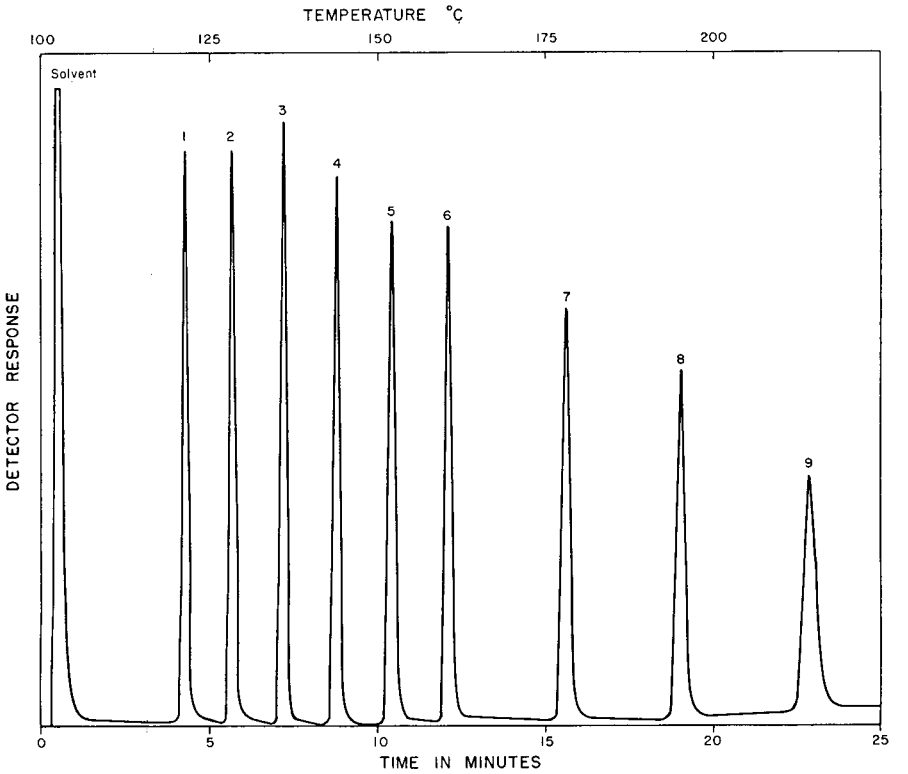


Fig. 1. Gas chromatogram of methyl esters of 2-fluorofatty acids resulting from linear temperature programming at 5°/min from 100° to 200°, after which isothermal conditions were maintained. The components are: 1 = methyl 2-fluoroheptanoate; 2 = methyl 2-fluorooctanoate; 3 = methyl 2-fluorononanoate; 4 = methyl 2-fluorodecanoate; 5 = methyl 2-fluoroundecanoate; 6 = methyl 2-fluorododecanoate; 7 = methyl 2-fluorotetradecanoate; 8 = methyl 2-fluorohexadecanoate; 9 = methyl 2-fluorooctadecanoate.

TABLE II

ISOTHERMAL RETENTION DATA FOR METHYL ESTERS OF FATTY ACIDS AND 2-FLUOROFATTY ACIDS TO EIGHT CARBON ATOMS

| *Methyl ester of* | *Relative time** |
|---|---|
| Acetic acid | 0.20 |
| Propionic acid | 0.25 |
| Butyric acid | 0.35 |
| 2-Fluoropropionic acid | 0.51 |
| 2-Fluoroacetic acid | 0.56 |
| 2-Fluorobutyric acid | 0.77 |
| Hexanoic acid | 1.00 |
| 2-Fluorovaleric acid | 1.29 |
| 2-Fluorohexanoic acid | 2.25 |
| Octanoic acid | 3.35 |
| 2-Fluoroheptanoic acid | 4.12 |
| 2-Fluorooctanoic acid | 7.74 |

* The values are retention times relative to methyl hexanoate. The observed value for this reference standard was 3.1 min at 85°.

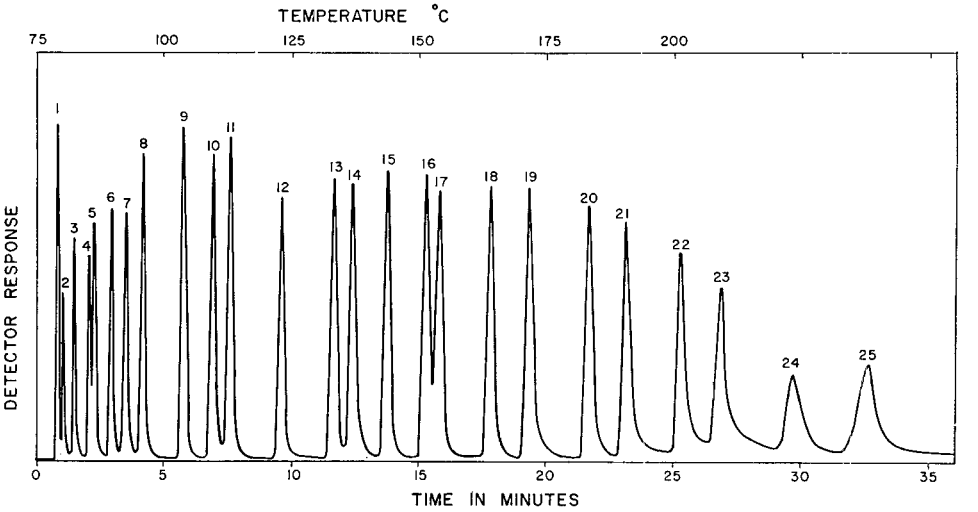


Fig. 2. Gas chromatogram of a mixture composed of methyl esters of fatty acids and methyl esters of 2-fluorofatty acids resulting from linear temperature programming at 5°/min from 75° to 200° after which isothermal conditions were maintained. The components are: 1 = methyl acetate; 2 = methyl propionate; 3 = methyl butyrate; 4 = methyl 2-fluoropropionate; 5 = methyl 2-fluoroacetate; 6 = methyl 2-fluorobutyrate; 7 = methyl hexanoate; 8 = methyl 2-fluorovalerate; 9 = methyl 2-fluorohexanoate; 10 = methyl octanoate; 11 = methyl 2-fluoroheptanoate; 12 = methyl 2-fluorooctanoate; 13 = methyl 2-fluorononanoate; 14 = methyl decanoate; 15 = methyl 2-fluorodecanoate; 16 = methyl dodecanoate; 17 = methyl 2-fluoroundecanoate; 18 = methyl 2-fluorododecanoate; 19 = methyl tetradecanoate; 20 = methyl 2-fluorotetradecanoate; 21 = methyl hexadecanoate; 22 = methyl 2-fluorohexadecanoate; 23 = methyl octadecanoate; 24 = methyl 2-fluorooctadecanoate; 25 = methyl eicosanoate.

TABLE III

ISOTHERMAL RETENTION DATA FOR METHYL ESTERS OF FATTY ACIDS TO TWENTY CARBON ATOMS AND 2-FLUOROFATTY ACIDS TO EIGHTEEN CARBON ATOMS

| *Methyl ester of* | *Relative time** |
|---|---|
| 2-Fluoroheptanoic acid | 0.10 |
| 2-Fluorooctanoic acid | 0.13 |
| 2-Fluorononanoic acid | 0.15 |
| Decanoic acid | 0.17 |
| 2-Fluorodecanoic acid | 0.21 |
| Dodecanoic acid | 0.26 |
| 2-Fluoroundecanoic acid | 0.28 |
| 2-Fluorododecanoic acid | 0.38 |
| Tetradecanoic acid | 0.49 |
| 2-Fluorotetradecanoic acid | 0.76 |
| Hexadecanoic acid | 1.00 |
| 2-Fluorohexadecanoic acid | 1.56 |
| Octadecanoic acid | 2.08 |
| 9-Octadecenoic acid | 2.08 |
| 9,12-Octadecadienoic acid | 2.30 |
| 9,12,15-Octadecatrienoic acid | 2.69 |
| 2-Fluorooctadecanoic acid | 3.26 |
| Eicosanoic acid | 4.43 |

* The values are retention times relative to methyl hexadecanoate. The observed value for this reference standard was 7.3 min at 180°.

in Fig. 2. Isothermal retention data for the methyl esters of the lower fatty acids and fluorinated fatty acids are included in Table II. Table III contains the corresponding data on the methyl esters of the higher fatty acids and 2-fluorofatty acids.

The chromatograms of Figs. 1 and 2 show that the mixtures of the methyl esters of 2-fluorofatty acids alone and admixed with unfluorinated fatty acids can be separated. For biological studies, it is generally more desirable to employ isothermal conditions, and consequently the retention data of Tables II and III were obtained. Since odd numbered fatty acids are uncommon in biological systems, they were excluded from our study, with the exception of propionic acid. This resulted in less overlapping and more easily interpretable results. Methyl octadecanoate and methyl 9-octadecenoate were not separated under the conditions reported. These esters have been separated on a column containing a higher percentage of liquid phase, but in such a column, overlapping occurs between some of the fluorinated and unfluorinated esters.

It should be noted that methyl fluoroacetate and methyl 2-fluoropropionate came off the column in reversed order as compared with the unfluorinated esters. The same observation was previously reported[1] for the corresponding fluorinated acids. On chromatographing methyl chloroacetate and methyl 2-chloropropionate under conditions similar to those employed for the corresponding fluoroesters, methyl 2-chloropropionate came off the column before methyl chloroacetate. Thus, it appears that this effect may not be peculiar to the fluorinated acids, but may be explained on the basis that the halogenoacetic acids and methyl esters are more polar than the halogenopropionic acids and methyl esters, and that on a polar column the polar effect exceeds the effect due to the boiling point.

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## Characterization of 3-oxo-$\Delta^4$-steroids by gas chromatography of enol heptafluorobutyrates

Characterization of hydroxysteroids by esterification or etherification, followed by gas chromatography is well documented in the literature. Similar methods for the characterization of oxosteroids are not so widespread. CHAMBERLAIN AND THOMAS[1,2] have suggested reduction with lithium aluminium hydride while VANDENHEUVEL AND HORNING[3] have proposed derivative formation with N,N-dimethylhydrazine. Recently ZMIGROD AND LINDNER[4] have reported the gas chromatography of oxosteroids as ethylene thioketals. This note is intended to demonstrate the potential of enol heptafluorobutyration for the specific characterization of 3-oxo-$\Delta^4$-steroids.

CLARK AND WOTIZ[5] first demonstrated that the heptafluorobutyrates of several hydroxysteroids were extremely sensitive to electron capture detection. Subsequently KNIGHTS[6] in discussions with WOTIZ and the present author pointed out that the conditions used for heptafluorobutyration would favour the formation of $\Delta^{3,5}$-enols from 3-oxo-$\Delta^4$-steroids. Recent reports on heptafluorobutyration of hydroxysteroids have described milder conditions[7,8].

In the present study conditions similar to those first reported by CLARK AND WOTIZ[5] were used. The steroid (*ca.* 2 mg) was dissolved in benzene (0.1 ml) and heptafluorobutyric anhydride (0.1 ml) and heated in a stoppered tube at 65° for 30 min. Excess reagent and solvent were removed by heating *in vacuo* at 100° for 1–2 min. The residue was dissolved in acetone for gas chromatography.

The free steroids and their derivatives were chromatographed on 1% SE-30 at 190° and on 3% QF-1 at 240° using a Pye series 104 gas chromatograph equipped

TABLE I

$\Delta R_{Mr}$ (HEPTAFLUOROBUTYRATION) VALUES FOR VARIOUS OXYSTEROIDS

| | *Compound* | $\Delta R_{Mr}$ (*heptafluorobutyration*) | |
|---|---|---|---|
| | | *3% QF-1 (240°)* | *1% SE-30 (190°)* |
| 1 | Androst-4-ene-3,17-dione | −0.49 | −0.15 |
| 2 | Androst-4-ene-3,11,17-trione | −0.49 | −0.14 |
| 3 | Pregn-4-ene-3,20-dione | −0.41 | −0.22 |
| 4 | Cholest-4-en-3-one | −0.53 | −0.19 |
| 5 | 17α-Acetoxyandrost-4-en-3-one | −0.47 | −0.14 |
| 6 | 17β-Acetoxyandrost-4-en-3-one | −0.51 | −0.14 |
| 7 | 20α-Hydroxypregn-4-en-3-one | −0.48 | −0.16 |
| 8 | 20β-Hydroxypregn-4-en-3-one | −0.48 | −0.23 |
| 9 | 17α-Hydroxyandrost-4-en-3-one | −0.55 | −0.27 |
| 10 | 17β-Hydroxyandrost-4-en-3-one | −0.45 | −0.19 |
| 11 | 3α-Hydroxy-5β-androstan-17-one | −0.03 | −0.09 |
| 12 | 3α-Hydroxy-5α-androstan-17-one | −0.06 | −0.13 |
| 13 | 3β-Hydroxy-5α-androstan-17-one | 0.00 | +0.02 |
| 14 | 3β-Hydroxycholest-5-ene | −0.01 | −0.04 |
| 15 | 3β-Hydroxyandrost-5-en-17-one | +0.03 | 0.00 |
| 16 | 3β-Hydroxypregn-5-en-20-one | 0.00 | −0.02 |
| 17 | 3-Methyloestradiol | 0.00 | −0.06 |
| 18 | Oestrone | −0.09 | −0.14 |
| 19 | Oestradiol | −0.13 | −0.17 |

with a flame-ionization detector. Single peaks were obtained in all cases, with the exception of the oestrone and oestradiol derivatives which were formed in only 50% yield.

Table I lists the $\Delta R_{Mr}$(heptafluorobutyration) values obtained on both columns. This parameter may be defined as the change in the logarithm of the retention time, produced by the esterification reaction[9,10].

On both columns the $\Delta R_{Mr}$ values for heptafluorobutyration of hydroxysteroids are small (compounds Nos. 11–17). Presumably the larger molecular weight of the derivative is approximately balanced by its increased volatility. $\Delta R_{Mr}$ values for 3-oxo-$\Delta^4$-steroids are significantly higher (Nos. 1–6), although the effect is more marked with the selective column (QF-1). Phenolic hydroxyl groups (Nos. 18, 19) apparently behave as enols on SE-30 and as secondary hydroxyl groups on QF-1. The low $\Delta R_{Mr}$ value for hydroxyl groups means that the same $\Delta R_{Mr}$ value would be found for 3-oxo-$\Delta^4$-steroids containing saturated hydroxyl groups (Nos. 7–10).

These results suggest a method for the specific characterization of 3-oxo-$\Delta^4$-steroids. A $\Delta R_{Mr}$ (heptafluorobutyration) value on QF-1, of —0.55 to —0.40 would suggest the presence of this functional group. The extreme sensitivity of the enol heptafluorobutyrates compared with the corresponding derivatives of saturated hydroxyl groups, and their suitability for the analysis of biologically important steroids will be discussed elsewhere.

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## Gas–liquid chromatography of steroid glucuronosides

Among the biologically important steroids amenable to gas–liquid chromatographic (GLC) analysis are the 17-ketosteroids, estrogens, testosterone and pregnanediol[1,2]. Certain of these compounds, the urinary levels of which reflect endocrine activity, are conjugated with glucuronic acid prior to their excretion. Methods for the determination of these steroids in urine usually call for chemical or enzymic hydrolysis of the conjugates to the free steroids which may then be determined by GLC, often as the trimethylsilyl (TMSi) ethers[1,2]. Although the size and complexity of the conjugated steroids themselves might be thought to preclude their direct separation by GLC, other types of compounds which appeared to present insurmountable challenges to this technique have been chromatographed successfully. For example, triglycerides of from 50 to 62 fatty acid carbon atoms have been separated[3], and a tetrasaccharide transformed to its fully trimethylsilylated ether (molecular weight 1676) has been eluted from a GLC column[4]. What may appear to be an excessively high molecular weight will not necessarily lead to an impractically long retention time, since it is the free energy of solution, rather than molecular weight, which determines retention behavior. Further, through the use of derivative formation compounds which possess exceedingly low vapor pressures may be converted to more volatile substances.

With this as a background, and using androstan-17-one-3α-yl-β-D-glucopyranosiduronic acid and 5-androstene-17-on-3β-yl-β-D-glucopyranosiduronic acid as model compounds, an investigation was carried out to determine the feasibility of the GLC of steroid glucuronosides.

Methylation of the carboxyl group (diazomethane in methanol–ether) was followed by trimethylsilylation of the hydroxyl groups of the sugar portion of the molecule by reaction with hexamethyldisilazane and trimethylchlorosilane in pyridine[4]. The GLC behavior of the reaction products and several reference standards is presented in Table I. The steroid glucuronoside derivatives are retained much longer than their parent steroids with the non-polar stationary phase SE-30 at 250°, a

TABLE I

| *Compound* | *Relative retention time*[a] | |
|---|---|---|
| | *SE-30* | *NGS* |
| Androstan-3α-ol-17-one | 1.00 | 1.00 |
| Androstan-3α-ol-17-one-TMSi | 0.94 | 0.22 |
| Androstan-17-one-3α-yl-β-D-glucopyranosiduronic acid methyl ester tri-trimethylsilyl ether[b] | 15.8 | 4.72 |
| 5-Androsten-3β-ol-17-one | 1.00 | 1.18 |
| 5-Androsten-3β-ol-17-one-TMSi | 1.15 | 0.34 |
| 5-Androsten-17-one-3β-yl-β-D-glucopyranosiduronic acid methyl ester tri-trimethylsilyl ether[c] | 29.0 | 11.0 |

[a] Experimental conditions: 1.5% SE-30 on 100-120 mesh Gas-Chrom P; 6 ft. × 4 mm glass U-tube; 250°; 18 p.s.i.; absolute retention time of androstan-3α-ol-17-one, 1.6 min. 2% NGS on 80-100 mesh Gas-Chrom P; 3 ft. × 4 mm glass U-tube; 240°; 11 p.s.i.; absolute retention time of androstan-3α-ol-17-one, 2.7 min.

[b] The parent glucuronoside was purchased from Mann Research Laboratories.

[c] The parent glucuronoside was generously provided by the Medical Research Council (Great Britain) through the Steroid Reference Collection Office of the National Institutes of Health.

reflection of the great differences in molecular weight. The volatility difference between a derivatized steroid glucuronoside and its parent steroid is smaller with the polyester neopentylglycol succinate (NGS) at 240° than with SE-30, probably because the presence of a polar hydroxyl group causes the steroid to interact strongly with the polar phase. Etherification would mask this group and reduce the polarity of the steroid. When such a reduction in polarity is effected by trimethylsilyl ether formation with androstan-3α-ol-17-one and 5-androsten-3β-ol-17-one, large increases in volatility are observed with NGS (Table I). Even with SE-30, a stationary phase generally noted for separations based upon molecular weight[1], the TMSi ether of androstan-3α-ol-17-one is actually eluted faster than the lower molecular weight parent steroid, and trimethylsilylation of 5-androsten-3β-ol-17-one does not lead to a large increase in retention time (see Table I). Indeed, trimethylsilylation results in a much smaller increase in retention time on non-polar phases than formation of butyryl esters, although the associated molecular weight changes are rather similar[1]. It is clear that the TMSi group possesses somewhat anomalous volatility properties. The glucuronosides may be considered steroids etherified by glucuronic acid at the 3-position (like TMSi ethers but with a much larger functional group), and the effect upon volatility of the introduction of such a large function into a steroid is difficult to predict. What is clear, however, is that the derivatized glucuronosides can be chromatographed under conditions which are not radically different from those normally employed in steroid GLC. The separation of the methyl ester tri-trimethylsilyl ether derivatives of the β-D-glucopyranosiduronic acids of androstan-3α-ol-17-one and 5-androsten-3β-ol-17-one with SE-30 is illustrated in Fig. 1, and the theoretical shape of the peaks indicates the excellent GLC properties of these compounds*.

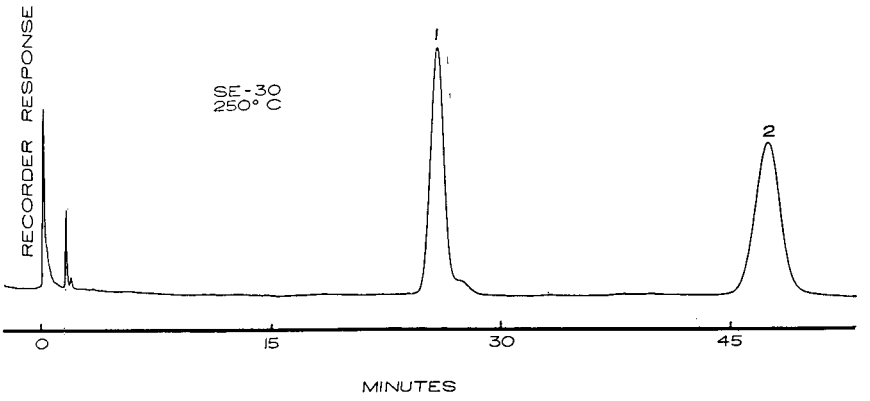


Fig. 1. Gas–liquid chromatographic separation of a mixture of the methyl ester tri-trimethylsilyl ethers of the β-D-glucopyranosiduronic acids of androstan-3α-ol-17-one (peak 1) and 5-androsten-3β-ol-17-one (peak 2). Column conditions given in Table I.

Although the two derivatized steroid glucuronosides differ only by a double bond and the stereochemistry of the substituent group at the 3-position, their retention

* When the products from esterification of the steroid conjugates were analyzed on SE-30 in each case only a small peak of non-theoretical shape was observed (shorter retention times than the fully derivatized compounds). GLC of equivalent amounts of the trimethylsilylated esters resulted in much larger symmetrical peaks.

behavior is widely different. With both stationary phases the derivatized glucuronoside of 5-androsten-3$\beta$-ol-17-one is retained much longer than the derivatized glucuronoside of androstan-3$\alpha$-ol-17-one (see Table I and Fig. 1), and this order of elution is not unexpected. Separation of closely related steroids, especially those differing in the configuration of a hydroxyl group at the 3-position, is generally increased by derivative formation (the axial isomer exhibiting a shorter retention time than the equatorial), the separation factor increasing with the steric requirements of the substituent group[1]. This effect can be seen in Table I, where with SE-30 the parent steroids possess the same retention times, but after trimethylsilylation a large difference in volatility is noted. NGS, a phase selective for equatorial hydroxyl groups and double bonds, retains 5-androsten-3$\beta$-ol-17-one longer than androstan-3$\alpha$-ol-17-one, but the effect is considerably increased by TMSi ether formation, although this separation factor and the corresponding one with SE-30 are much smaller than those observed for the derivatized glucuronosides.

Because of the paucity of the two steroid glucuronosides available to us it was not possible to obtain classical elementary analyses of their derivatives. The GLC behavior observed for these two compounds, however, is consistent with their possessing the assigned structures, and the data obtained by combined GLC–mass spectrometry[5,6] support the proposed chemical nature of the derivatives.

The application of the herein described separation technique to the identification and estimation of steroid glucuronosides in urine was beyond the scope of this investigation*. The essence of this communication is a further demonstration that experimental conditions may be established for the successful GLC of natural products of considerable structural complexity.

We are grateful to Dr. J. A. McCLOSKEY of Baylor University College of Medicine for obtaining and interpreting the mass spectra of the derivatized glucuronosides.

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* Work of this nature is presently being carried out, however (I. JAAKONMÄKI and E. C. HORNING, personal communication).

## Behaviour of 7,8-benzoquinoline as stationary phase in gas–liquid chromatography in the vicinity of the melting point*

7,8-Benzoquinoline has been found[1] to be a good stationary phase for the separation of *p*- and *m*-xylene. However, we have noticed great differences in retention times depending on whether the equilibrium temperature of the column has been approached from a lower or higher temperature. The experimental data obtained with two different instruments are collected in Tables I and II and plotted in Figs. 1 and 2. They were perfectly reproducible provided that the conditions of the heating were the same. Since the largest differences appear in the vicinity of the melting point, but slightly below it, we have investigated by various physical methods the behaviour of 7,8-benzoquinoline near this temperature. First of all, the melting point was checked. The literature values are 52, 51 and 50°[2], but we have observed that a highly purified

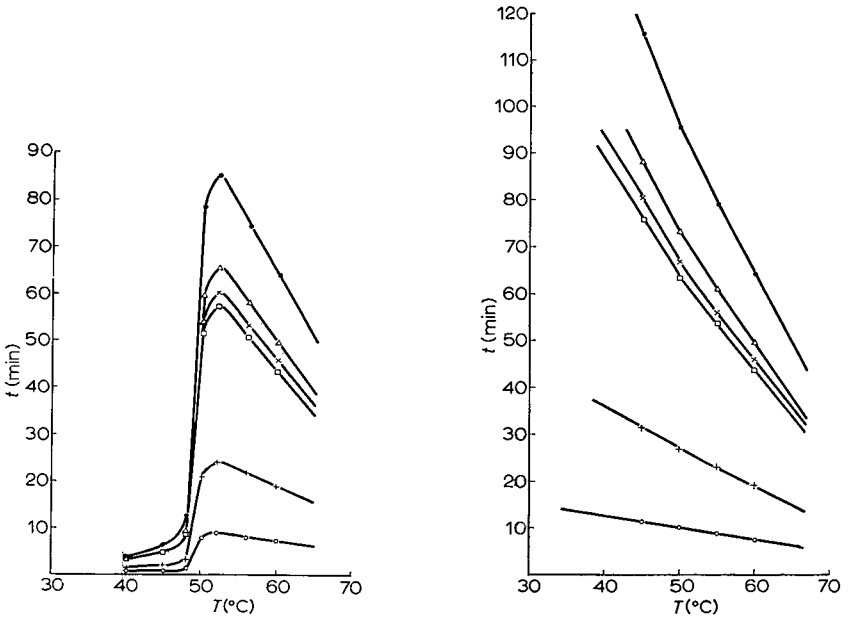


Fig. 1. Relative retention times as a function of increasing column temperature. (O) Benzene; (+) toluene; (□) ethylbenzene; (×) *p*-xylene; (△) *m*-xylene; (●) *o*-xylene.

Fig. 2. Relative retention times as a function of decreasing column temperature. (O) Benzene; (+) toluene; (□) ethylbenzene; (×) *p*-xylene; (△) *m*-xylene; (●) *o*-xylene.

sample actually shows pre-melting at 47° and the complete melting sets in at 52°. The substance regularly becomes undercooled and the crystallisation does not set in before 35°.

Differential thermal analysis shows (Fig. 3) three endothermal transitions with estimated temperatures 47, 52 and 55°. The n.m.r. spectrum is very instructive (Fig. 4). The relative intensity of the central line shows an increase from 47 to 50° with a possible inflection near 47°.

* Paper presented at the Gas Chromatography Discussion, Zagreb, Yugoslavia, November, 1966.

TABLE I

RELATIVE RETENTION TIMES WITH INCREASING COLUMN TEMPERATURE

| *Column temperature (°C)* | *40* | | *45* | | *48* | | *50* | | *52* | | *55* | | *60* | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *Instrument* | *Pye* | *PE 800* | *Pye* | *PE 800* | *Pye* | *PE 800* | *Pye* | *PE 800* | *Pye* | *PE 800* | *Pye* | *PE 800* | *Pye* | *PE 800* |
| *Retention of benzene (min)* | *0.79* | *1.89* | *0.95* | *3.00* | *1.42* | — | *7.90* | *8.53* | *9.00* | — | *8.21* | *7.27* | *7.42* | *6.16* |
| Benzene | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | — | 1.00 | 1.00 | 1.00 | — | 1.00 | 1.00 | 1.00 | 1.00 |
| Toluene | 1.80 | 1.92 | 2.17 | 2.32 | 2.34 | — | 2.66 | 2.52 | 2.68 | — | 2.66 | 2.44 | 2.57 | 2.38 |
| Ethylbenzene | | 4.08 | | 6.48 | 6.11 | — | 6.52 | 5.84 | 6.36 | — | 6.17 | 5.45 | 5.87 | 5.26 |
| *p*-Xylene | 4.00 | 4.75 | 5.34 | | | — | 6.82 | 6.19 | 6.72 | — | 6.48 | 5.78 | 6.20 | 5.56 |
| *m*-Xylene | | | | 7.00 | 6.56 | — | 7.58 | 6.69 | 7.30 | — | 7.04 | 6.24 | 6.68 | 6.00 |
| *o*-Xylene | 5.20 | 6.25 | 7.00 | 9.11 | 8.79 | — | 9.96 | 8.64 | 9.51 | — | 9.14 | 8.09 | 8.66 | 7.75 |

TABLE II

RELATIVE RETENTION TIMES WITH DECREASING TEMPERATURE

| *Column temperature (°C)* | *60* | | *55* | | *50* | | *45* | | *40* | |
|---|---|---|---|---|---|---|---|---|---|---|
| *Instrument* | *Pye* | *PE 800* | *Pye* | *PE 800* | *Pye* | *PE 800* | *Pye* | *PE 800* | *Pye* | *PE 800* |
| *Retention of benzene (min)* | *7.42* | *6.48* | *8.68* | *7.58* | *9.94* | *8.68* | *11.21* | *10.26* | *12.95* | — |
| Benzene | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | — |
| Toluene | 2.57 | 2.34 | 2.64 | 2.44 | 2.68 | 2.56 | 2.79 | 2.65 | 2.80 | — |
| Ethylbenzene | 5.87 | 5.12 | 6.15 | 5.54 | 6.38 | 5.90 | 6.79 | 6.26 | 6.30 | — |
| *p*-Xylene | 6.20 | 5.44 | 6.44 | 5.90 | 6.73 | 6.27 | 7.16 | 6.66 | 6.64 | — |
| *m*-Xylene | 6.68 | 5.85 | 7.02 | 6.36 | 7.35 | 6.84 | 7.86 | 7.26 | 7.36 | — |
| *o*-Xylene | 8.66 | 7.54 | 9.11 | 8.25 | 9.59 | 8.86 | 10.31 | 9.44 | 9.90 | — |

The results indicate that the transition of 7,8-benzoquinoline from solid to liquid goes through liquid crystal states. Similar phenomena were observed by BARRALL *et al.*[3] in the case of cholesteryl esters and by KELKER[4], DEWAR AND SCHROEDER[5,6] and other authors in the case of *p*-azoxy, *p*-alkoxy and similar compounds. Our results suggest that the good separating properties of 7,8-benzoquinoline for xylenes are due to its mesomorphic structure.

*Experimental*

7,8-Benzoquinoline samples from two sources were investigated: BDH Laboratory Reagent and Perkin Elmer column packing material (part. No. 154-0752).

Pye Argon Chromatograph and Perkin Elmer Model 800 instruments were used.

*Experimental conditions for the Pye Argon Chromatograph.*

Column packing: Embacel, 60–100 mesh, acid washed, with 25% 7,8-benzoquinoline

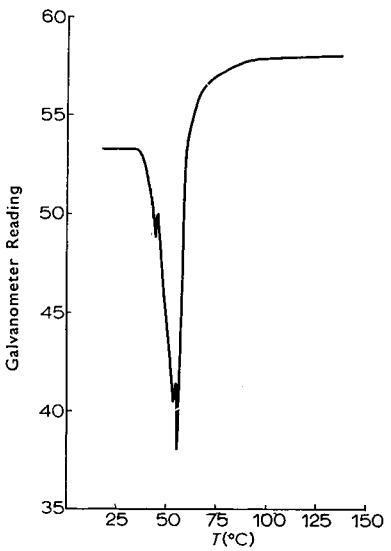


Fig. 3. DTA diagram of 7,8-benzoquinoline.

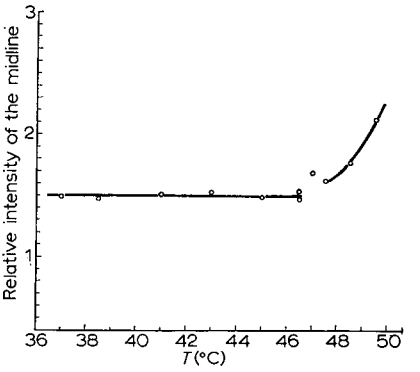


Fig. 4. The relative intensity of the central line in n.m.r. spectrum as a function of temperature.

Column diameter and length: 0.4 cm, 118 cm
Weight of column packing: 8.4 g
Column temperature: 40 to 60° ± 2°
Carrier gas: argon
Carrier gas flow: 50 ml/min; inlet pressure: 0.21 to 0.27 atm
Sensitivity: 10 ×
Detector voltage: 1500 V

*Experimental conditions for the Perkin Elmer Model 800 Chromatograph.*
Column packing: PE column X
Column diameter and length: 0.32 cm, 366 cm
Column temperature: 40 to 60° ± 2°
Carrier gas: argon
Carrier gas flow: 24 ml/min; inlet pressure: 2 atm
Detector: FID
Air pressure: 2.5 atm, hydrogen pressure: 1.0 atm
Attenuation: 500 and 1000

The melting points were determined with Kofler's polarizing melting microscope with a heating programme 2°/min. The errors of results were ± 1°.

An adapted differential thermal analysis instrument, with a heating programme 3°/min, and $Al_2O_3$ as reference material was used. The accuracy of temperature data was ± 2°.

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## Eine neue Methode zum Auffangen gas-chromatographischer Mikrofraktionen

Wertvolle Strukturinformationen lassen sich durch die spektroskopische Untersuchung von Mikrofraktionen gewinnen, wie sie von üblichen analytischen gaschromatographischen Trennsäulen eluiert werden können. Die Hauptschwierigkeit bei dieser Arbeitsweise besteht darin, die relativ geringen Substanzmengen, die mit Trägergas stark verdünnt aus der Säule austreten, quantitativ aufzufangen, d.h. innerhalb eines möglichst kleinen Volumens zu konzentrieren.

Für diesen Prozess des Aufkonzentrierens und Niederschlagens sind zahlreiche Methoden beschrieben worden, die sich hinsichtlich des benötigten Aufwandes und der erreichbaren Wirkung stark voneinander unterscheiden[1–35]. Ohne den Versuch einer kritischen Würdigung der verschiedenen Verfahren an dieser Stelle unternehmen zu wollen, kann festgestellt werden, dass bisher eine Methode fehlte, die die Vorzüge der Einfachheit, Wirksamkeit und vielseitigen Anwendbarkeit in sich vereinigt.

In unserem Laboratorium hat sich bei Strukturuntersuchungen an sehr verschiedenartigen Mehrkomponentengemischen eine Methode bewährt, die die o.g. Vorzüge sehr weitgehend für sich in Anspruch nehmen darf.

Als Auffanggefäss dient ein gewöhnliches U-Rohr aus Glas, dessen Innendurchmesser zwischen 1.5 und 2.5 mm liegen sollte. In dieses U-Rohr werden 2 bis 50 $\mu$l des Lösungsmittels gegeben, das für die anschliessende spektroskopische Untersuchung am besten geeignet ist. Das U-Rohr wird nunmehr in flüssiges Argon* (—185.7°) eingetaucht. Dabei erstarrt das Lösungsmittel in einer Form, die den Durchgang von Trägergas nicht verhindert.

Diese abgeschreckte und vermutlich mikrokristalline Lösungsmittelzone dient nunmehr als Filter, das leichtflüchtige Kohlenwasserstoffe (z.B. Propan) ebenso wie starke Nebelbildner (z.B. Hexandiol) mit überraschend hoher Ausbeute zurückzuhalten in der Lage ist.

Folgende Lösungsmittel wurden bisher eingesetzt: Tetrachlorkohlenstoff; Schwefelkohlenstoff; Chloroform; Deuterochloroform und Tetrachloräthylen.

Es ist anzunehmen, dass hinsichtlich der Wahl des Lösungsmittels weitgehend Freiheit besteht.

Der durch das Absorptionsrohr verursachte Strömungswiderstand ist abhängig von der Lösungsmittelmenge und vom Innendurchmesser der Kapillare. Bei der Auswahl geeigneter Bedingungen wird der Gasmengenstrom durch die Trennsäule nach Zuschalten des Absorptionsrohres nur unwesentlich vermindert. Eine Beeinträchtigung des Trennverhaltens wurde nicht beobachtet.

Folgende Dimensionen haben sich im routinemässigen Einsatz bewährt:

| | |
|---|---|
| Gesamtlänge des Absorptionsrohres | 180 mm |
| Aussendurchmesser des Absorptionsrohres | 7.5 mm |
| Innendurchmesser des Absorptionsrohres | 2.5 mm |
| Lösungsmittelmenge | 20 $\mu$l |

Auf die beschriebene Weise gelingt es, kleinste Substratmengen in geringen

* Flüssiger Stickstoff dürfte ebenso geeignet sein.

Lösungsmittelvolumina aufzufangen und mit den erhaltenen relativ konzentrierten Lösungen spektroskopische Untersuchungen durchzuführen.

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## An automatic fraction collector control and fraction counter

Numerous fraction collectors are commercially available for collection of chromatographic fractions. To the author's knowledge, however, none of these are completely automated to make the most efficient use of the limited number of tubes in a circular turntable. Furthermore, most of them make use of stepping relays which are subject to mechanical failure and possible overflow of inflammable solvent unless proper precautions are taken. An inexpensive (under $100.00) fraction collector control has been designed which eliminates the use of stepping relays and which has been in operation in the author's laboratory for over three years without a failure. This control will automatically (1) start the turntable after a predetermined number of forerun fractions have been syphoned into a waste container, (2) stop the turntable in the middle of a run and again empty a predetermined number of fractions into the waste container, (3) again start the turntable and collect the final fractions, and finally (4) shut off the flow of liquid from the chromatographic column, turn off all electric power to the equipment and record the elapsed time for the run. This control has made it possible to start a chromatographic separation at the close of a day's work and return the next morning to find it completed and the fractions ready for analysis. The fraction collector for which this control was designed has a large turntable with a single row of holes for tubes which is powered by a 0.6 A Bodine 1725 r.p.m. motor geared down to 24 r.p.m. Equal volume fractions are delivered from the column by means of a syphon and a stainless steel bellows pump is used to force solvent through a starch column[1]. Gravity alone is sufficient for a cellulose powder column[2].

*Construction and operation of control*

The wiring diagram for the control is shown in Fig. 1a. Fig. 1b shows the physical appearance of the control and Fig. 1c the upper and lower turntable control microswitches which are tripped by knobs attached to the edge of the turntable and extending above and below the table, respectively. All microswitches in the diagram are shown in their normal untripped positions. When the turntable is at rest the turntable microswitch is in the dotted position, actuated by a knob on the turntable motor shaft. When the turntable is in motion this switch assumes the normal position shown in the diagram. The power is 115 V a.c.

In operation the syphon contacts are closed by a salt solution or mercury and relay (3) closes. This activates counter (1) which counts down from the number of forerun fractions manually set on it each time the syphon empties until it reaches zero and activates its microswitch (shown immediately below it in the diagram). These fractions are emptied into a waste container placed below an empty hole in the turntable. The next signal from the syphon starts the turntable motor via the circuit: relay (3), upper turntable control microswitch, counter (1) microswitch, thermal delay relay (4) and turntable microswitch in dotted (rest) position. As soon as the turntable starts moving the turntable switch returns to its normal position and the thermal delay relay is by-passed. At approximately the mid-point of the turntable motion (1 sec) the thermal delay relay opens and when the knob on the turntable motor again trips the turntable switch it stops the turntable. The syphon then empties, the coils of the relays are inactivated and they both return to their normal

positions for the next fraction. This operation continues until the upper turntable control microswitch is thrown by a knob extending above the turntable and attached at a point where a wide band of pure solvent occurs. The next signal from the syphon now activates counter (2) via the circuit: relay (3), counter (2) microswitch, upper turntable control microswitch, counter (1) microswitch, thermal delay relay (4) and turntable microswitch (dotted rest position). The tube at this stop of the turntable is omitted so that the number of fractions set on counter (2) will be emptied into the same waste container which collected the forerun fractions. This solvent can be re-used in another run without redistillation. When counter (2) counts to zero it trips its microswitch (immediately below it in the diagram) and the turntable again starts turning via the circuit: relay (3), counter (2) microswitch, upper turntable micro-switch, counter (1) microswitch, thermal delay relay (4) and turntable microswitch (in dotted position). With the turntable in motion the turntable switch again by-passes the delay relay and the upper turntable control switch returns to its normal position powering the turntable motor directly through counter (1) microswitch, by-passing counter (2) microswitch. Fractions are then collected until the lower turntable control microswitch is tripped by a knob attached to the edge of the turn-table at the end of the set of tubes and extending down from the table. This shuts off all power to the system thus stopping the pump and clock and at the same time ac-tivates the electrocock which stops flow of liquid from the column.

The various components of the circuit can be conveniently housed in a sloping front cabinet 7 in. square and 6.5 in. high. The outlets in the cabinet are essentially in the same order as in the wiring diagram with the excepton of the upper and lower

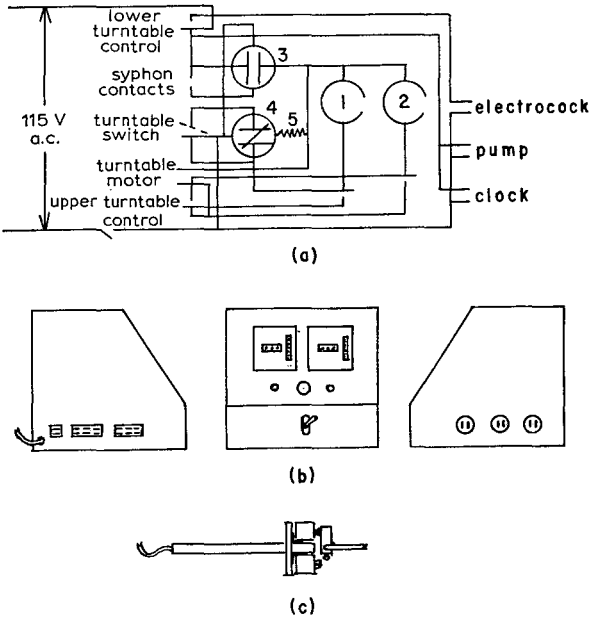


Fig. 1. (a) Wiring diagram for fraction collector control. (b) Physical layout of control. (c) Upper and lower turntable control microswitches.

turntable controls which are combined in one six-contact polarized outlet and six-wire cord to the turntable control microswitches. The turntable microswitch and turntable motor are fed by five wires in a second six-wire cord from a second six-contact polarized outlet. In Fig. 1b are shown two non-locking pushbutton DPDT switches, one immediately below each counter, and a pilot light in the center of the sloping panel. These are omitted from the wiring diagram for the sake of clarity. Each switch is wired so as to isolate the normally closed contacts of its counter microswitch and put them in a circuit with the 115 V pilot light so that the counter reset can be properly manipulated to put its microswitch into its normal position for the start of a run. When properly adjusted the pilot light goes on when either pushbutton switch is depressed.

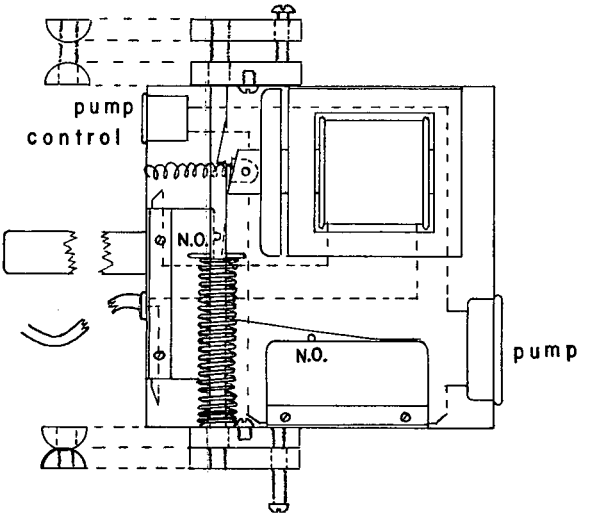


Fig. 2. Layout and wiring of electrocock.

*Construction and operation of electrocock*

For stopping flow of solvent from the column at the end of a run an electrocock was designed which would cut off its own power after closing. Fig. 2 shows the arrangement and wiring in a 3 × 4 × 4 in. box attached to a $^1/_2$ in. rod. The electrocock is shown in the closed position, the rubber tubing from the chromatographic column being pinched off between the lower rounded jaws. The electromagnet is an inexpensive 2 × 2 in. size with a 26 Ω coil for operation on 115 V a.c. The electrocock is opened by pressing on the upper jaw so as to compress the main spring and allow the core of the electromagnet to be drawn by its spring into the notch in the $^3/_{16}$ in. brass rod connecting the two jaws. The microswitches are closed by this action. Activation of the electromagnet draws its core in causing the jaws to snap shut and the microswitches to open. The microswitch in the pump circuit was included so that the pump would automatically start and stop when the electrocock was opened and closed without need for the rest of the fraction collector control power to be shut off. A single double pole microswitch would have served the same purpose. The upper jaws of the electrocock may be used to automatically start a flow by running a rubber

tube through them and then pressing them closed. They are adjustable for different thickness of tubing by removing the screw and screwing the jaw out or in on the brass connecting rod.

*Component list for collector control*

2 counters, 115 V a.c., SPDT, manual reset, Veeder Root BX-150703;
1 relay, 115 V a.c., SPDT, 2–3 V.A., Advance Electric GHP/1C;
1 relay, 28 V, 1 sec thermal delay, G-V HM-01;
1 wirewound adjustable resistance, 25 W, 750 Ω set for 600 Ω to supply delay relay coil from 115 V a.c. line;
2 non-locking pushbutton switches, DPDT, 3 A;
1 pilot light, 115 V a.c.;
2 AB sockets for chassis mounting, 6-contact, Cinch-Jones S-306;
2 CCT polarized plugs with cable clamps, 6-contact, Cinch-Jones P-306;
1 AB socket for chassis mounting, 2-contact, Cinch-Jones S-302;
1 CCT plug with cable clamp, 2-contact, Cinch-Jones P-302;
3 standard 2-pole female sockets with retaining rings, Amphenol.

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## Gas chromatography of hydroxyethyl derivatives

Hydroxyethyl derivatives are of diverse and widening interest in industry, agriculture, medicine and metabolic studies. The literature is fairly abundant with investigations concerning the role of C-hydroxylation, as well as N-hydroxylation, in the metabolic pathways for pesticidal carbamates[1–4] and 2-chlorotriazines[5–12]. N-(2-Hydroxyethyl) ethyleneimine has been proposed as a modifier for nitrogen plastics and other resins, and as an intermediate in the preparation of their polymers[13]. β-Hydroxyethyl hydrazine has been used as a growth regulator by pineapple growers to induce flowering and control the date of harvest[14,15]. A nitroimidazole derivative, 1-(2-hydroxyethyl)-2-methyl-5-nitroimidazole (metronidazole) is employed extensively as a specific agent against human trichomoniasis[16]. The technique of β-hydroxyethylation has been found useful for the characterization and enhancement of water solubility for complex molecules such as rutoside[17]. A number of *p*-acetylbenzenesulfonylureas utilized in diabetes therapy have been shown to be rapidly absorbed and metabolized in man by reduction to their corresponding *p*-α-hydroxyethyl derivatives[18].

TABLE I

GAS CHROMATOGRAPHY OF HYDROXYETHYL DERIVATIVES

R—$CH_2CH_2OH$

| *Compound No.* | *R* | *Mol. wt.* | *Name* | $t_r$°[a] | |
|---|---|---|---|---|---|
| | | | | *10% [b] C20M* | *4% [c] C20M* |
| 1 | $H_2N$— | 61 | Ethanolamine | 0.15 | 0.9, 2.75 |
| 2 | $CH_3NH$— | 75 | 2-(Methylamino) ethanol | | 2.10 |
| 3 | $H_2NNH$— | 76 | β-Hydroxyethylhydrazine | 0.30 | 2.15[d] |
| 4 | HS— | 78 | 2-Mercaptoethanol | | 3.65 |
| 5 | Cl— | 80 | 2-Chloroethanol | | 1.45 |
| 6 | $HC(=O)NH$— | 88 | N-(2-Hydroxyethyl) formamide | 6.7 | |
| 7 | $(CH_3)_2N$— | 89 | 2-(Dimethylamino) ethanol | | 0.60 |
| 8 | $(CH_3)_2CHNH$— | 103 | 2-(Isopropylamino) ethanol | | 2.90 |
| 9 | $CH_3C(=O)NH$— | 103 | N-(2-Hydroxyethyl) acetamide | 5.6 | |
| 10 | $CH_3C(=O)O$— | 104 | 2-Hydroxyethyl acetate | | 5.7, 9.4 |
| 11 |  | 112 | N-(β-Hydroxyethyl) pyrazole | | 3.50[d] |
| 12 |  | 115 | N-(β-Hydroxyethyl) pyrrolidine | | 5.6 |
| 13 |  | 122 | Phenethyl alcohol | | 2.8[d] |
| 14 |  | 123 | 2-(Pyridine) ethanol | 1.7 | |
| 15 |  | 129 | N-(β-Hydroxyethyl) piperidine | | 4.1 |
| 16 | $CH_2=C(CH_3)-C(=O)-O$— | 130 | 2-Hydroxyethyl methacrylate | | 1.70[d] |
| 17 |  | 130 | 1-Piperazine ethanol | 1.55 | 4.25[d] |
| 18 |  | 131 | N-(β-Hydroxyethyl) morpholine | | 1.95[d] |
| 19 |  | 143 | N-(2-Hydroxyethyl) cyclohexylamine | 1.25 | |
| 20 | —N(H)—N(H)—(bis) | 120 | N,N′-Bis(β-hydroxyethyl) hydrazine | | 2.20[d] |

[a] Retention time in minutes from solvent front.

[b] 10% Carbowax 20M on 60–80 mesh Chromosorb W; 8 ft. by 0.125 in. O.D. stainless steel column; column temperature 220°; 50 p.s.i.g. helium; 150 mA filament current; detector temperature 250°; hot wire detector.

[c] 4% Carbowax 20M terminated with terephthalic acid on 60–80 mesh HMDS-pretreated Chromosorb W; 6 ft. by 0.25 in. O.D. glass coil column; column temperature 73° and nitrogen carrier flow 63 ml/min except for those designated footnote d; hydrogen 45 ml/min, air 300 ml/min; detector temperature 200°; hydrogen flame detector.

[d] *Ibid* to footnote c, except: column temperature 110° and nitrogen carrier 81 ml/min.

Pyrolysis–gas chromatography has been employed as an analytical technique for the determination of the hydroxyethyl group in hydroxyethyl starch *via* measurement of the liberated acetaldehyde[19]. The present study reports the chromatographic behavior of twenty 2-hydroxyethyl derivatives of diverse structure, including the growth regulator β-hydroxyethyl hydrazine and discusses the faculty of silylation in elution enhancement of the derivatives.

*Experimental*

The hydroxyethyl compounds employed in this study were obtained from various commercial sources. Gas chromatography was carried out on a 10% Carbowax 20M column housed in an F & M Model 720 dual column oven containing a hot wire detector and coupled to an F & M Model 500 gas chromatograph; and on a 4% Carbowax 20M column housed in an F & M Model 1609 gas chromatograph containing a hydrogen flame detector. Specific analytical operating conditions are given in the footnotes to Table I.

*Results and discussion*

The chromatographic results for the hydroxyethyl derivatives are presented in Table I. The derivatives required the use of more than one column temperature. The low temperatures employed for the chromatography of most of the derivatives (73° and 110° on 4% Carbowax 20M), were not sufficient for several of the more strongly retained compounds (*viz.* compounds 6 and 9). Hence, one may qualitatively observe the varying degrees of interaction of the 2-hydroxyethyl substituents with

TABLE II

INFLUENCE OF SILYLATION ON CHROMATOGRAPHIC ELUTION OF HYDROXYETHYL DERIVATIVES

| *Compound No.* | *R* | *Name* | $t_r$° [a] | | % [b] |
|---|---|---|---|---|---|
| | | | $RCH_2CH_2OH$ | $RCH_2CH_2OSi(CH_3)_3$ | *Elution enhancement* |
| 16 | $CH_2=C(CH_3)-C(=O)-O-$ | 2-Hydroxyethyl methacrylate | 1.70 | 0.60 | 65 |
| 18 |  | N-(β-Hydroxyethyl) morpholine | 1.95 | 0.90 | 54 |
| 3 | $H_2NNH-$ | β-Hydroxyethylhydrazine | 2.15 | 0.60 | 72 |
| 20 | $-N(H)-N(H)-$ | N,N′-Bis(β-hydroxyethyl)-hydrazine | 2.20 | 0.60, 1.90 | 73, 14 |
| 13 |  | Phenethyl alcohol | 2.80 | 0.80 | 71 |
| 11 |  | N-(β-Hydroxyethyl) pyrazole | 3.50 | 0.55, 0.90 | 84, 74 |
| 17 |  | 1-Piperazine ethanol | 4.25 | 2.00 | 53 |

[a] For column and conditions see footnote d, Table I.

[b] Calculated by: $100 - \frac{[t_r°(RCH_2CH_2OSi(CH_3)_3)]100}{t_r°(RCH_2CH_2OH)}$

the polar polyglycol stationary phase. The carbonyl derivatives (compounds 6, 9, 10 and 16) illustrate to some degree the influence of dipole–dipole type interaction and its influence on retention. Hydrogen bonding, on the other hand, is also of considerable importance to solute retention, as may be seen for example, between compounds 2 and 7, or between compounds 17 and 15 or 17 and 18. The relatively large retentions observed for compounds 6 and 9 may well be attributed to hydrogen bonding *via* the tautomerized enol form of the solute.

The influence of hydrogen bonding *via* the hydroxyl proton on the chromatographic behavior of hydroxyethyl derivatives may be more strikingly observed by examination of the elution results on 4% Carbowax 20M at 110° between several hydroxyethyl compounds and their trimethylsilyl derivatives given in Table II. Trimethylsilylation was achieved by reaction with hexamethyldisilazane in a pyridine medium, initiated with trimethylchlorsilane. Elution enhancement *via* preparation of the silyl ether derivatives was generally in the order of 50–80%. The two peaks observed during chromatography of the silylated N,N'-bis(β-hydroxyethyl) hydrazine (compound 20) was believed to be due to a mixture of the mono- and disilylated derivatives.

In view of the important role of hydroxylation in the metabolic pathway of herbicides, other classes of alcoholic, as well as phenolic derivatives are presently under investigation.

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## A novel method for the identification of alcohols in complex mixtures

The successful analysis of alcohols in complex mixtures depends upon finding a suitable method for either extracting the alcohols or detecting them in the presence of other compounds.

In these laboratories, two published methods have been used in our studies on tobacco smoke condensate[1,2]. Both depend upon the conversion of the alcohols to derivatives, followed by isolation by extraction or column chromatography. The results obtained by these methods showed a measure of agreement, and support the results of other workers[3,4]. Both methods have the disadvantage of being lengthy and complicated. A new method was therefore sought which would be simple and rapid, and which would avoid as far as possible the prolonged action of heat and reagent on labile compounds.

A halogen-sensitive gas–liquid chromatography (GLC) detector was already in use in this department when the problem arose and the possibility of producing halogenated derivatives of alcohols and utilising the detector to examine them in the presence of other compounds seemed a worthwhile approach. The reaction of alcohols with chloroacetyl chloride to form halogenated esters was investigated

*Materials and method*

The work was carried out on a modified Pye Argon Chromatograph oven using an A.E.I. Ozotron type J halogen-sensitive detector[5].

The argon detector and aluminium heater bars were removed from the Pye oven to enable it to take a U-shaped $^1/_4$ in. glass column, and the oven top modified to take the injection system and detector shown in Fig. 1.

This detector has been investigated by other workers[6] and its performance, linearity and limits of detection determined. For this work it was found that high sensitivity was not required, and a heater supply of 4.6 V a.c. at 6.7 A and H.T. of 108 V d.c. was adequate for a 2 to 20 $\mu$g loading of the halogenated esters. Nitrogen was used as both carrier and dilution gas, the flows being maintained at 40 ml·min$^{-1}$ and 110 ml·min$^{-1}$, respectively. Injection was on column with no flash heaters.

All I.R. spectra were scanned on a Hilger and Watts H. 800 spectrophotometer, the samples being prepared as capillary films.

*Preparation of alkyl chloroacetates*

All the chloroacetates used could have been prepared by addition of chloroacetyl chloride to a solution of the alcohol in benzene at room temperature, but when dealing with complex mixtures the reaction mixture was actually refluxed. The method adopted for the preparation of all chloroacetates was as follows. The alcohol (1 g), or mixture was dissolved in benzene (10 ml, A.R.), the required amount of chloroacetyl chloride (at least twice the molar equivalent) was added dropwise, and the mixture boiled under reflux for one hour. After allowing the mixture to cool to room temperature a large excess (5 ml) of ethanol was added, and the mixture allowed to stand for a further 30 min. The mixture was made up to 50 ml with benzene and was then ready for chromatographic examination.

When this method was used with complex mixtures it was found necessary to determine the optimum amount of chloroacetyl chloride needed, since the presence

of a large excess of unreacted chloride at the end of the reaction leads to a very large ethyl chloroacetate peak, possibly inhibiting the detection of smaller peaks in the same region.

In early experiments the excess of chloroacetyl chloride was decomposed by washing the mixture with sodium bicarbonate. This was satisfactory, but the presence of alkali tended to hydrolyse some of the more reactive chloroacetates.

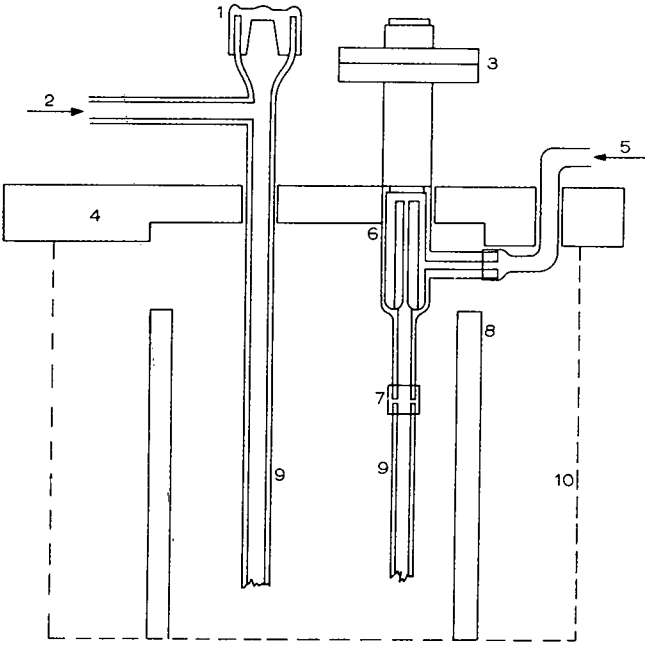


Fig. 1. Modified Pye argon chromatograph oven. 1 = Rubber serum cap; 2 = carrier flow; 3 = detector; 4 = oven top; 5 = detector flow; 6 = Pyrex adaptor; 7 = silicone rubber; 8 = aluminium heater tube; 9 = 2.2 m $^1/_4$ in. column; 10 = heating jacket.

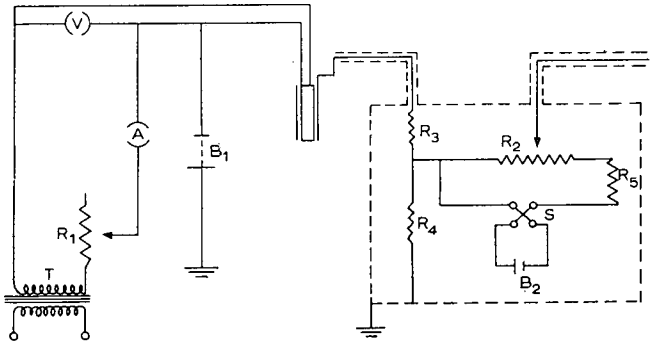


Fig. 2. Circuit used for the halogen-sensitive detector. $R_1$ = 1 Ω 10 A variable resistance; $R_2$ = 250 Ω 10 turn variable resistance; $R_3$ = 1 MΩ; $R_4$ = 1.5 kΩ; $R_5$ = 12 kΩ; S = backing-off polarity switch; T = 240/6 V constant voltage transformer; $B_1$ = 108 V H.T. battery; $B_2$ = 9 V backing-off battery; V = 0–10 V a.c. voltmeter; A = 0–10 A a.c. ammeter.

It is probable that phenols would react in a similar manner to alcohols, so that it might be necessary to remove these, and also amines, from a mixture prior to reaction with chloroacetyl chloride.

*Results*

Chloroacetates of most of the primary alcohols and many of the secondary and tertiary alcohols were prepared up to tridecanol. Chloroacetates were also prepared from several unsaturated alcohols, cyclopentanol, cyclohexanol, citronellol, menthol, tetrahydrofurfuryl, benzyl and β-phenylethyl alcohols. A chromatogram of some of these esters obtained at 200° is shown in Fig. 3. Attempts to produce a chloroacetate from furfuryl alcohol failed owing to the rapid polymerisation of the alcohol under acid conditions.

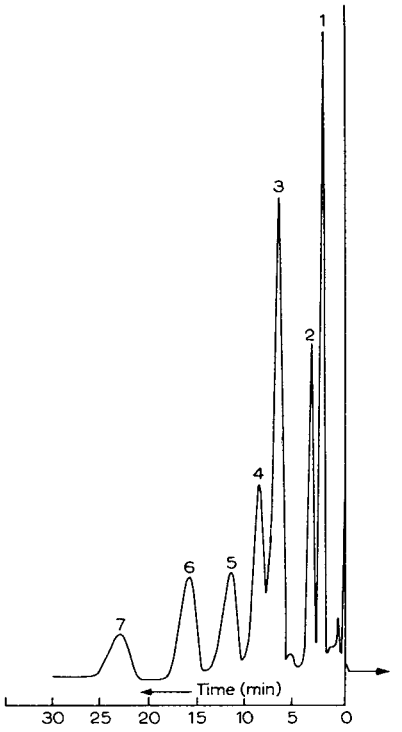


Fig. 3. Synthetic mixture run on 2.2 m 5% Reoplex 400 on 100/120 mesh Celite at 200°. 1 = Cyclopentyl chloroacetate; 2 = cyclohexyl chloroacetate; 3 = menthyl chloroacetate; 4 = tetrahydrofurfuryl chloroacetate; 5 = *n*-decyl chloroacetate; 6 = benzyl chloroacetate; 7 = β-phenylethyl chloroacetate.

All chloroacetates which were prepared were examined by I.R. spectroscopy. The spectrum of the ethyl chloroacetate was found to be identical with the spectrum of authentic ethyl chloroacetate purchased from B.D.H.*. This was the only chloroacetate commercially available, but the spectra of the other chloroacetates showed the type of absorption expected from α-halogeno esters.

* British Drug Houses Ltd., Poole, Dorset, Great Britain.

In the concentrated form many of the esters decomposed, but in dilute benzene solution they could be kept for several weeks.

A number of non-alcoholic neutral compounds were subjected to the reaction to determine whether any interference would be expected from such compounds if they were present in alcohol-containing mixtures.

Twenty-four compounds were investigated: acetaldehyde; acetone; acetophenone; amyl acetate; anisole; benzaldehyde; benzene; benzyl acetate; *n*-butyraldehyde; camphene; citral; cyclohexanone; cyclopentanone; dimethyl acetal; ethyl acetate; furfuraldehyde; hexaldehyde; isopropyl acetate; limonene; linalyl acetate; mesityl oxide; methyl amyl ketone; methyl benzyl ketone; 2-nonanone.

Of these cyclohexanone, hexaldehyde and butyraldehyde gave very small amounts of halogenated product possibly due to impurities, and acetaldehyde gave rather more interference than expected. The peaks obtained from the acetaldehyde reaction were eluted very early and did not appear from their retention times to be halogen esters.

As an example of the results obtained by this method, Fig. 4 shows a chromatogram of alcohol chloroacetates derived from tobacco smoke neutral condensate, *i.e.*, a condensate from which acids, bases and phenols have been extracted.

The method has not been extended beyond the tridecanol region owing to the

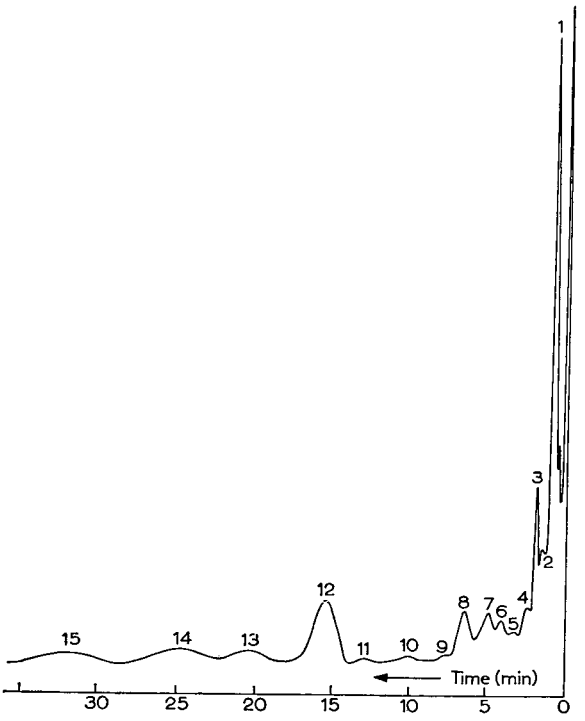


Fig. 4. Neutral smoke condensate sample on 2.2 m 5% Reoplex 400 on 100/120 mesh Celite at 200°. 1 = Isopropyl chloroacetate; 5 = isohexyl chloroacetate; 7 = *n*-hexyl chloroacetate; 10 = isooctyl chloroacetate; 11 = *n*-octyl chloroacetate; 14 = *n*-decyl chloroacetate; 15 = isoundecyl chloroacetate.

temperature limitations of the Pye argon chromatograph oven, but there appears to be no reason why this method should not be applicable to the higher molecular weight alcohols.

*Conclusion*

Reaction of neutral, alcohol-containing mixtures with chloroacetyl chloride, and analysis of the products by a gas–liquid chromatograph incorporating a halogen-sensitive detector, has been shown to offer a useful method for the identification of alcohols in such mixtures. The method is quick, and very sensitive. Satisfactory results were obtained with most of the alcohols studied, and interference from non-alcoholic compounds is negligible.

This method should also be applicable to the analysis of phenols, and the use of GLC with a halogen-sensitive detector may well have great potential value in the examination of other types of compound in complex mixtures if suitable halogenated derivatives can be prepared.

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## A chromatography–densitometry method for quantitative analysis of sugars of flavonoid glycosides

Quantitative analysis of the sugars of naturally occurring flavonoid glycosides is often needed in elucidation of the structures of these compounds. An improved method for quantitative determination of the microgram quantities of sugars obtained by hydrolysis of one milligram or less of pure flavonoid glycoside has recently been developed in our laboratory. In this method the sugars are separated by thin-layer chromatography on silica gel and individually quantitated directly on the chromatogram by determination of the density of the spots produced when the plate is sprayed with aniline–oxalic acid reagent[1]. This procedure is both less tedious and more reliable than methods which we have previously employed[2,3]. It appears generally applicable for determination of hexoses; we have employed it mainly for determination of rhamnose and glucose, the sugars occurring most commonly in flavonoid glycosides.

In a typical analysis, 0.25–1.0 mg of flavonoid glycoside is hydrolyzed by refluxing with 5 ml 3 % hydrochloric acid solution for 1–2 h. The liberated sugars are then separated quantitatively from the aglycone by passing the cooled hydrolysate through a 1.2 cm diameter column packed to a depth of 1.5 cm with an aqueous slurry of Polyclar AT polyvinylpyrrolidone (General Aniline and Film Corp., Grasselli, N.J.). The reaction vessel is rinsed 3 times with 5 ml portions of water which are added to the column, and the column is then washed with 30 ml additional water to elute the sugars. The aglycone, which remains on the column during water elution, is then eluted with 75 ml methanol and its quantity determined spectrophotometrically or by fluorescence.

The aqueous eluate containing the sugars from the Polyclar column is fed directly onto a 1.2 × 5 cm column of Amberlite IR-45 anion exchange resin (OH form), which is then washed with 25 ml additional water to insure complete elution of sugars. The resulting 75 ml of deionized sugar solution are evaporated to dryness in a round bottom flask on a rotary evaporator. The residue is transferred, in a total volume of about 15 ml of water, to a 25 ml conical flask; the solvent is again removed by evaporation; and the residue is dissolved in 500 $\mu$l of water. A sufficient quantity of this sugar solution to contain 1–10 $\mu$g of each individual sugar (usually 10 $\mu$l) is spotted in triplicate on a 200 × 200 mm thin-layer plate coated with a 250 $\mu$ layer of Adsorbosil 1 silica gel (Applied Sciences Laboratories, Inc., State College, Pa.). A 5 $\mu$g standard each of rhamnose and glucose is spotted in triplicate on the same thin-layer plate. The chromatogram is developed to a distance of 10 cm beyond the origin in *n*-propyl alcohol–ethyl acetate–water (7:1:2, v/v) solvent system and dried for 3 h in an air current. It is then sprayed with 10 ml of aniline–oxalic acid reagent, air dried 10 min, and heated at 105° for 20 min to develop the color.

The densities of the brown-yellow spots produced by reaction of the hexoses with the aniline–oxalic acid reagent are determined by scanning the thin-layer chromatogram with a Photovolt densitometer, Model 530, equipped with an Integraph integrator, Model 49 (Photovolt Corp., New York, N.Y.). A Wratten 47B filter is employed for the phototube to enhance sensitivity to the sugar-reagent spots, and the response selector switch of the recorder is set at position 6. Under these conditions, the density of the sugar-reagent spots on the chromatogram is a linear function of sugar concentration in the range 1–10 $\mu$g of rhamnose or glucose. The quan-

tities of these sugars in the sample are thus determined by comparing densities of standard and sample spots. Duplicate thin-layer chromatograms are usually run for each sample to minimize errors due to individual plate variation.

TABLE I

DETERMINATION OF SUGARS IN NARINGIN SAMPLES BY THE CHROMATOGRAPHY–DENSITOMETRY METHOD

| *Sample* | *Quantities (μg) determined by chromatography-densitometry method* | | *Quantities (μg) calculated from sample weight* | | *Ratio of determined to calculated values* | |
|---|---|---|---|---|---|---|
| | *Glucose* | *Rhamnose* | *Glucose* | *Rhamnose* | *Glucose* | *Rhamnose* |
| 1 | 263 | 267 | 292 | 269 | 0.90 | 0.99 |
| 2 | 296 | 288 | 300 | 273 | 0.99 | 1.05 |
| 3 | 288 | 245 | 299 | 272 | 0.96 | 0.90 |

Table I shows results obtained when this method was employed for determination of rhamnose and glucose in three samples consisting of approximately 1 mg each of authentic naringin. The method has been successfully applied in our laboratory for quantitative determination of sugars in previously unidentified flavanone triglycosides which were available only in very small quantities. The thin-layer chromatography–densitometry procedure employed in this method should be applicable for quantitative determination of microgram quantities of free hexoses occurring in small samples of biological materials as well as for quantitative determination of hexoses constituting the sugar moieties of other compounds in addition to the flavonoid glycosides.

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## A modified elution schedule for accelerated amino acid analysis

The resolution obtained with spherical resins when using the factory-recommended accelerated amino acid analysis elution schedules is inadequate for many plant samples. This difficulty is caused, in part, by the relatively large content of aspartic acid, amides, and ammonia. Moreover, in short column basic amino acid analyses, tyrosine, 3,4-dihydroxyphenylalanine and phenylalanine appear as a single peak, and tryptophan is resolved only as a broadly spreading peak. This paper describes changes in buffer pH and water jacket temperatures, as well as alterations in buffer and temperature change times which have eliminated these problems.

*Experimental**

*Apparatus.* A Beckman 120B amino acid analyzer was used at buffer flow rates of 50 ml/h and a ninhydrin reagent flow rate of 25 ml/h. Back pressures were 345 p.s.i. at 30° and 120 p.s.i. at 60° for the long and short columns, respectively. High pressure lines between the pumps and columns were 0.062 in. I.D. × 0.030 in. wall Teflon TFE (Penn. Fluorocarbon Co., Clifton Heights, Pa.). Adjustment of pH was done using a Beckman Expandomatic pH meter.

*Reagents.* Sodium citrate buffers were prepared according to the recommended procedures[1]. Modified pH's were obtained by adding either 50 % NaOH or conc. HCl to the appropriate buffer. Caprylic acid, rather than pentachlorophenol, was used as a preservative. All other reagents were prepared according to Beckman specifications.

*Procedure.* Basic amino acids were separated on a 20 × 0.9 cm PA-35 resin column. Elution was started with a pH 4.62 (0.38 *N*) buffer which was changed to a pH 5.28 (0.35 *N*) buffer after 30 min. The water jacket temperature during the entire analysis was 60°. In this procedure, if the ninhydrin pump was started at the same time as column elution there was not sufficient time to set the recorder baseline before the appearance, of the first peak. Therefore, this adjustment was made just prior to the short column analysis while pumping buffer through the long column.

A 57 × 0.9 cm PA-28 resin column was used for analysis of the acidic and neutral amino acids. After regeneration at 60°, the column was equilibrated with pH 3.47 (0.20 *N*) buffer; a reproducible amount of this same buffer was used to wash and fill the column after the sample had been driven into the resin column. Elution was started with a pH 3.12 (0.20 *N*) buffer at 30°. A temperature change to 55° was started after 105 min and a buffer change to pH 4.25 (0.20 *N*) after 215 min.

*Results and discussion*

Figs. 1 and 2 illustrate the resolution obtained with a synthetic mixture of amino acids and amides. Peak elution times for compounds not shown in the figures are: (57 cm column) taurine 44 min; urea 49 min; 3,4-dihydroxyphenylalanine 306 min; (20 cm column) anthranilic acid 50 min; kynurenine 55 min; 5-hydroxytryptophan 58 min; hydroxylysine 72 min; 5-hydroxytryptamine 91 min; carnosine and creatinine 109 min; 1-methylhistidine 115 min; L-3-methylhistidine 119 min; tryptamine 122 min.

* Reference to a company or product name does not imply approval or recommendation of the products by the U.S. Department of Agriculture to the exclusion of others that may be suitable.

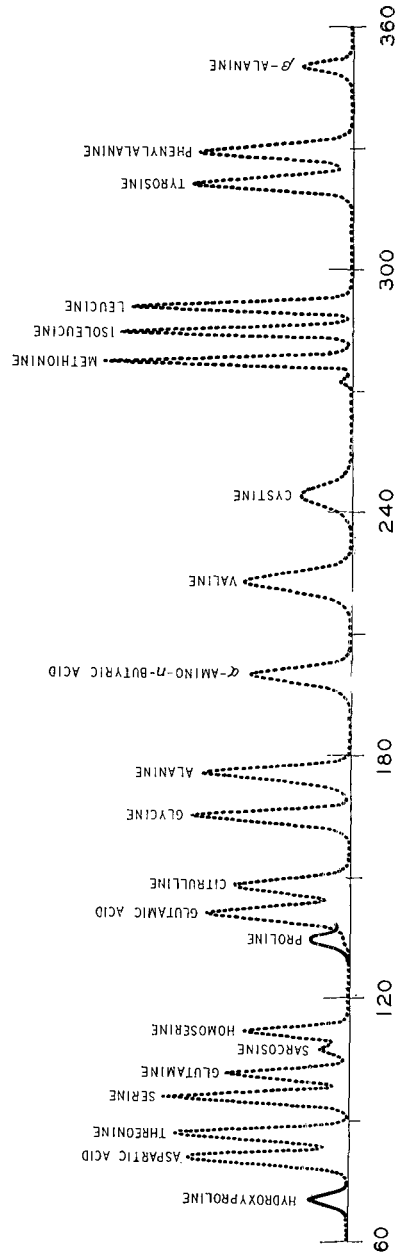


Fig. 1. Chromatogram of a synthetic mixture of acidic and neutral amino acids. 0.25 $\mu$mole of each compound was used except for hydroxyproline (0.10 $\mu$mole) and glutamine, sarcosine, homoserine, citrulline, α-amino-*n*-butyric acid and β-alanine (0.20 $\mu$mole).

The use of the pH 3.47 buffer for equilibration, as well as for filling, gives better reproducibility than if a buffer with pH higher than 3.28 is used for filling only. Automation for the addition of this buffer can be done by using a system similar to that described by HUBBARD[2] for accelerated regeneration and equilibrium.

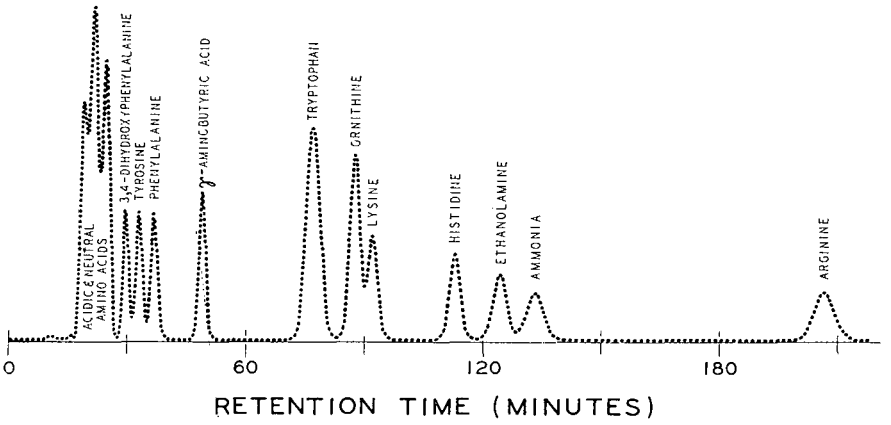


Fig. 2. Chromatogram of a synthetic mixture of basic amino acids. 0.125 μmole of each compound was used except for tryptophan (0.50 μmole) and ornithine (0.30 μmole).

In the analysis of basic amino acids, 3,4-dihydroxyphenylalanine, tyrosine and phenylalanine were separated with the pH 4.62 first buffer. Ethanolamine, ornithine and hydroxylysine can interfere with ammonia, lysine and tryptophan, respectively. However, the concentrations of the former group in most plant samples are such that they are unlikely to cause serious problems. This schedule is particularly useful for studies on aromatic amino acid metabolism.

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## The adsorption of azaaromatics on alumina: a comment on the paper of Klemm, Kloppenstein and Kelley

KLEMM, KLOPPENSTEIN AND KELLEY in a recent paper[1] have reported an extensive tabulation of $R_F$ values for the TLC separation on alumina of various azaaromatics related to pyridine. On the basis of these data these authors conclude that "...we continue to favor the concept of preferential edgewise adsorption of many azarenes...", a proposal first put forward by KLEMM *et al.* in 1961[2]. The hypothesis of edgewise adsorption in this chromatographic system has been challenged by the present author[3–5], on the basis of a theoretical analysis of a large body of linear isotherm retention volume values from column chromatography studies. If (and only if) planar adsorption is assumed, it is possible to quantitatively predict retention volumes for numerous pyridine derivatives by means of a previously developed theoretical equation. KLEMM *et al.*[1] question the validity of the latter equation in the adsorption of the azaaromatics, however: "This equation has been rigorously tested for aromatic hydrocarbons... (but) the applicability of this equation to the clarification of the geometry of adsorption of relatively non-hindered azarenes is more equivocal and seems not to have been rigorously tested".

The final test of any theory or correlational equation is in its ability to quantitatively account for a particular set of relevant experimental data. Since the comparisons offered by KLEMM *et al.*[1,2] are essentially of a qualitative nature, while the preceding analysis of the author[3–5] is quantitative, and since the quantitative data reported by the author are totally incompatible with nonplanar adsorption of the azaaromatics, the concept of nonplanar adsorption would seem to be clearly refuted. In the present communication we propose to show that our previous theoretical treatment (based on planar adsorption) is capable of quantitatively predicting the $R_F$ values recently reported by KLEMM *et al.*[1] for the azaaromatics. This further verifies the validity of our treatment in the case of the azaaromatic adsorbates and supports the underlying premise that these compounds adsorb in the plane of the alumina surface. We also hope to clarify some of the misunderstanding that has arisen concerning the configuration of the adsorbed azaaromatics.

For linear isotherm retention volumes $\underline{R}^c$ (ml/g) in column chromatography it has been shown (ref. 6 and prior references) that

$$\log \underline{R}^\circ = \log V_a + \alpha(S^\circ - \varepsilon^\circ A_s) + \Sigma\Delta_{eas} \qquad (1)$$

Here $V_a$ and $\alpha$ are adsorbent parameters determined by the alumina activity, $S^\circ$ is the adsorption energy of the solute (adsorbate) in a standard system, $A_s$ is the cross-sectional area of the solute, $\varepsilon^\circ$ is the adsorption energy of the eluent per unit area of surface (eluent strength), and the term $\Sigma\Delta_{eas}$ arises from certain "anomalous" adsorption effects which can be ignored here (*i.e.*, $\Sigma\Delta_{eas}$ equal zero). $S^\circ$ can in turn be expressed as a function of solute structure:

$$S^\circ = \sum^{i} Q^\circ_i + \sum^{j} q^\circ_j - f(Q^\circ_k) \sum^{i \neq k} Q^\circ_i. \qquad (2)$$

$Q^\circ_i$ refers to the adsorption energy of each solute group $i$, $q^\circ_j$ refers to a contribution to solute adsorption energy by interaction of two solute groups in a particular

geometry $j$, and $f(Q^\circ_k)$ is a localization function dependent upon the adsorption energy $Q^\circ_k$ of the strongest adsorbing solute group k (see Table II of ref. 3 for a list of values of $f(Q^\circ_k)$ *versus* $Q^\circ_k$). For the unsubstituted or methyl substituted azaaromatics (as reported by KLEMM *et al.*[1]) $Q^\circ_i$ for all aromatic carbons (—C=) equals 0.31, for methyl substituents $Q^\circ_i$ equals 0.06, and for the nitrogen atom(s) $Q^\circ_i$ (or $Q^\circ_n$) is dependent upon the adjacent substituents. Values of $Q^\circ_n$ for a number of nitrogen atom configurations have been tabulated (Table I of ref. 5), other values are reported in ref. 4, and still other values may be calculated from Figs. 2 and 3 of ref. 4. The only important $q^\circ_j$ terms in the present series of azaaromatics are those due to electronic activation of the nitrogen atom by a methyl group or another nitrogen atom contained in the same molecule[5]. For the azaaromatics studied by KLEMM *et al.*[1] we may rewrite eqn. (2) as follows:

*Monoazaaromatics*

$$S^\circ = Q^\circ_n + 1.86 + [1 - f(Q^\circ_k)]\,[0.06\, n_m + 0.31\,(n_a - 6)] + \sum^m C_e\, Q^\circ_n \qquad (2a)$$

*Diazaaromatics*

$$S^\circ = Q^\circ_n + 1.86 + [1 - f(Q^\circ_k)]\,[0.06\, n_m + 0.31\,(n_a - 6) + Q^\circ_{n'}] + \\ + \sum^m C_e\, Q^\circ_n + \sum^m C_e\, Q^\circ_{n'} + C_{e'}\,[Q^\circ_n + f(Q^\circ_k)\, Q^\circ_{n'}] \qquad (2b)$$

Here $n_m$ and $n_a$ refer, respectively, to the numbers of methyl groups and aromatic carbon atoms in the solute; $Q^\circ_{n'}$ is the $Q^\circ_i$ value of the less strongly adsorbed nitrogen atom in a diazaaromatic. The terms $\sum^m$ are summations over all methyl groups. The electronic interaction parameter $C_e$ depends upon the relative positions of a methyl group and the nitrogen atom with which it is interacting:

for *ortho* or *para* methyls in the ring of the nitrogen, $C_e = 0.095$

for *meta* methyls in the ring of the nitrogen, $C_e = 0.039$

for methyls in a ring adjacent to the ring of the nitrogen, $C_e \cong 0.034$.

The last term of eqn. (2b) can be estimated from the data of Table II of ref. 5; in the case of pyrazine derivatives, $C_{e'} \cong -0.42$. Eqns. (1) and (2) may be applied to TLC systems by means of the well known, approximate relationship (see refs. 7 and 8)

$$R_F\, f(g) = \frac{1}{1 + (W_a/V_s)\underline{R}^\circ} \qquad (3)$$

where $R_F$ and $\underline{R}^\circ$ are values from analogous thin-layer and column systems (same adsorbent activity, same eluent) for the same solute, $W_a$ and $V_s$ are the weight of adsorbent and volume of solvent (eluent) in a given volume of plate or column, and $f(g)$ is a constant (ideally equal one) for a given TLC system, normally equal to 1.1 $f(g)$ values greater than one arise from variations in the ratio $W_a/V_s$ in the direction of solvent flow on a plate (solvent gradient). With pre-equilibration of the plate in the solvent chamber, as practiced by KLEMM *et al.*[1] the *effective* solvent gradient will be increased, with increase in $f(g)$. In agreement with this expectation, it was found that a best fit of eqn. 3 to the data of KLEMM *et al.* gives $f(g)$ equal 1.25 for chloroform–

TABLE I

CALCULATION OF $R_F$ VALUES OF AZAAROMATICS IN SYSTEMS OF KLEMM *et al.*[1]

| *Solute*[a] | $Q^\circ_n$ | $S^\circ$ | $A_s$[b] | $(S^\circ - \varepsilon^\circ A_s)$ | $R_F$ | |
|---|---|---|---|---|---|---|
| | | | | | *Exptl.* | *Calc.*[c] |
| 1, I | — | 4.34 | 11.6 | 0.05 | 0.78 | 0.79 |
| 2, I | 0.7[d] | 7.22 | 16.0 | 1.30 | 0.71 | 0.73 |
| 3, I | 0.7[d] | 7.11 | 15.0 | 1.56 | 0.70 | 0.70 |
| 4, I | 0.7[d] | 7.01 | 14.0 | 1.82 | 0.70 | 0.66 |
| 5, I | 0.7[d] | 5.97 | 12.0 | 1.53 | 0.68 | 0.71 |
| 7, I | 2.3[d] | 6.65 | 12.0 | 2.21 | 0.64 | 0.59 |
| 10, I | 2.3[d] | 5.85 | 10.0 | 2.15 | 0.59 | 0.70 |
| 15, I | | 6.5[e] | 10.0 | 2.80 | 0.47 | 0.44 |
| 16, I | 3.3[f] | 7.22 | 13.0 | 2.41 | 0.45 | 0.54 |
| 17, I | 3.3[f] | 7.44 | 14.0 | 2.26 | 0.44 | 0.58 |
| 18, I | 3.8[d] | 6.46 | 10.0 | 2.74 | 0.41 | 0.45 |
| 19, I | 3.8[d] | 6.91 | 12.0 | 2.47 | 0.41 | 0.53 |
| 20, I | 3.8[d] | 7.02[g] | 12.0 | 2.58 | 0.41 | 0.50 |
| 21, I | | 6.0[e] | 8.0 | 3.04 | 0.40 | 0.37 |
| 22, I | 3.8[d] | 6.85 | 10.0 | 3.15 | 0.39 | 0.34 |
| 23, I | 3.8[d] | 7.02 | 11.0 | 2.95 | 0.37 | 0.39 |
| 24, I | 4.0[d] | 6.49 | 10.0 | 2.79 | 0.36 | 0.44 |
| 25, I | 4.0[d] | 8.29[h] | 13.6 | 3.26 | 0.35 | 0.31 |
| 26, I | 3.3[f] | 6.36 | 9.5 | 2.84 | 0.34 | 0.42 |
| 27, I | 4.0[d] | 6.37 | 8.0 | 3.41 | 0.33 | 0.27 |
| 28, I | 4.0[d] | 7.05 | 10.0 | 3.35 | 0.33 | 0.28 |
| 29, I | 4.0[d] | 7.05 | 10.0 | 3.35 | 0.32 | 0.28 |
| 30, I | 4.0[d] | 7.73 | 12.0 | 3.29 | 0.30 | 0.30 |
| 31, I | 4.0[d] | 7.73 | 12.0 | 3.29 | 0.30 | 0.30 |
| 32, I | | 7.6[e] | 10.0 | 3.90 | 0.25 | 0.15 |
| 33, I | 4.8[d] | 8.40[h] | 11.6 | 4.10 | 0.24 | 0.12 |
| 34, I | 4.8[d] | 6.89[g] | 9.6 | 3.34 | 0.21 | 0.28 |
| 35, I | 4.8[d] | 8.40[h] | 11.6 | 4.10 | 0.18 | 0.12 |
| 37, I | | 7.6[i] | 9.6 | 4.05 | 0.16 | 0.13 |
| 38, I | 4.0[d] | 9.66[j] | 14.0 | 4.48 | 0.13 | 0.08 |
| 39, I | 7.4[k] | 10.3[e] | 10.0 | 6.60 | 0.01 | 0.00 |
| 40, I | 7.4[k] | 10.87 | 12.0 | 6.43 | 0.01 | 0.01 |
| 41, I | 7.4[k] | 11.40 | 12.0 | 6.96 | 0.00 | 0.00 |
| 42, II | 0.7[d] | 7.62[l] | 19.6 | —2.0 | 0.69 | 0.70 |
| 43, II | 0.7[d] | 5.14[l] | 15.6 | —2.5 | 0.69 | 0.70 |
| 45, II | 3.8[d] | 6.92[l] | 15.6 | —0.7 | 0.68 | 0.70 |
| 22, II | 3.8[d] | 6.85 | 10.0 | 1.95 | 0.66 | 0.67 |
| 27, II | 4.0[d] | 6.37 | 8.0 | 2.45 | 0.65 | 0.65 |
| 48, II | | 8.3[e] | 10.0 | 3.40 | 0.63 | 0.54 |
| 51, II | | 7.0[e] | 8.0 | 3.08 | 0.55 | 0.59 |
| 54, II | | 7.7[e] | 8.0 | 3.78 | 0.52 | 0.47 |
| 56, II | 5.5[d] | 8.26 | 9.0 | 3.85 | 0.50 | 0.45 |
| 57, II | | 8.7[e] | 8.0 | 4.78 | 0.24 | 0.24 |
| 39, I | | 10.3[e] | 10.0 | 5.40 | 0.02 | 0.12 |

(*Continued on page 435*)

*Table 1 (continued)*

| *Solute*[a] | $Q^\circ_n$ | $S^\circ$ | $A_s$[b] | $(S^\circ - \varepsilon^\circ A_s)$ | $R_F$ | |
|---|---|---|---|---|---|---|
| | | | | | *Exptl.* | *Calc.*[c] |
| 61, III | 2.71[m] | 5.36 | 9.0 | 2.03 | 0.56 | 0.67 |
| 62, III | 2.71[m] | 5.66 | 10.0 | 1.96 | 0.53 | 0.68 |
| 65, III | 3.8[d] | 6.56 | 9.0 | 3.23 | 0.45 | 0.40 |
| 66, III | 3.8[d] | 6.73 | 10.0 | 3.03 | 0.43 | 0.45 |
| 67, III | 4.0[d] | 6.37 | 8.0 | 3.41 | 0.42 | 0.35 |
| 68, III | 4.0[d] | 6.54 | 9.0 | 3.21 | 0.39 | 0.40 |
| 69, III | 3.8[d] | 6.96 | 10.0 | 3.26 | 0.39 | 0.39 |
| 70, III | 4.0[d] | 6.54 | 9.0 | 3.21 | 0.39 | 0.40 |
| 72, III | 4.0[d] | 6.78 | 9.0 | 3.45 | 0.37 | 0.33 |
| 73, III | 4.0[d] | 6.78 | 9.0 | 3.45 | 0.35 | 0.33 |
| 74, III | 4.0[d] | 6.78 | 9.0 | 3.45 | 0.34 | 0.33 |
| 75, III | 4.0[d] | 6.95 | 10.0 | 3.25 | 0.33 | 0.39 |
| 76, III | 4.8[d] | 7.17 | 8.0 | 4.21 | 0.32 | 0.15 |
| 51, III | | 7.0[e] | 8.0 | 4.04 | 0.29 | 0.19 |
| 54, III | | 7.7[e] | 8.0 | 4.74 | 0.12 | 0.08 |

[a] Arabic numerals refer to solute (numbering system of KLEMM *et al.*[1]); roman numerals refer to chromatographic system (Table numbers of KLEMM *et al.*[1]): I = benzene–chloroform (1:1), 4.0% $H_2O$–$Al_2O_3$; II = butanone-2, 8.8% $H_2O$–$Al_2O_3$; III = benzene–chloroform (1:1), 4.4% $H_2O$–$Al_2O_3$.

[b] $A_s$ values calculated as described in ref. 12 unless otherwise noted.

[c] From eqns. 2a, 2b, 3, and 4, assuming the following parameters:

| *System* | *Log* $V_a$ | $\alpha$ | $\varepsilon^\circ$ | f($g$) |
|---|---|---|---|---|
| I | —1.85 | 0.63 | 0.37 | 1.25 |
| II | —2.53 | 0.59 | 0.49 | 1.43 |
| III | —2.00 | 0.62 | 0.37 | 1.25 |

[d] Table I (ref. 5).

[e] Table II (ref. 5).

[f] Figures 2 and 3 (ref. 4) ($Q^\circ_n = 1.13\ F_N$).

[g] For adsorption energy of phenyl group (and contribution to $A_s$), see ref. 14.

[h] f($Q^\circ_k$) for phenyl group equal 0.15 (rather than 0.45) because of separation of phenyl group from localized nitrogen (see Table I, ref. 5 and related discussion).

[i] $S^\circ$ calculation discussed in ref. 5.

[j] $C_e'$ estimated equal to value for 1,5-diazaanthracene (Table II, ref. 5).

[k] Combined value of $Q^\circ_i$ for both nitrogen atoms (Table I, ref. 5).

[l] $C_e'$ is estimated at 0.42 as in the case of *trans*-azobenzene (see ref. 5).

[m] Values reported in ref. 4 ($Q^\circ_n$ equal $1.13\ F_N$).

benzene solvent and 1.43 for 2-butanone solvent. As discussed previously[8] combination of eqns. 1 and 3 with the function $R_M'$ defined equal to log [$1/R_F$ f($g$) — 1] gives

$$R_M' = \log (V_a W_a / V_s) + \alpha(S^\circ - \varepsilon^\circ A_s) \qquad (4)$$

Application of eqn. (4) to the data of KLEMM *et al.*[1] yields the activity of the adsorbent used by these authors: Table I (ref. 1), 4.0% $H_2O$–$Al_2O_3$; Table II (ref. 1), 8.8% $H_2O$–$Al_2O_3$; Table III (ref. 1), 4.4% $H_2O$–$Al_2O_3$ (standard activity scale[8]). Assuming these three adsorbent parameters, it is possible to calculate $R_F$ values in the system of KLEMM *et al.* for almost all of the azaaromatics (59 $R_F$ values), using eqns.

(2a) or (2b) and (4). Table I summarizes these calculations along with pertinent details so that the steps involved may be easily followed. Similarly Fig. 1 compares experimental and calculated $R_F$ values. The adequacy of these calculated $R_F$ values is clearly evident from Fig. 1 and Table I. The standard deviation between the experimental and calculated $R_F$ values (52 different azaaromatic solutes) is $\pm$ 0.065 $R_F$ units. By way of evaluation, a similar correlation[8] of three previously reported TLC studies of hydrocarbons adsorbed on alumina[9–11] showed a standard deviation between experimental and calculated $R_F$ values of $\pm$ 0.06 units. Thus it appears that the present

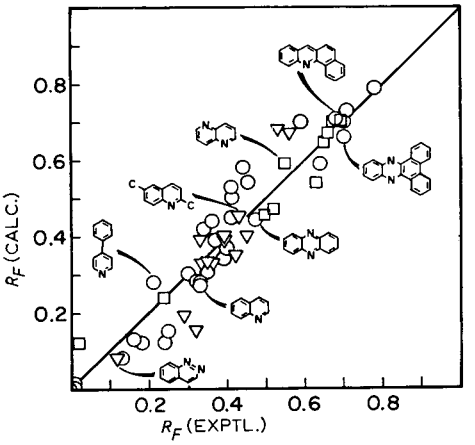


Fig. 1. Comparison of experimental and calculated $R_F$ values. ○ Data of Table I (ref. 1): benzene–chloroform (1:1); □ data of Table II (ref. 1): butanone-2; ▽ data of Table III (ref. 1): benzene–chloroform, 1:1.

theoretical treatment is essentially equivalent in its ability to correlate or predict $R_F$ values (and $\underline{R}°$ values) in the case of both hydrocarbons and azaaromatics. If it is conceded that the present correlational equations have been "rigorously tested for aromatic hydrocarbons"[1], then their "applicability... to the clarification of the geometry of adsorption of... (the) azarenes"[1] seems likewise assured. The number of different compounds successfully treated by eqns. (1–4) is now actually greater in the case of the azaaromatics than for the hydrocarbons.

The present theoretical model (the basis of eqns. 1 and 2) requires that all adsorbing groups in the azaaromatics (*i.e.*, aromatic carbon atoms, nitrogen atoms) be immediately adjacent to the alumina surface, with negligible differences in the distances of various parts of the molecule from the surface. Any significant "tilting" must markedly lower the adsorption energies of solute groups which are moved away from the surface. Previous studies have shown that there is *no* tendency toward reduced adsorption energies for such groups (*e.g.*, *para* substituted pyridines[4], azaaromatics of similar nitrogen geometry and varying ring size[3,4], diazaaromatics with nitrogen atoms on opposite sides of the solute molecule[1,5]). Similarly previous studies have used the technique of "eluent variation"[12] to measure the area $A_s$ required by large azaaromatics in adsorption on alumina; these areas are essentially identical with those calculated for planar adsorption, and markedly different from areas corresponding to edgewise adsorption. These prior studies plus the present correlation

of the data of KLEMM *et al.* appear to exclude completely the existence of tilted or edgewise adsorption in any of the fused azaaromatics, in the sense that certain solute atoms or groups, are not immediately adjacent to atoms of the alumina surface. Actually none of the arguments of KLEMM *et al.*[1,2] have been addressed to this specific point. Rather these authors have emphasized the probability of involvement of the nitrogen *n* electrons in bonding to the alumina surface, and the corresponding preference for an Al–N bond in the plane of the azaaromatic molecule. With the latter point the present author is in essential agreement, as noted in ref. 4 (however the possibility of modest deformation of an *n* electron N–Al bond out of the plane of the solute molecule seems completely consistent with previous data). The apparent divergency of these two views of the configuration of the adsorbed azaaromatics ("planar" adsorption, N–Al bond in plane of solute molecule) results from an overly simple conception of the alumina surface: *i.e.*, a simple plane formed by one face of an alumina crystal. The actual picture of the alumina surface is doubtless considerably more complex; *e.g.*, recent workers have postulated[13] a defect structure for active aluminum sites. As KLEMM *et al.*[1] have themselves observed "...the terms 'flatwise' or 'edgewise' need not be mutually exclusive or contradictory for it is readily conceivable that a molecule may be adsorbed both ways simultaneously...". In conclusion, we favor just such a configuration of the adsorbed azaaromatics, one with all atoms of the molecule adjacent to what might be termed the "major" plane of the alumina surface, and the nitrogen atom bonded to an Al atom lying in a different plane. One such possibility in the case of adsorbed pyridine is illustrated in Fig. 2.

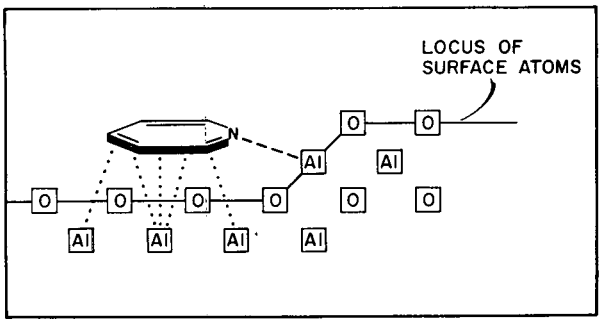


Fig. 2. Hypothetical representation of adsorbed pyridine on the alumina surface (cross-sectional view).

The author is grateful to Professor KLEMM for kindly making available a prepublication copy of his paper[1].

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## A simple device for filling a chromatographic column

The methods generally used, besides being tedious and time-consuming, do not always provide a uniform filling. The device described below removes the drudgery of column filling and enables even a novice to effect efficient and rapid filling under anhydrous conditions.

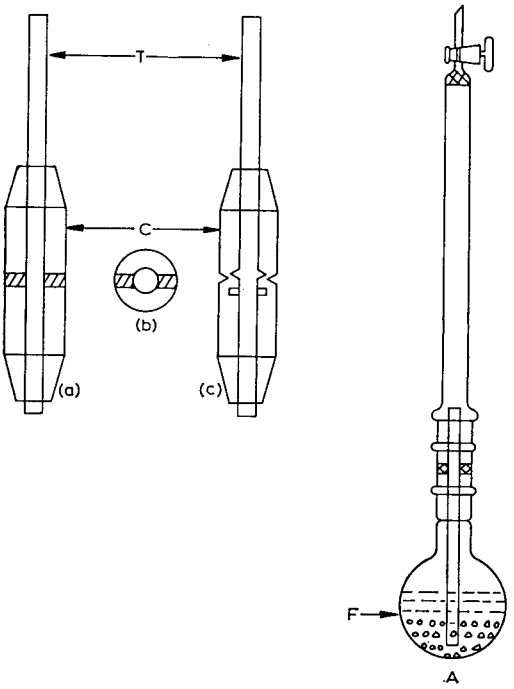


Fig. 1. Device for filling a chromatographic column. a and c = plan; b = sectional elevation. For further explanation of designations, see text.

The device (see Fig. 1) comprises a double cone C and a central tube T which is held appropriately mid-way. The size of the double cone C is such that its one end fits the chromatographic column and the other end fits the round-bottomed flask F, which contains the required quantities of the adsorbent and solvent. One end of the tube T slightly protrudes from the double cone C while the other end almost reaches the bottom of the flask F. The device, the column (carrying a cotton plug near the tap), and the flask F with its contents are assembled as shown in A. The tap is turned off and the contents of the flask are vigorously shaken. The tap is opened to release pressure, simultaneously displacing most of the air of the assembly A. The tap is again turned off and the contents of the flask are vigorously shaken once more, and the assembly A is quickly turned upside down. The slurry, so formed, rushes down through the annular space, while at the same time, the solvent vapour and any residual air of the column shoot through the central tube T thereby accelerating the flow of the slurry. The transfer is so fast—about fifteen seconds for small columns—that the process of sedimentation of the adsorbent does not begin until all the slurry has been delivered into the column. But soon afterwards the adsorbent begins to deposit producing a uniform column without channels.

There are two ways of holding the central tube T in the double cone C so that enough clearance is left in the annular space to allow the slurry to flow. The first is to fuse two equal lengths of thin glass rod, diagonally opposite, to the inside of the double cone C and the outside of the tube T. The device so produced is extremely efficient, but it is very fragile and somewhat difficult to make. Alternatively, the tube T can be held in a concentric position by three radially spaced dimples in the double cone C. In this case a ridge is made on the tube T which rests on one side of the row of dimples, and on the other side the tube T is held by a solvent-resistant collar. The device so made has comparatively less annular space, but it is fairly robust and easy to make.

The device could be adopted for filling, in a like manner, GLC columns, and for simultaneous filtering and transferring solutions of the Grignard reagents, phenyllithium, lithium aluminium hydride, etc., under anhydrous conditions. The latter operation, which is sometimes necessary for inverse additions, the Wittig reaction, etc., is carried out by inserting a small plug of glass wool in the annular space in the device, one end of which is attached to the flask F, carrying the reagent to be transferred, and the other to a separatory funnel (with its tap closed) to be used for adding the solution, and then inverting this assembly. The glass wool prevents most of the solid matter from running into the separatory funnel. *When using low-boiling solvents it is extremely important to eliminate all positive pressure from the assembly*, or else the system would blow off and precious compounds lost. In such cases, cooling the flask F, and use of gloves to prevent transfer of heat from hands is recommended.

The author wishes to thank Mr. N. E. ANTHONY for assistance in making the device.

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## Chromatographic identification of carbonyl compounds
## VII. Thin-layer chromatographic resolution of mixtures of keto acid methyl ester 2,4-dinitrophenylhydrazones*

Numerous solvent mixtures and adsorbents have been utilised in attempts to develop a method for the separation of 2,4-dinitrophenylhydrazone mixtures of keto acid methyl esters on thin layers, so that each component occurs in only one spot. The method developed has already been reported briefly in a preliminary communication[1], and is discussed below in greater detail, with consideration being given to the possible interference of the reagent with identification of the components, and an examination is made of the completeness of isolation of the components from aqueous ethanol.

*Experimental*

*Treatment and dissolution of keto acid methyl ester hydrazones.* Two hundredths of a millimole of each of the pure hydrazones of pyruvic, 2-oxobutyric, 2-oxoisovaleric, 2-oxoisocaproic, 2-oxo-3-methylvaleric, levulinic, 2-oxoglutaric and oxalacetic acid methyl esters were weighed and dissolved in a 10-ml volume of dioxan (for chromatography, E. Merck AG) and their mixture R containing 0.02 mmole of each component was likewise dissolved in 10 ml of dioxan.

A mixture of the same keto acid hydrazones, isolated from 4 l of the 8 wt. % aqueous ethanol solution by adsorption on carbon and selective elution (first the aldehyde hydrazones and then the keto acid hydrazones) from the latter (Part I), was treated with methanol containing hydrogen chloride to liberate the acid hydrazones from their pyridinium salts. After evaporation of the solvent, the residue was esterified with diazomethane in diethyl ether at 0° (*cf.* Part V). The resulting ester mixture, designated M, was isolated and dissolved in 10 ml of dioxan.

*Thin-layer chromatography and staining of the spots.* A 4-$\mu$l volume of the dioxan solution of each keto acid methyl ester hydrazone, 4 $\mu$l of the solution containing their mixture R and two different volumes, 4 and 40 $\mu$l, of dinitrophenylhydrazine solution (1 mg/ml) were applied to an activated thin layer of Silica Gel $HF_{254}$ (for thin-layer chromatography, E. Merck AG). The activation was carried out by heating at 120° for half an hour. Equal volumes (4, 2, 1 and 0.5 $\mu$l) of the solutions of the mixtures M and R were applied side by side to a second activated thin layer of Silica Gel $HF_{254}$. The plates were equilibrated in the vapour of the chromatographic solvent mixture composed of 18 volumes of petroleum ether (boiling range 60–80°, British Drug Houses Ltd.), 2 volumes of pyridine (Baker Analyzed Reagent, J. T. Baker Chemical Co.) and 1 volume of diethyl oxalate (purum, Fluka AG) in a closed chamber for half an hour before the run. The running time was 2 h in the closed chamber and then 2 h in the open chamber. The chromatograms were first photographed and then sprayed with a mixture containing 3 volumes of petroleum ether, 1 volume of pyridine and 1 volume of epichlorohydrin (purum, Fluka AG) and kept in a closed chamber above the same solvent mixture for one hour, after which the chromatograms were photographed again.

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* For Parts I–VI of this series, see *J. Chromatog.*, 27 (1967) 374, 380, 384; 28 (1967) 253, 259, 263.

*Results and discussion*

*Thin-layer chromatograms of the hydrazones of the keto acid methyl esters.* Fig. 1 shows the thin-layer chromatogram of the individual pure 2,4-dinitrophenylhydrazones of the keto acid methyl esters, their mixture R and dinitrophenylhydrazine. The photograph was taken one hour after the chromatogram had been sprayed with the (3:1:1) petroleum ether–pyridine–epichlorohydrin mixture. The spots that had turned red were encircled by continuous lines and those that had remained yellow by

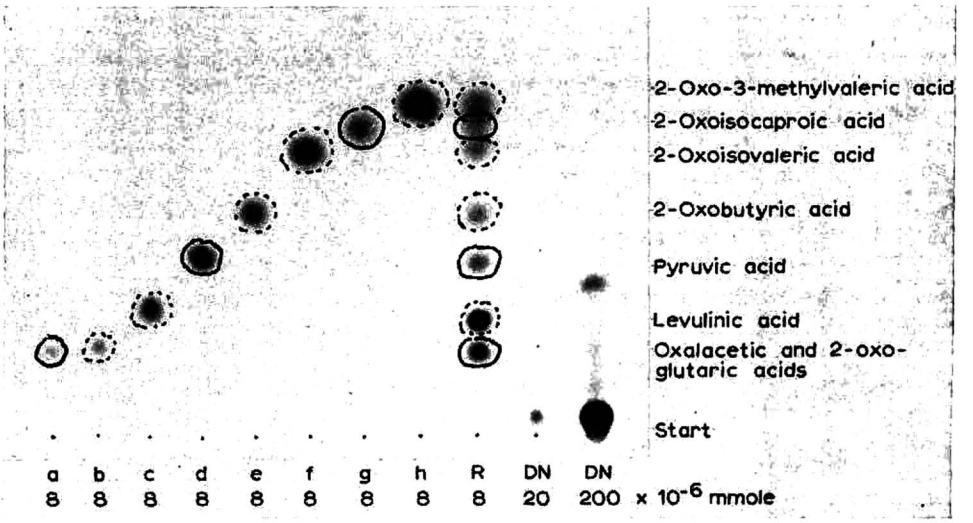


Fig. 1. Thin-layer chromatogram of pure 2,4-dinitrophenylhydrazones of oxalacetic (a); 2-oxoglutaric (b); levulinic (c); pyruvic (d); 2-oxobutyric (e); 2-oxoisovaleric (f); 2-oxoisocaproic (g); 2-oxo-3-methylvaleric (h) acid methyl esters; their mixture (R) and 2,4-dinitrophenylhydrazine (DN). The chromatographic solvent was petroleum ether–pyridine–diethyl oxalate (18:2:1) and adsorbent Silica Gel $HF_{254}$. Spots that became reddish within one hour after spraying the plate with a (3:1:1) mixture of petroleum ether–pyridine–epichlorohydrin are encircled by continuous lines, and spots which remained yellow by broken lines.

broken lines. Even the latter spots, except the spot of the levulinic acid derivative, gradually turned red during the course of several hours. It is possible, by this method, to identify the isomers 2-oxoisocaproic acid and 2-oxo-3-methylvaleric acid owing to the different rates of staining of their hydrazones; the derivative of the former changes colour more rapidly than that of the latter, although their $R_F$ values differ only slightly. Each keto acid methyl ester hydrazone gave only one spot when the petroleum ether–pyridine–diethyl oxalate (18:2:1) mixture was used as chromatographic solvent, but some of the hydrazones gave two successively migrating spots of stereoisomers when the ratio of the last two solvent components was varied. The spots of the dinitrophenylhydrazones of monomethyl 2-oxoglutarate and monomethyl oxalacetate overlap, but the spots of the other keto acid ester hydrazones are satisfactorily resolved.

Fig. 2 shows a similarly produced thin-layer chromatogram of a mixture M of keto acid methyl ester hydrazones isolated from the 8 wt. % aqueous ethanol solution and of the reference mixture R.

The amounts of the mixtures applied to the thin layer were varied similarly, and the intensities of the corresponding spots should have been equal if the adsorption and elution had been quantitative. There is no sign of a spot due to levulinic acid in the chromatogram of the mixture M. The possible existence of oxalacetic acid in small quantities among other keto acids cannot be determined by this method because monomethyl oxalacetate hydrazone migrates at the same rate as monomethyl

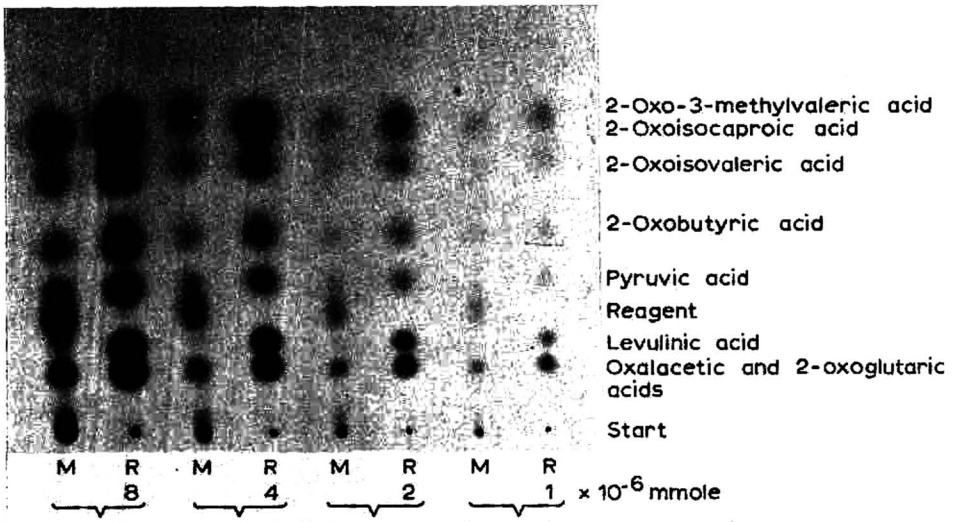


Fig. 2. Thin-layer chromatogram of the mixture M of 2,4-dinitrophenylhydrazones of keto acid methyl esters prepared by esterification with diazomethane of keto acid hydrazones isolated by adsorption on carbon from aqueous ethanol and elution from the carbon, and of the reference mixture R of pure keto acid methyl ester hydrazones. The chromatographic solvent was petroleum ether–pyridine–diethyl oxalate (18:2:1) and adsorbent Silica Gel $HF_{254}$. This thin-layer chromatogram was photographed before spraying.

2-oxoglutarate hydrazone. All the keto acid methyl ester hydrazones in the mixture M, except those of levulinic acid and oxalacetic acid, could be identified on the basis of their locations and different rates of staining of the spots. One additional spot due to dinitrophenylhydrazine is seen in the chromatogram of the mixture M (*cf.* Fig 1). The reagent, however, does not interfere with the identification of individual components.

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## Chromatographic identification of carbonyl compounds VIII. The carbonyl compounds in a fermented glucose solution*

The previously described thin-layer chromatographic method[1] (*cf.* Part VI) for the analysis of keto acid 2,4-dinitrophenylhydrazones has been applied for identification of the keto acids present in sugar solutions fermented by yeast[2,3]. The keto acids were converted into their 2,4-dinitrophenylhydrazones, which were isolated by extraction[2–4] In this study, the isolation of carbonyl compounds from a fermented solution was effected by the adsorption and elution technique developed (Part I) and in this case, in contrast to methods applied earlier, the aliphatic aldehydes were also


Fig. 1. Gas chromatogram of carboxylic acids produced by ozone oxidation of a mixture of 2,4-dinitrophenylhydrazones of aldehydes isolated by adsorption on carbon from a fermented glucose solution and elution from the carbon. Acetaldehyde is analysed as acetic acid, propionaldehyde as propionic acid, and so on. Conditions: column length, 4 m; internal diameter, 3 mm; liquid phase, NEGS containing phosphoric acid; solid support, acid-washed Chromosorb W; carrier gas, helium; flow rate, 75 ml/min; inlet pressure, 2.5 kp/cm$^2$; temperature 140°; detection by flame ionisation; sensitivity, 1; injected volume, 5 $\mu$l. 1 = Solvent; 2 = acetic acid; 3 = propionic acid; 4 = isobutyric acid; 5 = butyric acid; 6 = isovaleric acid and/or 2-methylbutyric acid.

isolated as their hydrazones. In the analyses of aldehydes and keto acids, the gas chromatographic methods developed previously (see Parts IV and V) were applied.

* For Parts I–VII of this series, see *J. Chromatog.*, 27 (1967) 374, 380, 384; 28 (1967) 253, 259, 263, 440.

*Experimental*

The fermentation was effected by the addition of 20 g of commercial baker's yeast (Rajamäki Factories of the Finnish State Alcohol Monopoly) to 2 l of 12 % glucose solution. In about 12 h at 30°, the glucose had been fermented to approximately 0.1 %, and the fermentation was interrupted by cooling. The yeast was separated on a Büchner funnel and washed. A 60-ml volume of 2,4-dinitrophenylhydrazine solution (2.5 g of the reagent in 1 l of 2 *N* hydrochloric acid) was added to the filtrate. No

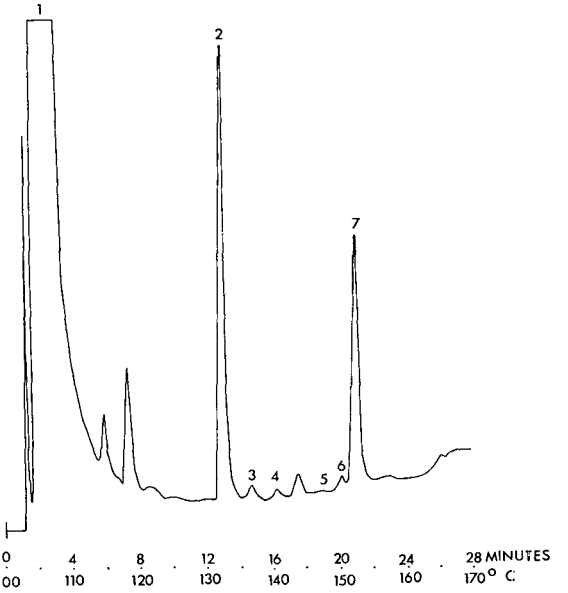


Fig. 2. Gas chromatogram of keto acid methyl esters liberated by ozone oxidation from a mixture of 2,4-dinitrophenylhydrazones of keto acid methyl esters produced by esterification with diazomethane of the keto acid hydrazones isolated by adsorption on carbon from a fermented glucose solution and elution from the carbon. Conditions: column length, 4 m; internal diameter, 3 mm; liquid phase, DEGA containing phosphoric acid; solid support, acid-washed Chromosorb W; carrier gas, helium; flow rate, 63 ml/min; inlet pressure, 1.8 kp/cm²; temperature programme, 100–170°/2.5°/min; detection by flame ionisation; sensitivity 8; injected volume, 5 $\mu$l. 1 = Solvent; 2 = methyl pyruvate; 3 = methyl 2-oxoisovalerate; 4 = methyl 2-oxobutyrate; 5 = methyl 2-oxo-3-methyl valerate; 6 = methyl 2-oxoisocaproate; 7 = unknown derivative of pyruvic acid.

precipitate was formed after standing overnight at room temperature. The hydrazones were isolated from the solution after its neutralisation with dilute ammonia by adsorption on activated carbon (*cf.* Part I). Aldehyde hydrazones were eluted from the carbon with methyl formate and dichloromethane, successively. The extract, dissolved in 0.5 ml of formic acid, was ozonizated and analysed by isothermal gas chromatography on a NEGS column. The keto acid hydrazones were then eluted from the carbon with an azeotropic pyridine–water mixture at reduced pressure. The keto acid hydrazones extracted were first liberated from their pyridinium salts by treatment with methanol containing hydrogen choride and then esterified with diazomethane in ether at 0°. The mixture of the methyl ester hydrazones was dissolved for ozonation and subsequent gas chromatography in 0.5 ml of a dichloromethane–methanol (1:4,

v/v) mixture. A temperature programme and a DEGA column were used in the chromatographic run.

*Results*

Gas chromatograms showing the resolution of derivatives of carbonyl compounds formed in glucose fermentation by baker's yeast under anaerobic conditions are shown in Figs. 1 and 2. The aldehydes were identified as carboxylic acids (Fig. 1) and the keto acids as their methyl esters (Fig. 2). On the basis of the chromatogram in Fig. 1, the aldehyde present in greatest amount in the fermented solution was acetaldehyde and the other aldehyde components were propionaldehyde, isobutyraldehyde, butyraldehyde and isovaleraldehyde and/or 2-methylbutyraldehyde. Pyruvic acid is the dominating keto acid (Fig. 2); it gives rise to two peaks (*cf.* Part V, Fig. 1) of which that designated "2" is due to methyl pyruvate, and that designated "7" is due to an unknown derivative of pyruvic acid formed in the ozonization of methyl pyruvate dinitrophenylhydrazone. The trace components are the same as have previously been found present in fermented liquors by thin-layer chromatography of their dinitrophenylhydrazones, *viz.* 2-oxobutyric acid, 2-oxoisovaleric acid and 2-oxoisocaproic acid[2]. There is only a very small peak due to 2-oxo-3-methylvaleric acid in the chromatogram of Fig. 2; this acid was, however, identified when a greater detector sensitivity was employed. There is one unidentified peak between the peaks 4 and 5 in Fig. 2. The keto acids in the fermented glucose solution were also analysed by thin-layer chromatography of their hydrazones. 2-Oxoglutaric acid, which cannot be identified in the gas chromatographic method used, was then found to be present in an amount of the same order of magnitude as pyruvic acid (*cf.* ref. 2).

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## A technique for the removal of the active silica gel layer from Eastman Chromagram sheet and its subsequent replication

The material now known as Eastman Chromagram* sheet was first described by LESTIENNE *et al.*[1] at the Thin-Layer Chromatography Conference held in Brussels, Belgium, in September 1964. It consists of a 100 $\mu$ thick, polyvinyl alcohol-bound layer of silica gel on 200 $\mu$ polyethylene terephthalate.

There are times when it would be advantageous to be able to remove the chromatogram developed on the silica gel from its relatively thick (0.008 in.) plastic support. For example, many chromatograms are retained as elements of permanent test records and, as such, are included in data books of various kinds. Anything that can be done to reduce the thickness of the chromatograms obviously decreases the ultimate bulkiness of the book in which they are stored.

We have found that by firmly pressing No. 810 Magic Mending Tape** over the chromatogram and subsequently removing it, the silica gel layer can be transferred almost intact from the supporting plastic to the adhesive layer of the tape. Since the thickness of the 3M tape is of the order of 0.0027 in., the thickness of the chromatogram has been reduced by over 0.005 in.

Further, another strip of tape can be pressed over the now-transferred silica gel and the two strips of tape subsequently separated, splitting the chromatogram, and producing a mirror image of the original. This process can be repeated several times to produce multiple, though obviously less distinct, copies. We have, using this technique, produced up to sixteen replicas of a single ink chromatogram. Using this approach it is possible with one separation to provide samples for inclusion in several notebooks, etc. or for inter-laboratory exchange.

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*Janesville, Wisc. (U.S.A.)*

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* Product of Distillation Products Industries, Division of Eastman Kodak Company, Rochester, N.Y. 14603.

** Product of Minnesota Mining and Manufacturing, St. Paul, Minn.

## On the relationship between amount of substance and spot size in thin-layer chromatography

PURDY AND TRUTER[1], and TRUTER[2] discussed the possible relationships between the amount of substance per spot and the resulting spot size in thin-layer chromatography. They quoted the experiences of STAHL *et al.*[3] showing that, for thin-layer chromatography on silica gel, log weight is not always proportional to spot area.

Out of 10 substances tested by PURDY AND TRUTER[1] on silica gel, the best linear fit was with the relationship between log weight and the square root of the area. The relation between log weight and area gave a less good fit with the actual results.

An "unexpected feature", however, was that some of the substances gave reasonably good linear fits both with the areas and with the square roots of the areas.

The present author studied the relationship between amount of substance and spot area for the common sugars and organic acids, as well as for some amino acids, whereupon some other "unexpected features" turned up.

*Technical procedures*

Cellulose, MN 300 was spread on 12 × 16 cm plates as described by NYBOM[4]. Solvents were, for acids and sugars: ethyl acetate–formic acid–water (3:1:1) and for the amino acids: isopropanol–formic acid–water (20:1:5). Spots were developed with: bromphenol blue (0.016 % BFB + 0.085 % NaAc added to the solvent before separation) for acids; benzidine (*cf.* LINSKENS[5]), for sugars; and isatin[6] and ninhydrin (0.2 % ninhydrin in 5 ml collidine + 20 ml acetic acid + 75 ml methanol) for amino acids. The spots were copied on millimetre tracing paper, and the areas were determined by counting the millimetre squares under weak magnification.

*Results*

It was found that different relationships existed for log weight as a function of the area, depending upon the thickness of the cellulose layer.

Figs. 1 and 2 show this for citric acid and sucrose, but the same general tendency characterized malic acid, as well as glucose and fructose to the same degree.

Slits, 200, 400, 600, 800 and 1,000 $\mu$ wide, were used with the Balsgård spreader[4]. Layers intermediate in thickness gave curves falling between those of both extremes.

As was evident from the figures, a thin layer gave a linear relationship between log weight and area, whereas a thick layer gave a line relating log weight and the square root of the area.

The 350 $\mu$ layers, generally used with cellulose powder, gave reasonably linear fits for the relationship log weight–area for all sugars and acids tested (*cf.* Fig. 7).

As can be seen from Figs. 1 and 2, the spots for corresponding amounts were considerably bigger on the thin layer than on the thick layer. Thus, the thinner layer is more sensitive and will reveal smaller amounts. On the other hand, 48 $\mu$g is too large a quantity to be properly separated on the thinner layer.

When studying the amount–spot size relationships for amino acids, something similar occurred, although this time the same layer thickness, 350 $\mu$, was used in all cases, but the spots were revealed by two different methods, *viz.* with isatin and with ninhydrin.

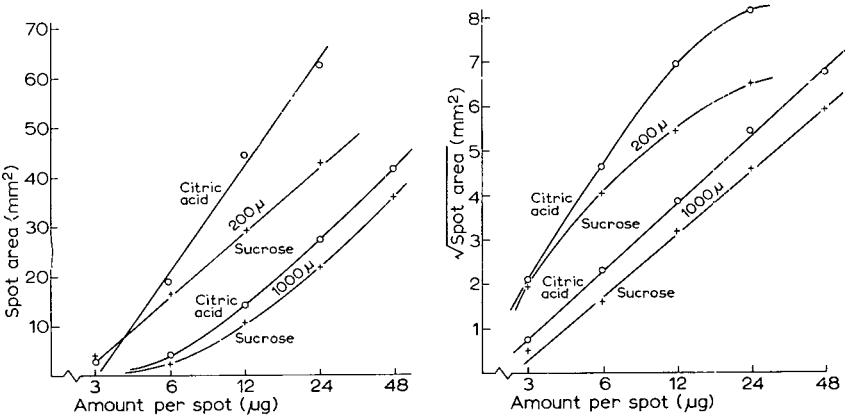
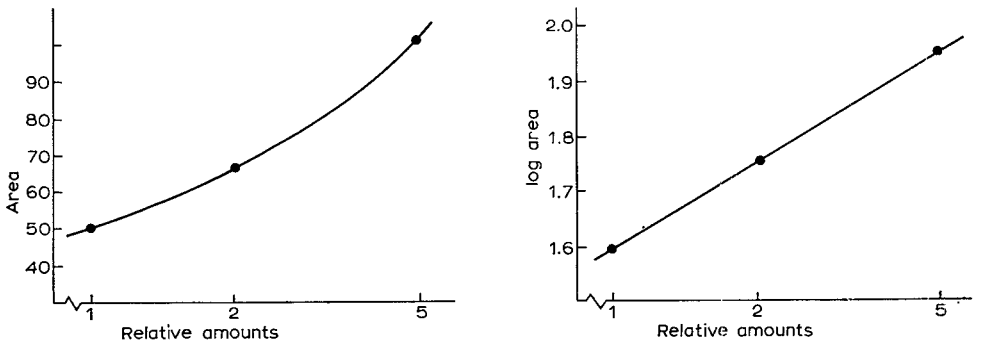



Fig. 1. The relationship between amount of substance per spot, on a logarithmic scale, and the resulting spot area. (O—O—O—) Citric acid; (+—+—+—) sucrose. Bent curves are obtained for the thick layer (1,000 $\mu$) and straight lines for the thin layer (200 $\mu$).

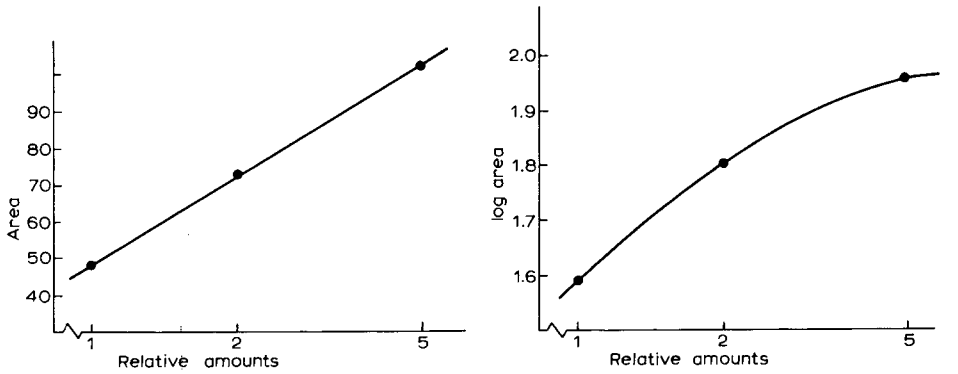
Fig. 2. The same material as in Fig. 1 with the square of the spot area along the ordinate. The thicker layer now gives straight lines, in contrast to the bent curves obtained for the thin layer.

As shown in Figs. 3 and 4, the isatin development gave a linear fit for the relationship log amount–log area, whereas ninhydrin, in Figs. 5 and 6, gave a straight line for the relationship log amount–area. Now, isatin is a less sensitive reagent than ninhydrin, spots containing corresponding amounts becoming considerably bigger with ninhydrin. Thus, one may say that isatin simulates a thicker layer, whereas ninhydrin behaves as if the layer was thinner—both with regard to spot size and with regard to the relationship between amount and spot size. Other amino acids behaved in the same way.

It should be mentioned that the relationship log weight–square root of the area is intermediate in curvature to log weight–area and log weight–log area. Another relationship that might work with very thick layers and insensitive reagents is the relationship weight–area (non-logarithmic scales).




Figs. 3 and 4. Three different amounts of $\beta$-alanine on a logarithmic scale give a straight line relationship with log area when developed with isatin.




Figs. 5 and 6. After ninhydrin development, on the other hand, a straight line relationship is obtained between log amount and the areas of the spots.

*Quantitative routine analysis*

For the quantitative analysis, the spots are encircled as carefully as possible and traced on to semitransparent millimetre paper. Preparatory investigations have to be made in order to find out the actual relationship between amount per spot and spot size.

For the common sugars and organic acids, as shown in Fig. 7, straight lines are obtained if the logarithm of the weight of substance per spot is plotted against the surface area of the spots in a coordinate system.

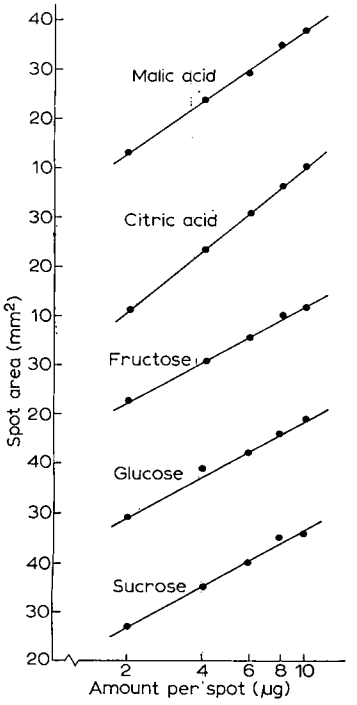



Fig. 7. Calibration curves for the common sugars and organic acids, showing the straight line relationships between log amount per spot and spot area.

Thus, the area $A = m \cdot \log w + c$, where $m$ and $c$ are constants, and $w$ is the amount of substance in the spot. The area may be expressed in $mm^2$ and the weight in $\mu g$.

The constants $m$ and $c$ vary considerably from plate to plate, especially $c$; $m$ being more constant. In one experiment with glucose, with six different plates, $m$ varied between 41 and 56, with an average at *ca.* 50. The constant $c$ varied between 4 and 21, with an average at *ca.* 13. This interplate variation with regard to $m$ corresponds to errors of $\pm 25$ %, whereas the variation in $c$ gives errors of $\pm 40$ %.

These constants, therefore, have to be determined separately for each plate, and at least two standard spots containing different known amounts are necessary per plate.

If the areas of these standard spots are determined for a TLC plate, the amounts in the unknown spots may be calculated according to the following formula:

$$\log w_x = \log w_{ds} + \log d \frac{A_x - A_{ds}}{A_s - A_{ds}},$$

where:

$w_x$ = amount of substance in the spot from an unknown sample,
$w_{ds}$ = amount of substance in the smaller diluted standard spot,
$d$ = the relation (dilution factor) between the two standard spots,
$A_x$ = surface of the unknown spot,
$A_{ds}$ = surface of the smaller standard spot,
$A_s$ = surface of the larger standard spot.

This arithmetic method is a modification of the procedures described by TRUTER[2].

It is often convenient to use standard spots containing 2 and 6 $\mu g$, respectively, of the substance in question. The dilution factor $d$ will then be 3. The above formula then reduces to:

$$\log w_x = 0.778 \frac{A_x - A_{2s}}{A_{6s} - A_{2s}},$$

where $2s$ and $6s$ denote the spots having 2 and 6 $\mu g$ of standard substance, respectively. To reduce the errors, which will be of the order of magnitude of 10–20 % for each determination, one should have two of each of the standard spots (2 and 6 $\mu g$) per plate. There will then be room enough for 8 spots from the samples to be analysed.

If it should turn out during the preparatory work that the relationship between log weight and area does not follow a straight line, one will have to try substituting areas with the square roots of the areas or with the logarithms of the areas. After such proper modifications, the above formulae can again be used in these cases.

Summing up, it is concluded that there does not appear to be any true, or *a priori* expected, theoretical relationship between amount per spot and spot size in thin-layer chromatography. The relationship will vary from substance to substance, depending on the chemical properties, and it will vary with the thickness of the layer as well as with the method used for revealing the spots. It will probably also depend on the absolute amount of substance in the spots, varying from one concentration range to another (*cf.* RANDERATH[7]).

However, for a fixed set of conditions, the determination of spot size lends itself fairly well for an, at least approximate, quantitative determination of the contents of the spots.

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## A thin-layer chromatographic method for distinguishing between natural rubber and synthetic polyisoprene

With the advent of increasing production of synthetic polyisoprene and subsequent use in commercial vulcanizates, there was an obvious need for a simple, reliable method for distinguishing between natural rubber and synthetic polyisoprene, to be used in a programme of research undertaken by the Association.

The present analytical methods, *e.g.*, the Weber test, infra-red spectroscopy etc., will only identify the polymers as *cis*-polyisoprene and will not differentiate between natural rubber and synthetic polyisoprene. Several methods have been published[1,2] which depend on the identification of a minor constituent of natural rubber not found in synthetic polyisoprene. The disadvantages of these methods are that they are time-consuming and present some uncertainty when dealing with rubber of unknown origin.

Natural rubber contains about 1 % w/w of extractable lipid materials[3,4] consisting of phospholipids, sterols, tocopherols, tocotrienols, carotenes, squalene etc., whereas synthetic polyisoprene will only contain ingredients added during manufacture. A new approach to this problem is based on examination of a solvent extract of the rubber by the technique of thin-layer chromatography, in order to characterize and distinguish between the lipid components in natural rubber and the additives in synthetic polyisoprene.

*Experimental*

Five grams (± 0.01 g) of the rubber sample (raw, gum or vulcanized: thinly sheeted on a mill) were extracted for 12 h with Analar acetone under reflux. After removal of the acetone by distillation the extract was dried at 105° for 10 min, dissolved in Analar carbon tetrachloride and diluted to 5 ml in a graduated flask. Silica gel (50 g) (Kieselgel G nach STAHL; neutral grade) was used as the substrate,

being slurried with distilled water (100 ml) and applied to clean, grease-free plates using a Shandon applicator. The plates were activated by heating in an air-oven at 105° for 30 min, followed by cooling and storing in a desiccator.

Suitable aliquots (5 $\mu$l) of the extract were applied to the activated plate using Drummond microcap pipettes and a multiple-spotting template.

A solvent system of 40–60° petroleum ether–ether (50:50, v/v), Analar grades, was used for ascending elution of the plates at a constant temperature of 20 ± 1°. The plates were developed in tanks lined with filter paper and sealed with masking tape, to ensure complete saturation of the tank atmosphere with solvent vapour. The length of the run was 150 ± 5 mm, the time taken for the solvent front to travel this distance being 35 min.

After elution the chromatograms were dried and then sprayed with phosphomolybdic acid solution (10 % w/v in methanol). The lipids in natural rubber gave a blue spot on a yellow background. One of these components, $\beta$-sitosterol, is only detected after heating at 105° for 10 min and it is detection of this component that forms the basis of the described method.

*Results*

*Raw rubbers.* Various grades of natural rubber (RSS1, RSS3, RSS5, pale crepe, SMR 5–50, SMR Heveacrumb 5–50 etc.) and synthetic polyisoprene (Cariflex, Natsyn 200, 400 and 2000) were examined by the described technique. The chromatograms obtained showed that the natural rubber extracts contained a component of $R_F$ value 0.40 ± 0.02 units, only being detected on heating the plate at 105° for 10 min. This component has been identified as being $\beta$-sitosterol by several workers[5,6] and confirmed in these laboratories by comparison of the $R_F$ value obtained from an authentic sample of $\beta$-sitosterol (recrystallized × 6; m.p. 133°).

No corresponding component was detected in the synthetic polyisoprene samples. However a purple spot was detected ($R_F \approx 0.9$) being due to the presence of a phenolic type of antioxidant added during manufacture. Due to the uncertainty of the antioxidant type used by different manufacturers, no definite $R_F$ values can be quoted to characterize the presence of synthetic polyisoprene.

*Gum and vulcanized rubbers.* In order to establish whether any interference was observed from extractable compounding ingredients, several samples of gum and vulcanized rubbers containing different ingredient recipes were examined by the described technique, typical examples being:

| | | |
|---|---|---|
| (1) Polymer | 100 parts | (natural rubber or synthetic polyisoprene); |
| Zinc oxide | 5 parts | |
| Stearic acid | 1 part | |
| Tetramethyl thiuram disulphide | 1.2 parts | |
| Dibenzothiazol-2-yl disulphide | 1.1 parts | |
| Sulphasan R | 0.5 parts | |
| Antioxidant | 1.0 parts | |

Cured at 140°/40 min.

| | | |
|---|---|---|
| (2) Polymer | 100 parts | (natural rubber or synthetic polyisoprene) |
| Zinc oxide | 5 parts | |
| Stearic acid | 2 parts | |
| Cyclohexylbenzothiazol-2-yl sulphenamide | 0.4 parts | |
| Sulphur | 2.0 parts | |
| HAF black | 50.0 parts | |
| Antioxidant | 2.0 parts | |

Cured at 140°/30 min.

In all samples containing natural rubber as the raw polymer, positive identification of $\beta$-sitosterol ($R_F$ 0.40 $\pm$ 0.02) was possible. No component of corresponding $R_F$ value was detected in the synthetic polyisoprenes, although the phenolic antioxidant could be detected ($R_F \approx 0.9$).

An interesting observation was that the relative concentration of $\beta$-sitosterol expressed as the spot area per gram of natural rubber was reasonably constant (3.0 $\pm$ 0.5 mg; tracing and weighing), irrespective of the natural rubber grade, compounding ingredients or curing conditions. Even raising the temperature of cure from 140° to 160° for 40 min had little or no effect on the relative concentration of $\beta$-sitosterol.

It seemed possible that the technique could be made semi-quantitative by estimating the concentration of $\beta$-sitosterol (expressed as spot area) relative to a known weight of natural rubber.

Consequently six blends of natural rubber/synthetic polyisoprene were prepared, the raw polymer composition having the following ratios of natural rubber to synthetic polyisoprene: (1) 100:0 %; (2) 75:25 %; (3) 50:50 %; (4) 25:75 %; (5) 5:95 %; (6) 0:100 %.

Then 100 parts of raw polymer were mixed with 5 parts zinc oxide, 2 parts stearic acid, 2.5 parts sulphur, 0.5 parts cyclohexyl benzothiazol-2-yl sulphenamide, 50.0 parts HAF carbon black, and 2.0 parts antioxidant; and cured at 140°/60 min.

Each sample was examined by the previously described experimental procedure. A typical chromatogram (Fig. 1) shows the presence of the $\beta$-sitosterol component.

The thin-layer chromatogram can be evaluated semi-quantitatively by measurement of the spot area of the $\beta$-sitosterol component, which is related to the volume of extract applied (and hence weight of natural rubber) by the following expression[7]:

$$\sqrt{A} = m \log W + c$$

where $A$ = spot area; $W$ = weight of extract applied, and $m$ and $c$ are constants for the individual compound.

From the results obtained a linear relationship was found for the square root of the spot area and the $\log_{10}$ of the concentration of natural rubber over the ranges 0–100 % w/w natural rubber, as shown in Fig. 2. The limit of detection is about 5 % natural rubber in a natural rubber/synthetic polyisoprene blend. If the $\beta$-sitosterol component cannot be detected and the presence of *cis*-polyisoprene has been shown by other methods, *e.g.*, the Weber test or I.R. spectroscopy, it can be concluded that the natural

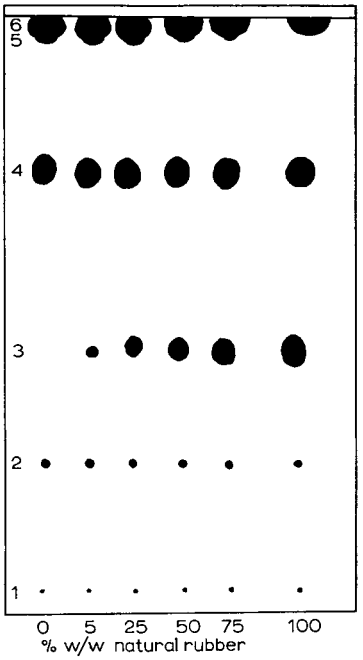


Fig. 1. A chromatogram of solvent extracts of natural rubber/synthetic polyisoprene blends in a vulcanized stock. (1) = Origin; (2) = unknown; (3) = β-sitosterol; (4) = antioxidant of *p*-phenylenediamine type; (5) = phenolic antioxidant in synthetic polyisoprene; and (6) unresolved components.

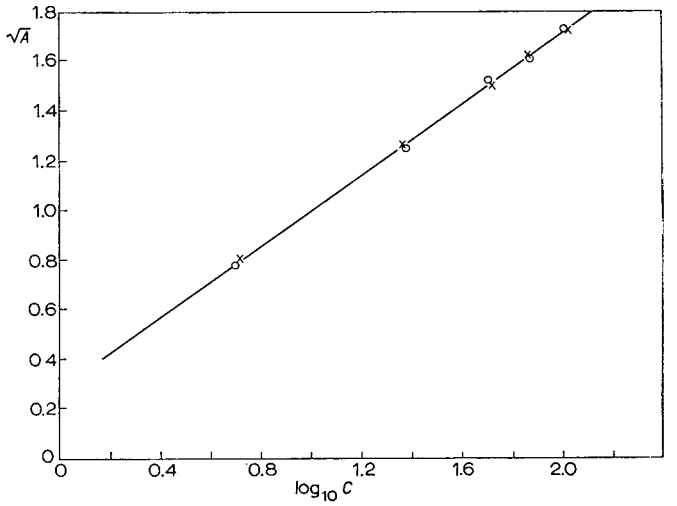


Fig. 2. The relationship between the square root of the spot area ($A$) of β-sitosterol and the logarithm (base 10) of the concentration ($C$) expressed as % w/w natural rubber, in a natural rubber/synthetic polyisoprene blend. XO = Duplicate analysis.

rubber content is less than 5 % w/w and that the polymer is substantially synthetic polyisoprene. This will be confirmed by detection of purple spots due to the presence of phenolic antioxidants added to the raw polymer.

*Applications*

The main applications will be in laboratories dealing with polymer identification and will enable a differentiation of natural rubber (NR)/synthetic polyisoprene to be made. This can be made semi-quantitative if accurate blends of NR/polyisoprene can be prepared.

The technique will also be useful in examination of small rubber samples (50–100 mg), where solvent extraction followed by dissolving of the residue in 1 drop ($\approx$ 0.05 ml) of carbon tetrachloride will enable thin-layer chromatography to be performed on a qualitative basis.

The equipment required is relatively cheap, the technique is simple to perform and the time required for the analysis (excluding the extraction procedure which can be performed overnight) is 40–45 min.

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## Separation of polymyxin B, D, E and M by thin-layer chromatography

The antibiotics of the polymyxin group are peptides with very similar structures[1,2], consequently their separation and identification presents quite a difficult analytical problem. Though investigations by paper chromatography of the amino acid components of their hydrolysates[1,2] affords a sure means of identification, this is a lengthy process and successful only with isolated products. The paper chromatography method with isopropylamine proposed by MISTRETTA[3] deserves special mention since it gives a good separation of intact polymyxin B and D, but the long time needed for development prevents quick evaluation, NASH AND SMASHEY[4] suggest the use of impregnated paper for this purpose.

Our object was to find a suitable method for the rapid and reliable separation and identification of polymyxin antibiotics when present as a preparation or in a nutrient medium.

*Experimental*

Experiments were carried out for the direct separation, and identification by TLC without hydrolysis of four types of antibiotic substances of the polymyxin group. For this a 15:5:1:2 mixture of acetone–water–acetic acid–2 *N* $NH_4OH$ proved to be the most suitable, a 100 $\mu$ layer of Merck's Kieselgel G was used as the adsorbent. For the location of the spots, a ninhydrin spray and biological reactions were used. The standard preparations available were the following: polymyxin B sulphate (Pfizer); polymyxin D sulphate, isolated here; polymyxin E sulphate (Colistin, Laboratoire Roger Bellon); and polymyxin M sulphate from the U.S.S.R. These were also identified by their amino acid components, through paper chromatography according to the literature[1]. Table I lists the amino acid components of the four polymyxins investigated.

TABLE I

| *Polymyxin* | *α,γ-Diamino-butyric acid* | *Phenyl alanine* | *Leucine* | *Serine* | *Threonine* |
|---|---|---|---|---|---|
| B | + | + | + | − | + |
| C | + | − | + | + | + |
| E | + | + | + | − | + |
| M | + | − | + | − | + |

*Investigation of the purified solid substances.* In the first part of our work the $R_F$ values of the four standard substances were determined separately, and then in a mixture. Glass plates 20 × 5 or 20 × 20 cm carried the Kieselgel G layer, and 20 to 50 $\mu$g samples of the polymyxin sulphates dissolved in water were spotted on them. Development with a 15:5:1:2 mixture of acetone–water–acetic acid–2 *N* $NH_4OH$ took 90 min.

Spots were developed on the plates dried at 80°, with a 0.25 % solution of ninhydrin in *n*-butanol. $R_F$ values, as shown in Fig. 1, were as follows: polymyxin B = 0.45; D = 0.51; E = 0.95; and M = 0.36.

Fig. 2 shows chromatograms, prepared in the same way, of the polymyxins used as standards, the spots being made visible on plates inoculated with a *Bordetella bronchiseptica* strain. Here the samples were 5 to 10 $\mu$g of polymyxin sulphates.

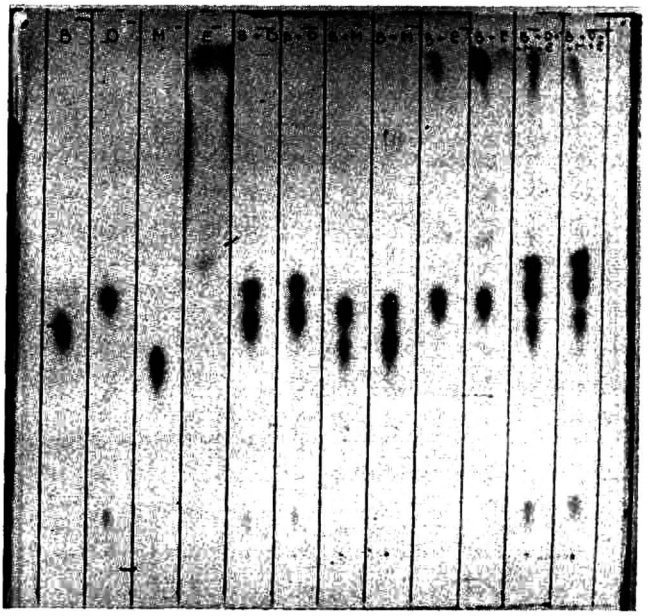

Fig. 1. Separation of polymyxin B, D, E, and M. Spots made visible through application of a 0.25% solution of ninhydrin in *n*-butanol saturated with water.

The figures show that pure polymyxin E dissolved in distilled water has two biologically active spots at lower $R_F$ values than the principal component ($R_F$ 0.95). One of these scarcely separates from the principal spot. No such phenomenon was observed with the other standard substances.

*Identification of the active substance of a fermentation liquor containing polymyxin B.* Since ninhydrin is positive to other substances present in fermentation broths the detection of the polymyxins was carried out by biological testing. Fermentation broths were acidified to pH 3 with solid oxalic acid and centrifuged for about 10 min. The supernatant liquid was neutralized with 2 *N* sodium hydroxide and its polymyxin B content was approximately determined biologically. A sample of this liquid,

Fig. 2. Separation of polymyxin B, D, E, and M. Spots made visible on an agar plate inoculated with *Bordetella bronchiseptica*.

calculated as containing about 5 to 10 $\mu$g of polymyxin B was then spotted on to a plate at the starting point, together with similar samples of the standards. In addition to the fermentation liquor, mixtures of the fermentation liquor with several standard substances were also applied at the start line. Development was carried out as described in the preceding part. To ensure that no trace of solvent remained, the plates were dried at 80° for 1 h, then held in ammonia vapour for 5 min to neutralize any acetic acid possibly still present, since this would interfere with the biological testing on the agar plates inoculated with *Bordetella bronchiseptica*. A sheet of filter paper was

Fig. 3. Chromatography of fermentation broth containing polymyxin B, and of fermentation broths admixed with standard polymyxin substances. Spots made visible on an agar plate inoculated with *Bordetella bronchiseptica*.

laid on the agar plate and the chromatoplate was put on top. The latter and the filter paper were removed after 1 h and the agar plate was then incubated at 37° for 16 to 18 h. Fig. 3 shows that the active substance of the fermentation broths is distinct from all the standards, with the exception of polymyxin B. Thus the active substance produced is identical with it.

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## Fingerprints of DNS-labeled protein digests on a millimicromole scale

The two-dimensional separation of protein hydrolysates on sheets of filter paper by combined electrophoresis and chromatography as developed by INGRAM[1] has become a standard technique for the study of similarities and differences among proteins. This so-called fingerprinting method has more recently been adapted to thin-

layer electrophoresis and chromatography by WIELAND *et al.*[2] In both cases the location of the fragments is normally detected with ninhydrin. The sensitivity of this reagent, which can vary considerably for different peptides, limits the minimum amount of protein digest required for a fingerprint to about 0.1 micromoles for separations on paper and to about 0.01 to 0.02 micromoles for the thin-layer method.

In this communication a new type of fingerprinting technique is described which requires as little as 1 to 2 millimicromoles of the enzymic hydrolysate of a protein. This has been achieved by labeling the peptides with the highly fluorescent DNS-residue according to GRAY AND HARTLEY[3]. This marker not only allows the detection of millimicromole quantities of peptides but also renders them more hydrophobic thereby facilitating their separation by two-dimensional chromatography on Kieselgel plates with organic solvent systems.

DNS-Cl* and chymotrypsin-free trypsin were purchased from Calbiochem, horse heart cytochrome c from Boehringer (Mannheim). Cytochrome c isolated from tuna fish hearts was a generous gift from Dr. D. M. BLOW (Cambridge, England). Both cytochromes were hydrolyzed with 5 % trypsin (w/w) at pH 8.5 and 37° for 2 h. The isolation of human $\gamma$-globulin and of a Bence-Jones protein, the reduction and alkylation of these proteins and the preparation of the L-chain of $\gamma$-globulin followed published procedures[4–6]. The reduced and alkylated globulins were dialyzed extensively against 0.01 *M* phosphate buffer (pH 8.2) and then digested with trypsin for 20 h at pH 8.2 and 37°.

The protein hydrolysates were incubated with a twenty-fold excess of DNS-Cl (w/w) at pH 8.0 overnight at room temperature. The reaction mixture, which contained appreciable amounts of DNS-OH, was applied to a Dowex 50-X8 column (0.2 × 2 cm) previously equilibrated with 0.01 *N* acetic acid (pH 3.5) (see ref. 3). The large excess of DNS-OH was eluted with the same medium. The labeled peptides could be displaced from the column with 1 *M* ammonia in 25 % acetone and were taken to dryness. Aliquots of this eluate were used for the separation on thin layer plates. The traces of DNS-OH and the DNS-$NH_2$ as well as some unknown side products still present in the peptide fraction served as convenient markers in the comparison of different fingerprints.

Thin layer plates were prepared with Kieselgel G (Merck) and dried for 1 h at 120°. The composition of the solvent mixtures used for the two-dimensional separation is given in the legends to the figures. Between the two runs, the plate was dried for 10 to 15 min at 100°. The labeled peptides could be detected on the wet plate by their intense yellow to orange fluorescence under a U.V. lamp and were marked on the rear side of the plate. DNS-OH and some unknown side products are easily discernible by their bluish fluorescence. Preliminary experiments have shown that the marked areas can be scratched off the plate and the peptides recovered by elution with 25 % dioxane or acetone.

Fig. 1a and b show a comparison of the DNS-labeled tryptic digests of horse and tuna fish cytochrome c. These fingerprints were obtained with 20 $\mu$g of hydrolysate, which is equivalent to about 1.5 millimicromoles of protein. From the known sequences of these proteins[7,8] and from earlier studies with the fingerprinting techni-

* Abbreviations: DNS-Cl = 1-Dimethylamino-naphthalene-5-sulphonyl chloride; DNS-OH = 1-dimethylamino-naphthalene-5-sulphonic acid; DNS-$NH_2$ = 1-dimethylamino-naphthalene-5-sulphonyl amide.

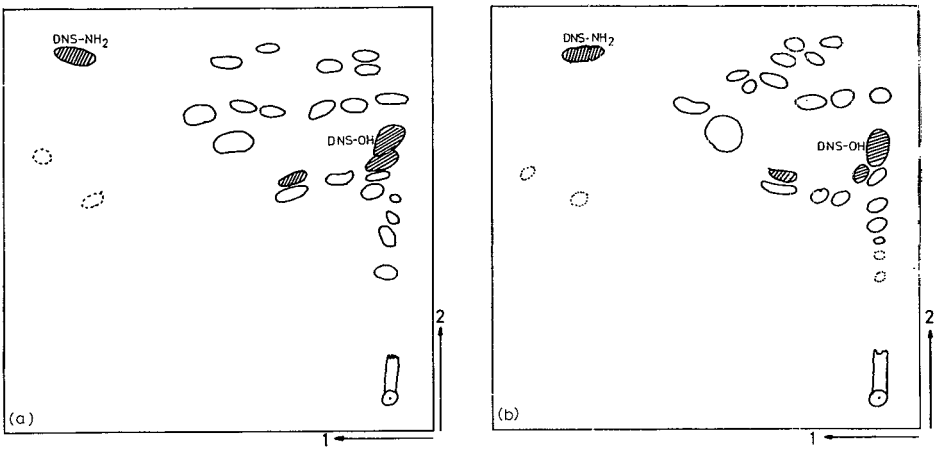


Fig. 1. Two-dimensional chromatography of DNS-labeled tryptic digests of (a) horse heart and (b) tuna fish heart cytochrome c. Solvent systems: (1) benzene–pyridine–acetic acid (80:20:5, v/v/v); (2) methyl acetate–isopropanol–conc. ammonia (45:35:20). The shaded spots represent DNS-OH, DNS-$NH_2$ and unknown side products.

que on paper[9], the number of major fragments to be expected is about 20 for horse and 18 for tuna fish cytochrome c, respectively. Due to incomplete hydrolysis of the clusters of basic amino acids occurring in these proteins, this number can vary with the time of the digestion. Minor components are therefore always observed. Two-dimensional chromatography on thin layer plates revealed 21 and 20 major and a few minor spots for horse and tuna fish cytochrome c, respectively. This is in good agreement with the expected numbers. The hemopeptides, barely visible as pink spots at these low concentrations, showed some tailing at the origin and could not be differentiated. Among the tryptic peptides six should be identical in both cytochromes[8]. On

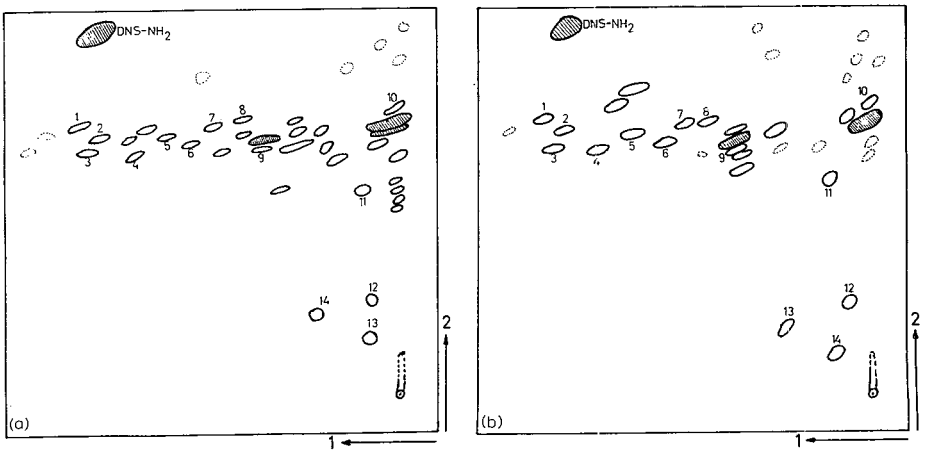


Fig. 2. Two-dimensional chromatography of DNS-labeled tryptic digests of (a) the L-chain of human $\gamma$-globulin, (b) a Bence-Jones protein. Same solvent systems as in Fig. 1. The spots occupying identical positions in both fingerprints were numbered 1–14.

the thin layer plates, according to our judgment, seven to nine spots were identical. Obviously, some of the differences are too small to be detectable by this method. It is, however, a general drawback of all fingerprinting techniques that only relatively drastic changes can be detected unambiguously and that the number of differences observed is generally smaller than the actual number.

A comparison of the tryptic digests of 20 $\mu$g (about one millimicromole) each of the L-chain of human $\gamma$-globulin and of a Bence-Jones protein is shown in Fig. 2a and b. Those peptides which upon close examination of a number of runs seemed identical were numbered 1–14. Some of the differences have to be attributed to the fact that the L-chain of normal $\gamma$-globulin is a mixture of serological types I and II. The Bence-Jones protein used in this study had N-terminal aspartic acid and is therefore presumably type I (ref. 10). After tryptic hydrolysis 21 major fragments could be detected on the thin layer plates. This is in good agreement with the investigations of HILSCHMANN AND CRAIG[11], who found 20 and 21 tryptic peptides for two other Bence-Jones proteins of type I.

In different experiments with a labeled hydrolysate the same overall picture was obtained. For a detailed comparison, however, only runs where both plates were prepared and dried simultaneously and all steps were done in parallel have been used.

This new fingerprinting technique requiring only millimicromole quantities of the enzymic hydrolysate of a protein is at least ten times more sensitive than previous methods. It should therefore facilitate the investigation of structural similarities and differences among proteins which bear an evolutionary, functional, serological or other relationship. It is the method of choice if only small amounts of a protein are available. Using this technique[12] it has already been possible to obtain fingerprints of blood clotting factors VII and X, where the preparation of larger amounts as required for other types of peptide separation would be extremely laborious.

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## Chromatographic separation of the 1-alkoxy-2(3)-hydroxy-3(2)-aminocyclohexanes and their derivatives*

In connection with the continuation of our studies on the mode of cleavage of the stereoisomeric 1-alkoxy-2,3-epoxycyclohexanes[1] it became necessary to develop methods for the separation and quantitative estimation of the proportions of the positionally isomeric amines (or suitable derivatives) which could result from the action of ammonia on each oxide. To this end, the paper, thin-layer and vapor phase chromatographic separation of the stereoisomeric 1-alkoxy-2-hydroxy-3-aminocyclohexanes[2], their N-acetyl[2], O,N-diacetyl[2,3] and O,N,N-triacetyl derivatives[3] were examined and the most suitable method was chosen for application to the separation of the position isomers referred to earlier. Results are summarized in Table I.

*Experimental*

(*a*) *Paper chromatography*

The compounds were applied as 200 $\gamma$ spots (10 $\lambda$ of a 2 % solution in absolute methanol) to 9 × 22 in. sheets of Whatman No. 1 paper and developed for approximately 17 h by descending flow in *n*-butanol–acetic acid–water (4:1:5). The chromatograms were dried for 1.75 h at 30° in a forced air oven. Detection of compounds having unsubstituted amino groups was effected by spraying the chromatograms with a 0.04 % solution of bromcresol green (sodium salt) in ethanol. All other compounds were located by dipping the chromatograms in a 0.1 % solution of iodine in petroleum ether.

(*b*) *Thin-layer chromatography*

Each compound, 5 $\gamma$ (1 $\lambda$ of a 0.5 % solution in absolute methanol) was applied to 2 × 8 in. strips of Gelman chromatography media Type A (alumina gel on micro glass fibers). The chromatograms were developed (25 min) in hexane–ethyl acetate–methanol (10:1:0.5), unless otherwise specified in Table I, following which they were dried for 2–3 min at 100°. Spraying with a 1 % solution of sodium dichromate in 50 % sulphuric acid and subsequent charring of the chromatograms by heating them on a hot plate produced excellent detection of all the compounds, first as white spots on a yellow-orange background and, on continued heating, as brown spots on a white background.

(*c*) *Vapor phase chromatography*

The instrumentation was the same as mentioned earlier[4] for the vapor phase chromatographic separation of the alkoxybromocyclohexanols but the column packings, temperatures and helium flow rates were different. For column No. 1 Chromosorb P (225 ml, 45–60 mesh) was washed first with concentrated hydrochloric acid (250 ml) for 1 h with occasional stirring, with water (4 × 250 ml), with 10 % sodium hydroxide (250 ml) for 6 h, with water (4 × 250 ml), and finally dried at 130° for 6 h. This material was impregnated with 5 % methanolic potassium hydroxide, dried *in vacuo* at room temperature and again impregnated with 25 % polyethylene glycol 20,000 in chloroform. Column No. 2 was packed with Fluoropak 80 which had been impregnated with 10 % SE 30. The compounds were injected as 1–2 mg samples in 50 $\mu$l of absolute methanol.

* Issued as DCBRL Report No. 519.

*Discussion*

It is a recorded fact[5] that amines tend to tail badly during vapor phase chromatography on solid supports containing polar sites, presumably due to hydrogen bonding. Unless the Chromosorb P used as the support in some of these experiments was pretreated in a manner similar to that reported by other workers[6,7] it was impossible to elute the amines listed in Table I. Consideration has been given to both the solid support and the stationary phase as the cause of amine tailing[8]. However, it was found that if a common stationary phase (polyethylene glycol 20,000) was used on two different supports, Chromosorb P and Fluoropak 80, complete retention occurred with the former, while elution occurred with the latter. This result indicates that the solid support and not the stationary phase is responsible for the tailing of the amines in the experiments described herein. Column No. 2 was used chiefly for the acetyl derivatives of the amines since even after pretreatment of the solid support Column No. 1 retained virtually all the acetyl compounds.

To determine the relative merits of the three chromatographic methods from the data in Table I, the compounds are considered in groups which consist of stereoisomers and/or position isomers. Paper chromatography with the solvent system used was very effective for the separation of the methoxyaminocyclohexanols (compounds 1, 2 and 3), the ethoxyaminocyclohexanols (11 and 12), the aminocyclohexanediols (19, 20, 21) and somewhat less effective for the aminocyclohexanols (25, 26). However, the acetyl derivatives of all of these compounds moved too rapidly and produced diffuse spots. The latter circumstance made it impossible to distinguish between mixtures of mono- and di-N-acetylamino derivatives (*e.g.* 6 and 9) with this solvent system, but did provide an excellent means of detecting the presence of unreacted amines in their acetylated derivatives.

Thin-layer chromatography was extremely successful for the separation of the acetylated compounds and was especially useful for revealing the contamination of the di-N-acetyl derivatives with their mono-N-acetyl analogues (*e.g.* 6 and 9). By this means it was established that the mono-N-acetyl compounds were present in large amounts in all of the di-N-acetyl derivatives which had been prepared three years previously[3] and which were judged at that time to be free of their mono-N-acetyl analogues on the basis of elemental and acetyl group analysis, and the infrared and n.m.r. spectra of the compounds. It was thus clearly demonstrated that the di-N-acetylamines undergo a slow and spontaneous degradation to their less highly acetylated analogues. The fact that di-N-acetylisopropylamine had undergone complete degradation to mono-N-acetylisopropylamine during this period of storage suggests that some of the lower molecular weight di-N-acetylamines may prove useful as mild reagents for transacetylation. The chromatographic procedures reported herein provide simple analytical methods for studying the kinetics of the decomposition of di-N-acetylamines. Thin-layer chromatography was more effective than either of the other methods examined for the separation of mixtures containing acetylaminocyclohexanols and the corresponding O-acetyl derivatives (*e.g.* 4 and 6). This procedure also provided a better separation for the 1-methoxy-2(3)-acetoxy-3(2)-bromocyclohexanes than the method reported earlier[4]. Although some of the $R_F$ values quoted for the thin layer chromatographic experiments are close, satisfactory separations of the compounds were obtained because of the very compact nature of the spots. It was found that the Gelman support for thin-layer chromatography is very

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TABLE I

| Compound | Compound No. | Vapor phase chromatography, retention time (min) | | $R_F$ | |
|---|---|---|---|---|---|
| | | Column 1[a] | Column 2[b] | Thin-layer chromatography | Paper chromatography |
| 1α-Methoxy-2β-hydroxy-3α-aminocyclohexane | 1 | 55.5 | 2.5 | | 0.49 |
| 1α-Methoxy-2α-hydroxy-3β-aminocyclohexane | 2 | 48.5 | 2.5 | | 0.56 |
| 1α-Methoxy-2α-amino-3β-hydroxycyclohexane | 3 | 68.0 | 3.5 | | 0.61 |
| 1α-Methoxy-2β-hydroxy-3α-acetylaminocyclohexane | 4 | | 15 | 0.06 | 0.78 |
| 1α-Methoxy-2α-hydroxy-3β-acetylaminocyclohexane | 5 | | 14 | 0.12 | 0.82 |
| 1α-Methoxy-2β-acetoxy-3α-acetylaminocyclohexane | 6 | | 16 | 0.29 | 0.85 |
| 1α-Methoxy-2α-acetoxy-3β-acetylaminocyclohexane | 7 | | 18 | 0.23 | 0.87 |
| 1α-Methoxy-2α-acetylamino-3β-acetoxycyclohexane | 8 | | 18 | 0.70 | 0.91 |
| 1α-Methoxy-2β-acetoxy-3α-diacetylaminocyclohexane | 9 | | 31.5 | 0.61 | 0.90 |
| 1α-Methoxy-2α-acetoxy-3β-diacetylaminocyclohexane | 10 | | 21.5 | 0.90 | 0.91 |
| 1α-Ethoxy-2β-hydroxy-3α-aminocyclohexane | 11 | 54.5 | 33.5[c] | | 0.58 |
| 1α-Ethoxy-2α-hydroxy-3β-aminocyclohexane | 12 | 44 | 30.5[c] | | 0.63 |
| 1α-Ethoxy-2β-hydroxy-3α-acetylaminocyclohexane | 13 | | 18 | 0.32 | 0.83 |
| 1α-Ethoxy-2α-hydroxy-3β-acetylaminocyclohexane | 14 | | 16.5 | 0.03 | 0.86 |
| 1α-Ethoxy-2β-acetoxy-3α-acetylaminocyclohexane | 15 | | 18 | 0.59 | 0.88 |
| 1α-Ethoxy-2α-acetoxy-3β-acetylaminocyclohexane | 16 | | 18.5 | 0.49 | 0.89 |
| 1α-Ethoxy-2β-acetoxy-3α-diacetylaminocyclohexane | 17 | | 34.5 | 0.74 | 0.90 |
| 1α-Ethoxy-2α-acetoxy-3β-diacetylaminocyclohexane | 18 | | 24 | 0.93 | 0.91 |
| 3α-Amino-1α,2β-cyclohexanediol | 19 | | 3.5 | | 0.36 |
| 3β-Amino-1α,2α-cyclohexanediol | 20 | | 2.5 | | 0.40 |
| 2α-Amino-1α,3β-cyclohexanediol | 21 | | 4.5 | | 0.45 |
| 3α-Acetylamino-1α,2β-diacetoxycyclohexane | 22 | | 26.5 | 0.60[d] | 0.87 |
| 3β-Acetylamino-1α,2α-diacetoxycyclohexane | 23 | | 25.0 | 0.67[d] | 0.88 |
| 2α-Acetylamino-1α,3β-diacetoxycyclohexane | 24 | | 30 | 0.48 | 0.89 |
| *trans*-2-Aminocyclohexanol | 25 | 30 | 10[c] | | 0.58 |
| *cis*-2-Aminocyclohexanol | 26 | 27.5 | 10.5[c] | | 0.61 |
| *trans*-2-Acetylaminocyclohexanol | 27 | | 8.0 | 0.19 | 0.83 |
| *cis*-2-Acetylaminocyclohexanol | 28 | | 7.5 | 0.26 | 0.85 |
| 1α-Acetoxy-2β-acetylaminocyclohexane | 29 | | 9.0 | 0.53 | 0.89 |
| 1α-Acetoxy-2α-acetylaminocyclohexane | 30 | | 11.5 | 0.37 | 0.89 |
| 1α-Acetoxy-2β-diacetylaminocyclohexane | 31 | | 17.0 | 0.44 | 0.93 |
| *trans*-2-N-Ethylacetylaminocyclohexanol | 32 | 14.5 | | 0.47 | 0.90 |
| 1α-N-Ethylacetylamino-2β-acetoxycyclohexane | 33 | 21.5 | 70 | 0.73 | 0.92 |
| Cyclohexylamine | 34 | 3.5 | 0.5 | | 0.72 |
| Acetylaminocyclohexane | 35 | | 3.5 | 0.68 | 0.91 |
| Diacetylaminocyclohexane | 36 | | 6.5 | 0.94 | 0.92 |
| Ethylacetamide | 37 | 17 | 3.5[c] | 0.45 | 0.85 |
| Ethyldiacetamide | 38 | 17 | 8.0[c] | 0.89 | 0.89 |
| Isopropylacetamide | 39 | 14.5 | 4.0 | 0.52 | 0.87 |
| Benzyldiacetamide | 40 | | 2.5, 12.0 | 0.54, 0.88 | 0.91 |

[a] Conditions: column 165°; collector 158°; detector 185°; injector 180°; He flow 300 ml/min. [b] Conditions: column 170°; collector 160°; detector 185°; injector 180°; He flow 300 ml/min. [c] Column temperature reduced to 110°, He flow to 200 ml/min. [d] Solvent: toluene–ethyl acetate–acetic acid (100:5:1, v/v).

satisfactory since it can be treated with sulphuric acid and exposed to high charring temperatures without the danger of breakage experienced by the use of glass plates.

Vapor phase chromatography on column 1 provided an excellent method for separation of the alkoxyaminocyclohexanols and aminocyclohexanols. Most noteworthy in this connection is the capability of the procedure to separate the three methoxyaminocyclohexanols on a preparative scale, thereby providing a suitable means for the isolation of the difficultly accessible 1 α-methoxy-2 α-amino-3 β-hydroxycyclohexane[1] in a pure condition. This column was not satisfactory for separation of the acetylated derivatives but the latter compounds were resolved when column 2 was used. The relatively short retention times achieved by the use of this column made for rapid analysis and the method was convenient for checking the products from the degradation of the di-N-acetylamino compounds. This column was not satisfactory, however, for the separation of the alkoxyacetylaminocyclohexanols from their O-acetyl derivatives (*e.g.* 13 and 15) but these compounds are readily separable by the thin-layer chromatographic method mentioned earlier.

It is evident from Table I that by selecting one of the three chromatographic methods examined, any individual member of any of these groups of isomers can be separated. These results underline the importance of concurrent examination of several chromatographic techniques when difficulty is encountered in the separation of isomers by more conventional means.

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## Stabilization of xanthophyll and carotene by ethoxyquin during thin-layer chromatography

The use of thin-layer adsorption chromatography (TLC) for the quantitative and qualitative determination of xanthophyll and carotene is limited due to the rapid oxidation and isomerization of these compounds during TLC. Employment of impregnated and reverse-phase TLC[1] as well as saturated solvent chambers[2] has eliminated some of these losses, however, these procedures are more complex and require more time and equipment than the more common technique of adsorption chromatography. Recently, a procedure was described for retarding autoxidation of lipids

during TLC by the addition of antioxidants to the developing solvent[3]. Previously, ethoxyquin (6-ethoxy-2,2,4-trimethyl-1,2-dihydroquinoline) has been shown to be an effective antioxidant for carotene[4] and xanthophyll[5] in dehydrated alfalfa meal. This present paper describes the stabilization of these carotenoids during adsorption TLC by the addition of ethoxyquin.

*Materials and methods*

A slurry of Silica Gel G*–calcium hydroxide (1:6 by wt ) in 50 ml of water, was applied with a mobile applicator as a 250 $\mu$ layer to glass plates, 20 cm × 20 cm, and activated at 105° for 30 min. After preparation the plates were left in the laboratory atmosphere with a relative humidity of approximately 50 % until used.

Extracts of dehydrated alfalfa meal were prepared according to the procedure of KOHLER *et al*[6] and concentrated under reduced pressure to a final concentration equivalent to 0.5 g meal/ml of extract. Aliquots of the extracts were applied to the chromatoplates while under dim light, and the chromatograms developed in the dark at room temperature with benzene–1-butanol (100:2, v/v). Five levels of redistilled ethoxyquin were added to the developing solvent (Table I).

TABLE I

% OF CAROTENOIDS RECOVERED FROM THIN-LAYER CHROMATOGRAMS

| *Carotenoid* | *Spectral adsorption maxima*, mμ* | $R_F$ | *mg of ethoxyquin per 200 ml developing solvent* | | | | |
|---|---|---|---|---|---|---|---|
| | | | *0* | *150* | *450* | *900* | *1500* |
| Neoxanthin | 466, 438, 418 | 0.07 | 35 | 97 | 99 | 99 | 97 |
| Xanthophyll No. 1 | 470, 443, 426 | 0.23 | < 1 | 79 | 91 | 100 | 100 |
| Violaxanthin | 469, 441, 417 | 0.31 | < 1 | 73 | 81 | 80 | 82 |
| Xanthophyll No. 2 | 471, 445, 424 | 0.48 | < 1 | 58 | 87 | 97 | 95 |
| Lutein | 474, 446, 421 | 0.60 | 59 | 96 | 100 | 100 | 100 |
| β-Carotene | 477, 449, 421 | 0.87 | 84 | 88 | 94 | 97 | 100 |

* In hexane–acetone (7:3).

Six carotenoid spots were separated as shown in Fig. 1. Following drying of the developed plate, initial carotenoid content was determined by immediately scraping the spots from the chromatoplate and eluting the carotenoids with hexane–acetone (7:3). Related developed and dried plates were stored in the dark with exposure to the air for 2 h, prior to the scraping and elution of the carotenoid spots. The absorbance of the filtered eluates was measured in a spectrophotometer at 475 m$\mu$ for the xanthophyll (neoxanthin at 445 m$\mu$) and 436 m$\mu$ for the β-carotene, employing the absorption coefficients of 236 and 196, respectively.

*Results and discussion*

The use of ethoxyquin as an antioxidant during thin layer chromatography has been useful in the quantitative determination of carotenoids. At the higher levels of ethoxyquin almost complete stability was obtained. Lutein was the most stable of the hydroxylated carotenoids, however, carotene was more stable without antioxidant treatment than any of the xanthophylls. The least stable of the xanthophylls was violaxanthin. This is apparently due to violaxanthin being a dihydroxy diepoxide

* Reference to a company or product name does not imply approval or recommendation of the product by the U.S. Department of Agriculture to the exclusion of others that may be suitable.

and hence more labile. Neoxanthin, a trihydroxy, monoepoxide[7], suffered only a 3% loss at the high level of ethoxyquin, However, due to isomerization the main spectral peak was shifted from 465 mμ to 440 mμ. The absorbance measured at 445 mμ did not show the effect of this shift.


Fig. 1. Separation of dehydrated alfalfa xanthophylls and carotene by TLC. 1 = β-Carotene; 2 = lutein; 3 = xanthophyll No. 2; 4 = violaxanthin; 5 = xanthophyll No. 1; 6 = neoxanthin.

In the solvent system employed, only a very slight separation of zeaxanthin from lutein, and cryptoxanthin from β-carotene was achieved. Since zeaxanthin and cryptoxanthin account for less than 10% of the total xanthophylls in dehydrated alfalfa[8], they were not isolated in the present study, but were included in the lutein and β-carotene fractions, respectively.

In addition to acting as an antioxidant the added ethoxyquin may also act as an antacid to counteract any acidity of the Silica Gel G particles. This, of course, would prove very advantageous during chromatography of the acid labile epoxide containing xanthophylls.

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## Dünnschicht-Chromatographie von Nitroguanylhydrazonen

Die Abscheidung und Charakterisierung von Carbonylverbindungen erfolgt im allgemeinen in Form schwerlöslicher 2,4-Dinitrophenylhydrazone. Als Reagens wird eine Lösung von 2,4-Dinitrophenylhydrazin in Salzsäure, Schwefelsäure, Phosphorsäure oder Perchlorsäure benutzt. Es gibt jedoch zahlreiche carbonylhaltige Substanzen, die von den stark sauren Lösungsmitteln angegriffen werden. Um diese Schwierigkeit zu umgehen, wurde Diäthylenglykoldimethyläther in Gegenwart katalytischer Mengen Säure als Reaktionsmedium empfohlen[1]. Ein ebenfalls schonendes Verfahren zur Isolierung von Carbonylverbindungen besteht in der Umsetzung mit N,N'-Nitroaminoguanidin in schwach essigsaurer Lösung[2–4]. Dieses Reagens wurde von uns mit Erfolg zur Analytik von Fulvosäurefraktionen benutzt[5]. Für Strukturuntersuchungen komplexer Naturstoffgemische ist vor allem eine vollständige Trennung der gebildeten Nitroguanylhydrazone entscheidend. Es war daher naheliegend, verschiedene Lösungsmittelsysteme und Adsorbentien auf ihre Verwendungsmöglichkeit für die Dünnschicht-Chromatographie zu überprüfen (Tabelle I).

Die Adsorptionsaffinität aromatischer Nitroguanylhydrazone wird unabhängig

TABELLE I

$R_F$-WERTE DER NITROGUANYLHYDRAZONE

| | $R_F$-Werte × 100 | | | | | | |
|---|---|---|---|---|---|---|---|
| | Adsorbens*/Laufmittel** | | | | | | |
| | M 1 | A 2 | M 3 | M 4 | A 5 | A 6 | A 7 |
| Benzaldehyd | 27 | 56 | 44 | 64 | 46 | 61 | 57 |
| Salicylaldehyd | 15 | 25 | 13 | 43 | 13 | 24 | 32 |
| *p*-Hydroxybenzaldehyd | 17 | 19 | 12 | 40 | 18 | 27 | 25 |
| Protocatechualdehyd | 0 | 7 | 0 | 5 | 0 | 0 | 0 |
| Anisaldehyd | 35 | 68 | 26 | 60 | 62 | 65 | 61 |
| Veratrumaldehyd | 30 | 64 | 21 | 56 | 65 | 65 | 59 |
| Vanillin | 10 | 35 | 9 | 31 | 18 | 27 | 18 |
| *o*-Nitrobenzaldehyd | 39 | 59 | 33 | 67 | 44 | 48 | 46 |
| *m*-Nitrobenzaldehyd | 22 | 59 | 6 | 58 | 38 | 57 | 59 |
| *p*-Nitrobenzaldehyd | 23 | 66 | 10 | 63 | 59 | 69 | 65 |
| Zimtaldehyd | 35 | 65 | 57 | 69 | 54 | 70 | 63 |
| Acetophenon | 45 | 84 | 60 | 73 | 60 | 71 | 56 |
| 3,4-Dihydroxyacetophenon | 4 | 12 | 0 | 8 | 0 | 0 | 0 |
| *p*-Methoxyacetophenon | 37 | 76 | 37 | 67 | 68 | 67 | 67 |
| 2-Hydroxy-4-methoxyacetophenon | 19 | 62 | 16 | 52 | 77 | 78 | 68 |
| 4-Hydroxy-2-methoxyacetophenon | 44 | 52 | 69 | 83 | 61 | 67 | 53 |
| Acetovanillon | 15 | 43 | 14 | 38 | 29 | 47 | 28 |
| *p*-Methylacetophenon | 42 | 80 | 61 | 73 | 37 | 71 | 50 |
| 3,4-Dihydroxypropiophenon | 4 | 15 | 0 | 8 | 0 | 0 | 0 |
| Benzophenon | 50 | 87 | 63 | 70 | 36 | 54 | 48 |
| Cyclohexanon | 57 | 71 | 67 | 76 | 50 | 40 | 30 |

* Adsorbens: A = Aluminiumoxid D; M = Magnesiumsilikat.

** Laufmittel: 1 = Essigester–Aceton (5:1); 2 = Benzol–Dioxan–Eisessig (90:25:4); 3 = Chloroform–Isoamylalkohol–Eisessig (70:30:3); 4 = Aceton–*n*-Propanol (1:1); 5 = Chloroform–Diäthylamin (9:1); 6 = Chloroform–Aceton–Diäthylamin (5:4:1); 7 = Essigester–Diäthylamin (9:1).

vom Adsorbens und Laufmittel durch die zunehmende Zahl an Hydroxylgruppen erheblich vergrössert. Methoxylgruppen können die $R_F$-Werte sowohl verringern als auch erhöhen, führen jedoch bei hydroxylhaltigen Verbindungen in *o*- und *p*-Stellung zur Ketonhydrazongruppierung stets zu einer Abnahme der Adsorption. C-Methylgruppen sind ohne wesentlichen Einfluss auf das chromatographische Verhalten. Nitroguanylhydrazone von Aldehyden zeigen gegenüber denen von Ketonen eine grössere Adsorptionsaffinität. Der Austausch der am Benzolkern substituierten Aldehydfunktion gegen die O=CH—CH=CH— Gruppierung führt zu grösseren $R_F$-Werten.

*Methodik*

Für die Herstellung der Dünnschichtplatten (20 × 20 cm) werden Aluminiumoxid D (Chemiewerk Greiz-Döhlau) bzw. Magnesiumsilikat "Woelm" benutzt. Bei Verwendung von Kieselgel als Adsorbens sind die Trenneffekte gering. Die Platten beschichtet man manuell mit 12 g Aluminiumoxid in 14 ml Wasser oder 5 g Magnesiumsilikat in 15 ml Wasser nach der von LEES UND DE MURIA[6] angegebenen Methode und aktiviert anschliessend 30 Min. bei 120°. Die Nitroguanylhydrazone werden in 0.1-proz. dimethylformamidhaltiger Lösung aufgetragen und bei Kammersättigung bis zu einer Laufhöhe von 13 cm chromatographiert. Zur Detektion verwendet man Rhodamin B[7] im U.V.-Licht. Falls die ursprünglichen Carbonylverbindungen säurestabil sind, können die Platten mit 20-proz. HCl intensiv besprüht und anschliessend 20 Min. auf 120° erhitzt werden. Dabei erfolgt hydrolytische Spaltung unter Rückbildung der Ausgangsverbindungen, die sich mit 2,4-Dinitrophenylhydrazin anfärben lassen.

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## The detection of thiram by thin layer chromatography

During work involving the wheat coleoptile straight growth bioassay, it was found necessary to test the wheat seed for the presence of the fungicide Thiram, bis(dimethylthiocarbamoyl) disulphide. Chloroform washings of the seed and Thiram standards in chloroform were spotted on to thin layers of Silica Gel G, developed in methanol–water (1:1) in an S chamber[1] and sprayed with a sodium azide and iodine solution (3 g sodium azide in 100 ml 0.1 *N* iodine)[2]. The decolorization of the spray by spots containing Thiram was temporary but clear enough to warrant further investigations of sensitivity.

It was found that solvents containing considerable amounts of water could not be used to develop spots containing 5 $\mu$g or more of Thiram without severe streaking. A solvent comprising chloroform–carbon tetrachloride (3:1) saturated with water was most convenient, enabling adequate loading and producing well defined spots after a short development time, on thin layers air dried overnight at 35°. Although the sodium azide and iodine spray is very sensitive for Thiram on silica gel, the background colour fades so rapidly that spots with $<$ 1 $\mu$g become invisible in seconds. Immediate subsequent spraying with a 2 % starch solution stabilizes the remaining colour. Much more sensitive detection with more permanent colour and higher contrast can be obtained by spraying the thin layers first with starch solution until they are opalescent and then with the sodium azide and iodine solution[3]. This procedure immediately shows up Thiram as white spots on a blue-black background, although 15–30 min may be required before spots containing less than 1 $\mu$g are fully developed. The blue-black background fades to light brown in several hours but may be darkened again by simply spraying with water if maximal contrast is needed for photographic recording.

Provided care is exercised in limiting the size of the spot to *ca.* 1 mm diameter during application, as little as 0.01 $\mu$g may be detected with this system. $R_F$ values measured from the spot centre vary little over a thousand-fold concentration range—from 0.190 for 0.01 $\mu$g to 0.196 for 10 $\mu$g samples. The spots expand during migration, and the spot diameter, if initially limited to 1 mm, may be used for visual estimation of unknown amounts of Thiram with an accuracy of $\pm$ 0.5 $\mu$g, by comparison with a range of concentration standards.

A commercial preparation of 75 % Thiram applied to wheat and barley seed at the rate of *ca.* 1 part per 500 (2 ounces to the bushel) could be detected clearly in 1 g samples of whole or finely ground seed. By washing the seed for 1 min in 10 ml chloroform and preparing chromatograms of the washing as described above, spots corresponding to Thiram were observed. Untreated samples produced no spot. The chromatograms prepared from the barley sample, which had been stored for several months after treatment, also showed faint bands indicative of some chemical change or degradation in Thiram induced by prolonged contact with the seed. Complex formation of Thiram with amino acids is suggested in recent work[4,5].

Treated and untreated *Brassica* seed samples were also tested after Thiram had been applied at the rate of 1 part per 5,000. Although this seed had a high content of sulphur compounds which could possibly interfere with the test and the rate of application was some ten-fold less than that commonly used in agricultural practice, only one spot, corresponding to Thiram, was detected.

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## Solvents for anthocyanidin chromatography

Two kinds of acidic solvents are used in anthocyanidin chromatography; the organic solvents (generally, butanolic or phenolic) and aqueous solvents (a mixture of water, mineral and organic acids). Although certain butanolic and phenolic solvents give a good "spread" of $R_F$ values for certain anthocyanidins, the spread cannot be fully exploited in identification because the anthocyanidins often fade rapidly and small amounts of anthocyanidins are not detected.

Anthocyanidins are generally stable in aqueous acidic solvents, but there are few such solvents satisfactory for anthocyanidin identification. In Forestal solvent, it is very difficult to differentiate peonidin from pelargonidin, petunidin from cyanidin, and occasionally, cyanidin from malvidin. The formic acid solvent of HARBORNE[1] (a modification of the solvent originally proposed by ENDO, as quoted by ABE AND HAYASHI[2]), although useful, gives a poor spread between cyanidin and malvidin, and malvidin and peonidin. The acetic acid–hydrochloric acid–water (5:1:5) solvent of ABE AND HAYASHI[2] is not satisfactory with partially hydrolyzed anthocyanins because the $R_F$ value ranges of anthocyanins and anthocyanidins overlap.

Because of the shortcomings of these solvents, new solvents were added. The composition of these and some standard solvents is listed in Table I. The range and average of $R_F$ values of standard anthocyanidins in the solvents are listed in Table II. The first line in each column gives the range of $R_F$ values obtained in several runs. The number of runs is given in brackets. One sample of peonidin in HARBORNE's formic acid solvent showed a pink fluorescent contaminant, probably an unknown anthocyanidin, whose $R_F$ values are included.

The *iso*-PrOH solvent of ABE AND HAYASHI[2] is excellent for differentiating peonidin from pelargonidin. Anthocyanidins, in this solvent, fade quite rapidly. Also, it cannot be used as a general solvent for anthocyanidin chromatography because malvidin and petunidin and also cyanidin and peonidin resolve at about the same chromatographic loci.

TABLE I

COMPOSITION OF SOLVENTS USED FOR ANTHOCYANIDIN CHROMATOGRAPHY

| *Abbreviation* | *Composition*[a] | *Development period (h)* |
|---|---|---|
| Forestal | HAc–HCl–$H_2O$ (30:3:10), S | 16 |
| FA–HARBORNE | HCOOH–HCl–$H_2O$ (5:2:3), S | 7.5 |
| FA–4 *N* HCl | HCOOH–4 *N* HCl (2:1), S | 7.5 |
| HAc–HCl[b] | HAc–HCl–$H_2O$ (15:3:82), S | 6 |
| Propionic | PA[c]–HCOOH–HCl–$H_2O$ (2:5:1:6), U | 10 |
| 3:1:8 | HAc–HCl–$H_2O$ (3:1:8), S | 8 |
| 5:1:5 | HAc–HCl–$H_2O$ (5:1:5), S | 11 |
| *Iso*-PrOH[d] | *i*-PrOH–5% HCl (55:45), S | 20 |
| Aq–HCl[b] | HCl–$H_2O$ (3:97), S | 4 |
| BAW | *n*-BuOH–HAc–$H_2O$ (4:1:5), U | 13 |

[a] S = Single phase; U = upper phase.
[b] Anthocyanin solvent of HARBORNE[1] extended to anthocyanidins.
[c] PA = propionic acid.
[d] Isopropyl alcohol solvent of HAYASHI[3], ABE AND HAYASHI[2].

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TABLE II

RANGES AND AVERAGE OF $R_F$ VALUES OF AUTHENTIC ANTHOCYANIDINS[a,b]

| *Solvent* | $R_F \times 100$ | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | *Pelargonidin*[c] *1 OH* | *Peonidin*[d] *1 OH, 1 $OCH_3$* | *Contaminant*[f] | *Malvidin*[d–g] *1 OH, 2 $OCH_3$* | *Cyanidin*[c] *2 OH* | *Petunidin*[d,g] *2 OH, 1 $OCH_3$* | *Delphinidin*[d] *3 OH* | *Apigenidin*[d] *1 OH* | *Luteolinidin*[d] *2 OH*[e] |
| Forestal | 62–74 | 60–69 | | 56–64 | 45–55 | 42–50 | 34–36 | 81–83 | 65–70 |
| | (18) | (13) | | (11) | (14) | (12) | (4) | (3) | (4) |
| | 67 | 64 | | 60 | 48 | 45 | 35 | 82 | 67 |
| FA–Harborne | 32–40 | 25–33 | | 24–30 | 22–27 | 18–23 | 13–15 | 60–62 | 44 |
| | (17) | (12) | | (11) | (15) | (11) | (13) | (2) | (2) |
| | 36 | 29 | 5 | 26 | 24 | 20 | 14 | 61 | 44 |
| FA–4 *N* HCl | 47–55 | 44–51 | | 42–64 | 33–38 | 30–35 | | | |
| | (10) | (10) | | (10) | (12) | (12) | | | |
| | 51 | 48 | 10 | 44 | 37 | 33 | 21 | 69 | 56 |
| HAc–HCl | 15–19 | 12–13 | | 8–10 | 10–12 | 7–8.5 | | 27–30 | 18–21 |
| | (15) | (5) | | (10) | (13) | (10) | | (3) | (3) |
| | 18 | 12 | | 9 | 11.5 | 8 | 7 | 29 | 20 |
| 3:1:8 | 25–28 | 19–21 | 3 | 13–16 | 15–18 | 11–13 | 9 | | |
| | (12) | (7) | | (11) | (15) | (10) | (3) | | |
| | 26 | 20 | 3 | 15 | 16 | 12 | 9 | 38 | 27 |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Propionic | 45–50<br>(14)<br>46 | 39–43<br>(8)<br>40 | 8 | 33–38<br>(9)<br>35 | 28–32<br>(13)<br>30 | 24–29<br>(12)<br>26 | 20<br>(3)<br>20 | 65–67<br>(3)<br>65 | 51–53<br>(3)<br>52 |
| *Iso*-PrOH | 54–73<br>(13)<br>68 | 45–52<br>(6)<br>50 | | h | 45–42<br>(12)<br>48 | h<br>33 | | | |
| 5:1:5 | 49 | | | 38 | 38 | 31 | | | |
| Aq–HCl | 4.5–5<br>(2)<br>5 | 2.5–3<br>(20)<br>3 | | 2–2.25<br>(3)<br>2 | 2.5–3<br>(5)<br>2.5 | 2–2.5<br>(3)<br>2 | 1.5[i] | 7 | 4.25 |
| BAW | 80–82<br>(2)<br>64 | 61–68<br>(2)<br>64 | | | 52 | 60 | 46 | | |

[a] In general, the $R_F$ values of each anthocyanidin in each solvent are in three rows; in the first row, the range of $R_F$ values; the number of runs in parentheses, in the second row; the average in the third row.

[b] The chromatography was carried out at 20 ± 1°.

[c] Provided by Dr. R. M. ACHESON, University of Oxford, England.

[d] Provided by Dr. J. B. HARBORNE, John Innes Horticultural Institution, Hertfordshire, England, (see g, h, and i).

[e] OH at 3-position absent

[f] $R_F$ values of the fluorescent pink contaminant.

[g] The malvidin and petunidin as a mixture.

[h] Malvidin and petunidin do not separate in the *iso*-PrOH solvent.

[i] Delphinidin with a fluorescent pink contaminant at $R_F$ 0.04 in aq. HCl.

The formic acid–4 *N* HCl (2:1) solvent developed in this laboratory is a good one for routine use and has a greater spread in $R_F$ values than the formic acid solvent of HARBORNE[1].

The use of the HAc–HCl solvent originally proposed by HARBORNE[1] for anthocyanins was extended to anthocyanidins. In studying hydrolysis and stability of anthocyanins, anthocyanins and anthocyanidins are most usefully run in one solvent. The HAc–HCl solvent is excellent for this purpose and has been extensively used. $R_F$ values of anthocyanins are generally above 0.20 and those of anthocyanidins below 0.20. The solvent gives sharp resolution and minimal variation in $R_F$ values. The HAc–HCl solvent, like the *iso*-PrOH solvent, differentiates pelargonidin and peonidin.

$R_F$ values of anthocyanidins in a given solvent are usually predictable on the basis of structure. Although the basic constituents of the HAc–HCl solvent are the same as those of Forestal solvent, the order of $R_F$ values of malvidin and cyanidin, as found in Forestal solvent, is reversed in the HAc–HCl solvent. This is particularly useful in identifying malvidin.

The 3:1:8 solvent of ABE AND HAYASHI[2] usually gives good results. Although it is useful in characterizing anthocyanins, it is strongly acidic and may partially hydrolyze these compounds. The order of $R_F$ values for malvidin and cyanidin in Forestal solvent is reversed in this solvent. $R_F$ value ranges for anthocyanidins are much less in this solvent than in Forestal.

The new propionic acid solvent is an excellent solvent. There is a uniform spread of approximately 0.05 $R_F$ value units between the common anthocyanidins and $R_F$ values are conveniently in multiples of 5.

The 1% aqueous HCl solvent of HARBORNE[1] is very good for the purification and characterization of anthocyanins but $R_F$ values for anthocyanidins are very low. Nonetheless, it is useful in studying the products of partial hydrolysis of anthocyanins. The colors of anthocyanins and anthocyanidins, both in visible and ultraviolet light, are very sharp in this solvent.

The authors are grateful for gifts of delphinidin, peonidin and pelargonidin and a mixture of malvidin and petunidin to Dr. J. B. HARBORNE and of cyanidin to Dr. R. M. ACHESON.

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## Rapid thin-layer chromatography of porphyrins and related compounds, and its application to the study of porphyrias*

Rapid methods of thin-layer chromatography (TLC) have been worked out in which porphyrins, chlorins, chlorophyll and related compounds are separated. The procedures are more rapid than our earlier methods[1]. A glass fiber reinforced silica gel sheet, made by Gelman Co.**, is used as the separating medium, and kerosene–chloroform and aqueous lutidine are used as the respective solvents for methyl esters of porphyrins and coproporphyrin (copro) isomers. A linear relationship between $R_F$ values of the porphyrins and the number of ester groups can be obtained in 5 min and the separation of copro I, II and III (or IV) is achieved in 1 h.

Based upon the findings that different types of porphyria have different porphyrin patterns[2], the method is applied to differentiate major types of porphyria by a simple test tube chromatography of the porphyrins isolated from urine, or other porphyric materials, from such patients. Estimation of copro I and III isomers in such samples can be done in 30 min, which is much faster than the time required by paper chromatography (PC) (overnight)[3,4], or by JENSEN's TLC (2 h)[5].

*Experimental*

The Gelman sheets, ITLC-Type SG, are produced by combining silica gel with a slurry of microfilaments of glass fiber. With a thickness of about 0.3 mm, this glass fiber media is strong enough to withstand the chromatographic process.

*Separation of methyl esters of porphyrins and related compounds.* A rectangular museum jar, 10.5 × 5 × 15 cm high, with a ground glass cover was used as the developing chamber, and a mixture of 3 ml of kerosene and 7 ml of chloroform (U.S.P.) was used as the solvent. A 6 × 12 cm sheet cut out from the original 8 × 8 in. size, was spotted with chloroform solutions of samples along a pencilled base line 12 mm from one of the shorter edges. Because of the less compact nature of the Gelman sheet, the spotted area may easily spread over 2 mm in diameter. Therefore, repeated applications of dilute solutions should be avoided. The spotted sheet was inserted immediately after the solvent mixture was introduced with a long stem funnel into the chamber. In 5 min, the ascending solvent reached about 7 cm above the base line of the leaning chromatogram. The developed sheet was taken out, dried, and examined as usual.

For a single porphyric sample, a 2.5 × 15 cm test tube and a small watch glass were used. Two milliliters of the solvent mixture were introduced into the tube without touching the wall. A Gelman strip, 1.8 × 12 cm, spotted with an unknown and a proper marker, was then inserted. Methyl esters of crude porphyrins such as those originating from porphyria patients, were spotted with copro and uroporphyrin (uro) esters as references. The developing time was also 5 min.

*Separation of copro isomers.* A tall museum jar, 8 × 4.5 × 20 cm high, was used for this purpose. A mixture of 7 ml of 2,6-lutidine and 2 ml of water was the solvent. A necessary ammonia atmosphere was provided by 2 supported tubes of concentrated ammonium hydroxide. The supporting frame was made from a piece of No. 16 nichrome wire about 50 cm long. It was bent into a U-shape of 7–8 cm base. The 2

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** Gelman Instrument Co., Ann Arbor, Mich., U.S.A.

ends were made into rings, each large enough to support a 12 × 75 mm test tube around its rim. The positions of the rings were adjusted in the jar, so that when the frame was leaning against the wall, the 2 tubes were hanging vertically at the upper corners of the jar. To help stabilize the tube, a piece of glass rod of appropriate diameter, and 5–5.5 cm long, was slid down into each tube. Concentrated ammonia was added with a pipet, to about 1 cm below the rim of the tube. Because of the glass rod, only about 2 ml of ammonia per tube is needed. The solvent mixture, previously cooled to room temperature, was then added through a long funnel into the chamber. It was left undisturbed for 30 min to attain equilibrium. A 6 × 18 cm sheet was spotted with freshly prepared ammoniacal solutions of copro isomers along the 12 mm high base line. The spreading effect of the sheet is much more pronounced with ammonia. Sample spots should be applied in equal volumes of approximately equal concentrations for better quantitative results. Using a pair of forceps, the spotted sheet was inserted quickly but steadily into the chamber, with its back leaning against the wall opposite the ammonia tubes. The solvent ascended about 14 cm in 1 h. In routine analysis of the naturally occurring copro I and III isomers, the developing time was reduced to 30 min.

TABLE I

$R_F$ VALUES (× 100) OF PORPHYRINS AND RELATED COMPOUNDS

*Methyl esters* (*Solvent*: *kerosene–chloroform, 3:7 ml*)

| °C | *Porphyrins* | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | *Proto* | *Meso* | *Deutero* | *Hemato* | *Copro* | *Penta-carbo-xylic* | *Hexa-carbo-xylic* | *Hepta-carbo-xylic* | *Uro* |
| 20 | 67 | 67 | 67 | 3 | 50 | 40 | 28 | 18 | 9 |
| 23 | 68 | 68 | 68 | 3 | 50 | 40 | 29 | 19 | 10 |

| | *Chlorins and chlorophyll* | | | |
|---|---|---|---|---|
| | *Pyropheophorbide-a* | *Mesochlorin-$e_6$* | *Uch** | *Chlorophyll-a* |
| 20 | 60 | 62 | 4 | 73 |

| | *Metalloporphyrins* | | | |
|---|---|---|---|---|
| | *Cu-deutero* | *Cu-copro* | *Cu-uro* | *Zn-uro* |
| 20 | 72 | 56 | 3 | 3 |

*Free coproporphyrins* (*Solvent*: *2,6-lutidine–water, 7:2 ml*; *$NH_3$ atmosphere*)

| | *I* | *II* | *III* | *IV* |
|---|---|---|---|---|
| 23 | 39 | 57 | 48 | 48 |

* Uch, a urinary chlorin isolated from a congenital porphyria patient[6].

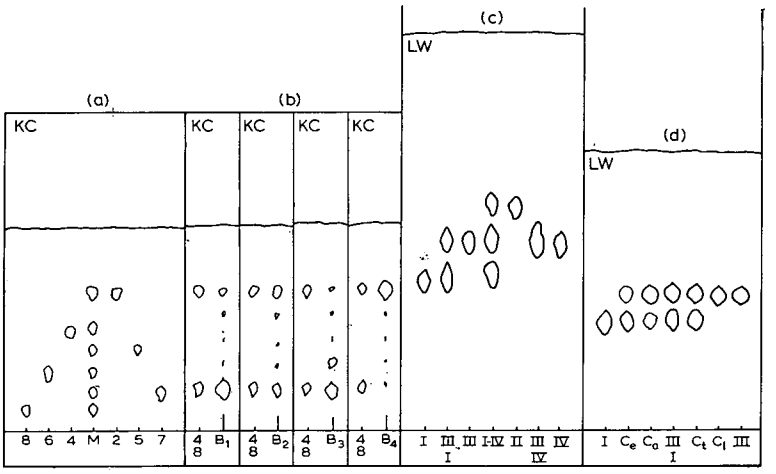


Fig. 1. Thin-layer chromatograms of porphyrins. (a) KC, kerosene–chloroform (3:7, v/v), with solvent front marked after 5 min development at 23°. 2 = Dimethyl ester of protoporphyrin; 4 = copro ester; 5, 6 and 7 = penta-, hexa- and heptacarboxylic porphyrin esters, respectively; 8 = uro ester; and M = artificial mixture of the esters. (b) Porphyrin patterns of major types of porphyria. $B_1$ = Total porphyrin esters prepared from urine sample of an erythropoietic porphyria patient; $B_2$ = from acute intermittent porphyria; $B_3$ = cutanea tarda; and $B_4$ = lead poisoning case. (c) LW, lutidine–water (7:2, v/v), with solvent front after 1 h. I, II, III, and IV = Free copro isomers of respective types; I–IV = mixture of the four; and I, III, etc. = mixtures of specified isomers. (d) Solvent front after 30 min. $C_e$ = Copro from ertythropoietic porphyria patient; $C_a$ = acute intermittent; $C_t$ = cutanea tarda; and $C_l$ = lead poisoning.

*Results and discussion*

The $R_F$ values of porphyrins, chlorins, chlorophyll and metalloporphyrins are listed in Table I. The chromatograms showing separations of porphyrin esters and isomers are given in Fig. 1. From Table I, the $R_F$ values of the methyl esters, revealing a linear function with the number of ester groups of the porphyrins, are quite constant at room temperature. The difference in the relative positions of porphyrins in Fig. 1a and b is due to the difference in the two experimental set-ups. Different batches of the Gelman sheets have also shown some differences in $R_F$ values. Aside from some non-uniformity occasionally encountered, the sheet behaves almost like filter paper without the worry of its being deactivated on storage.

Fig. 1b shows the results obtained from urine samples from different porphyria patients. The uro is predominant in the erythropoietic or congenital porphyria ($B_1$), while the copro dominates in the lead poisoning porphyrinuria (B4). In the acute intermittent type of porphyria, the copro and uro are eliminated in comparable amounts ($B_2$), and in porphyria cutanea tarda, the excretion of the heptacarboxylic porphyrin is greatly enhanced ($B_3$).

Fig. 1c has reproduced the paper chromatographic result of FALK[3], with a better separation of the copros. Besides the much shortened developing time, the $R_F$ values are reproducible even with the 30 min short runs. As in PC, the lutidine system does not separate copro III and IV.

Fig. 1d shows some typical isomeric compositions of copro isolated from various types of porphyria patients. The copro in the erythropoietic porphyria is mainly of

the type I, while that in acute and lead poisoning cases is mainly of the type III. In cutanea tarda, the copro has a wider range of the percentage composition, with an average of about 65 % III.

The minimum detectable amount of fluorescent porphyrin esters is 0.002 μg, and 0.1 μg is a convenient quantity to work with. In the case of copro isomers, 0.05–0.1 μg is only barely detectable, because of the spreading spots, and the easy fading of fluorescence on drying.

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## Thin-layer chromatography of isatins and corresponding isatin-N-Mannich bases

Recently we have synthesized a series of isatin-N-Mannich bases for pharmacological screening. These results are reported elsewhere[1]. During the course of this investigation it became necessary to develop a suitable method for the separation of isatins from their corresponding Mannich bases. Thin-layer chromatography was chosen because of its rapidity and simplicity. In this communication the results of thin-layer chromatography of these compounds are reported.

*Materials*

Commercially available isatin, 5-bromoisatin and 5-methylisatin were used.

Isatin-N-Mannich bases were prepared by condensing equimolar proportions of isatin, formaldehyde and appropriate secondary amine[1].

*Solvent system*

Benzene–ethyl acetate–diethylamine (75:20:5, v/v).

*Visualization*

Most of the spots were readily visible as such but the optimum visualization was achieved by the use of an ultraviolet lamp.

*Method*

Pre-coated chromatogram sheets (20 × 20 cm; type K301R, Silica Gel with fluorescent indicator) from Distillation Products Industries were used without activation. Samples were dissolved in acetone and applied to the sheets. The spots were placed on a line 1.5 cm from the lower edge of the sheet at intervals of 2 cm. The

TABLE I

$R_F$ VALUES OF ISATINS AND ISATIN-N-MANNICH BASES

| *Compound* | $R_F \times 100$ | | |
|---|---|---|---|
| | *Run I* | *Run II* | *Mean of I and II* |
| Isatin | 44 | 42 | 43 |
| N-Morpholinomethylisatin | 65 | 61 | 63 |
| N-Piperidinomethylisatin | 74 | 67 | 70.5 |
| N-3-Azabicyclo[3,2,2]nonanomethylisatin | 75 | 67 | 71 |
| N-3-Azabicyclo[3,2,1]octanomethylisatin | 74 | 68 | 71 |
| N-Hexamethyleneimino-methylisatin | 71 | 66 | 68.5 |
| 5-Bromoisatin | 44 | 38 | 41 |
| N-Morpholinomethyl-5-bromoisatin | 68 | 68 | 68 |
| N-Piperidinomethyl-5-bromoisatin | 67 | 66 | 66.5 |
| N-3-Azabicyclo[3,2,2]nonanomethyl-5-bromoisatin | 71 | 68 | 69.5 |
| N-3-Azabicyclo[3,2,1]octanomethyl-5-bromoisatin | 67 | 69 | 68 |
| 5-Methylisatin | 38 | 40 | 39 |
| N-Piperidinomethyl-5-methylisatin | 69 | 69 | 69 |
| N-Morpholinomethyl-5-methylisatin | 68 | 73 | 70.5 |

rectangular tank was saturated for 2 h and lined with filter paper. The solvent front was 10 cm and the running time was about 20 min.

*Discussion*

Chromatographic data for three isatins and eleven isatin-N-Mannich bases are summarized in Table I. Although the $R_F$ values of Mannich bases are somewhat close to each other, there is a significant difference when compared to the parent isatin. The method is therefore very useful for the separation and identification of isatins from their corresponding Mannich bases. The method is quite sensitive in that even a trace of isatin, if present in the Mannich base as an impurity, results in the formation of two spots. Thus the method is also useful to ascertain the purity of the Mannich bases.

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## Thin-layer chromatography of anthocyanins from blackcurrant juice

In recent years a number of workers have studied the separation of anthocyanins by thin-layer chromatography (TLC) using a variety of adsorbents. Thus NYBOM[1] used cellulose powder, BIRKHOFER *et al.*[2] polyacrilonitrile–polyamide, and TANNER *et al.*[3] silica gel. ASEN described the use of layers composed of cellulose and silica gel mixtures[4], and more recently BIRKHOFER *et al.* have used alumina[5].

In the present communication, a simple method is described for the rapid resolution and characterisation of the anthocyanins of blackcurrant juice. Kieselgel G (E. Merck, Darmstadt) was found to be a suitable adsorbent, and separations of the glycosides of cyanidin and delphinidin were obtained within one hour. The aglycone cyanidin was also resolved satisfactorily.

*Experimental*

Kieselgel G (110 g) was agitated with 500 ml acidified methanol ($CH_3OH$–0.5 *N* HCl, 80:20, v/v) to remove metallic ions, filtered, washed with 400 ml distilled water and dried in a shallow dish for 8 h at 80°. The material was sieved to pass 100 mesh and stored in a glass container. The quantity of Kieselgel G produced was sufficient to coat twenty 20 × 20 cm TLC plates. Five plates (20 × 20 cm) were coated with a 250 $\mu$ layer prepared from a slurry of 25 g adsorbent and 56 ml distilled water. The plates were dried for 25 min at 110° and cooled in a desiccator. Samples were always applied to freshly prepared plates. The solvent system ethyl acetate–ethyl methyl ketone–formic acid–water (6:3:1:1, v/v) was used, and the development tank was allowed to equilibrate overnight before use. Several variations[6] of this solvent system were investigated, but were found to be less satisfactory. Plates were run three times in the same direction with intermediate drying. The solvent was allowed to ascend for 5 cm on the first development and for 10 cm on the last two runs. The shortened first development prevented diffusion of the spots.

Extracts of blackcurrant juice in *n*-butanol were separated on cellulose columns by the procedure of CHANDLER AND HARPER[7]. Several bands were eluted and concentrated by rotary evaporation *in vacuo*. The fractions containing the glycosides of cyanidin and delphinidin were subjected to preparative TLC. Concentrated fractions containing 240 $\mu$g were applied in 30 $\mu$l of solvent as narrow bands to the base line of the plate. After development and subsequent evaporation of the solvents, the separated bands were scraped off into filter funnels packed with prewashed pledgets of glass wool, and the glycosides were then eluted with acidified methanol. The eluates were clarified by centrifugation, the absorbances read at 525 m$\mu$, and the concentration of the anthocyanins determined by the technique of SWAIN AND HILLIS[8]. The recovery of anthocyanins was calculated by reference to standard curves prepared from pure cyanidin and delphinidin glycosides.

*Results and discussion*

Recoveries of 98 ± 2 % of cyanidin glycosides, and 94 ± 4 % of delphinidin glycosides were obtained in four determinations of each glycoside. Up to 600 $\mu$g glycosides could be separated when applied to the plate as a thin streak, and not more than 80–90 $\mu$g could be resolved satisfactorily when applied as a single spot. The separation achieved with a range of concentrations of cyanidin-3-rhamnoglucoside

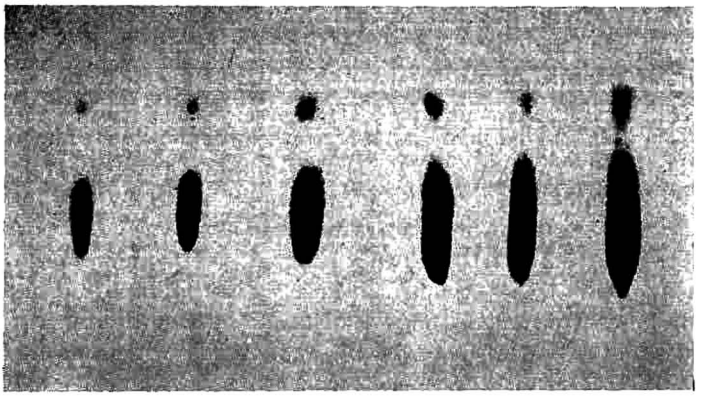

Fig. 1. Separation of cyanidin-3-rhamnoglucoside (lower) from cyanidin-3-glucoside (upper) on TLC plates coated with a 250 $\mu$ layer of Kieselgel G. Developing solvent: ethyl acetate–ethyl methyl ketone–formic acid–water (6:3:1:1, v/v). First development for 5 cm, and redeveloped twice for 10 cm. Concentrations (left to right) were 5, 10, 30, 50, 90 $\mu$g mixed glycosides.

contaminated with cyanidin-3-glucoside from a fraction of blackcurrant juice is shown in Fig. 1. The cyanidin glycosides migrated in front of the delphinidin glycosides, and in each case the glucosides had higher $R_F$ values than the corresponding rhamnoglucosides. No hydrolysis was observed when the eluted glycosides were centrifuged and concentrated under a gentle stream of nitrogen, and the technique could be readily used to prepare small amounts of pure anthocyanins. TLC of partially hydrolysed cyanidin glycosides[7] showed that the aglycone cyanidin was well resolved from the unhydrolysed material, and moved close to the solvent front. The aglycone faded about 5 min after evaporation of the solvents, but with rapid handling the aglycone could be recovered by elution with a suitable acidic solvent[9]. By contrast delphinidin was not well resolved from the glycosides, and it faded too rapidly for satisfactory recovery.

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## A solvent equilibrator inside the descending chromatography tank

### Automatic application of mobile phase

For paper chromatography in volatile systems composed of two solvent phases such as are used for the separation of steroids[1], the prepared paper sheets generally require some hours' equilibration in the tank. To start the run, a volume of upper phase solvent pre-equilibrated with the lower phase is drawn from a nearby separator and introduced into the trough from which the paper hangs inside the tank. During the transfer the solvent frequently turns cloudy, indicating a disturbance of equilibrium. Furthermore, automatic starting becomes desirable when it is necessary to introduce the solvent at inconvenient times during the night so as to finish the run within the following day.

Few of the automatic starting systems described in the literature are adaptable for two-phase solvent chromatography. To ensure a completely closed system for such volatile solvents, ABELSON AND FOX[2] linked the solvent reservoir directly with the tank through a tap, which was automatically operated by means of a battery-powered electrical circuit comprising a solenoid and anti-flash unit triggered by a timer. It would be more desirable to equilibrate the running solvent actually inside the tank and without the complication of an electrical system. For this purpose, the apparatus shown in Fig. 1 was devised in this laboratory. It consists of a siphon inside a reservoir tube shaped to fit into the solvent trough of a chromatography tank, the measurements given being appropriate to a tube for the 12-inch Shandon model. Charging the equilibrator tube with solvent through the side-arm is greatly facilitated by inclining the tube at about 45° to the vertical. The volumes of the two phases are so chosen that only the upper (mobile) phase feeds the internal siphon when the tube is placed in the dry trough to clamp the paper in position. The tank is sealed by a glass lid with a centre hole to accommodate the side-arm which carries a sleeve to make the tank sufficiently air-tight. During equilibration the side-arm is kept closed; but momentary application of pressure to it, such as by a rubber bulb, starts the run by causing upper phase solvent to siphon into the trough. At the end of the run, suction is applied at the side-arm to drain the solvent in the trough back through the siphon. The tube is then removed and the paper lifted out.

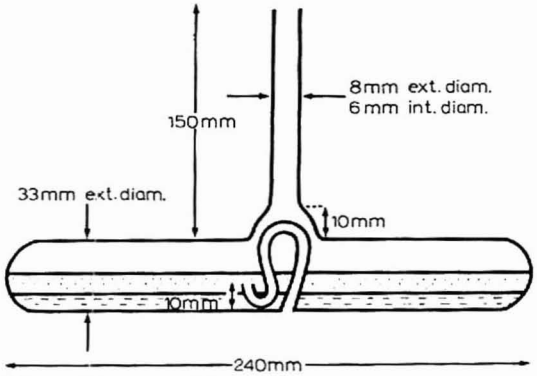


Fig. 1. Solvent equilibrator.

An adaptation of the simple alarm-clock device of KRABISH AND SJÖVALL[3] permits automatic addition of the running solvent (Fig. 2). When the alarm rings, the winding screw turns to lower the sinker into the reservoir and siphons the water it contains into the funnel and *via* its overflow tube into the separator. The air displaced transfers this volume of running solvent from the equilibrator (shown here in cross-section) into the trough and thereby starts the run. When the run is completed, some of the water in the separator is drawn off by opening the tap and the resultant reduction in pressure drains the solvent in the trough back into the equilibrator tube through the siphon.

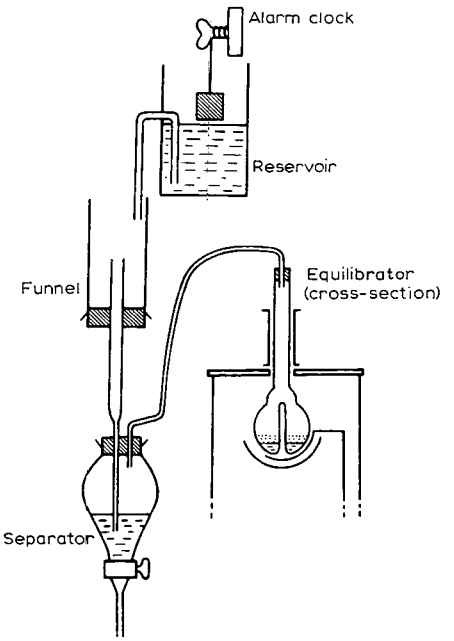


Fig. 2. Arrangement for automatic starting of chromatography.

The equilibrator is robust and made entirely of glass. It can be constructed to fit most standard chromatography apparatus. There are no mechanically moving parts inside the tank. The central position of the siphon in the tube minimises the chance of lower phase solvent entering its orifice while easing the tube into the trough. By its upwards extension into the side-arm the siphon is increased in height to avoid inadvertent starting during equilibration. A feature of the equilibrator is that it allows drainage of the solvent from the trough after the run; this facilitates withdrawal of the paper chromatogram. Without material alteration the equilibrator may be used equally well for monophasic systems.

Grateful thanks are due to G. FRANGLEN whose alarm clock "sleep conserver" stimulated this work, to Prof. N. H. MARTIN in whose department the research that required long chromatography runs was carried out, and to the British Empire

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## Paper chromatography of higher esters of fatty acids with saccharose

Analyses of the samples taken in the course of the reaction of methyl esters of fatty acids with saccharose in dimethylformamide or dimethylsulfoxide[1–14], and those of the finished product for determining the mono- and diester content, are based on the specific rotation of the butanol solution[3]. This method is not reliable, assuming the presence of only two components, the mono- and diester. In fact, the reaction is more intricate, as higher esters of saccharose or esters of fatty acids together with split products of saccharose, especially glucose, form comparatively easily, as shown by earlier work studying the kinetics of the reesterification of saccharose dipalmitate with saccharose[15].

These classic methods may serve as orienting tests, but they do not comply with the requirements for accurate determinations of the composition of the product or of more detailed studies of the reaction. Therefore our attention was called to analytical studies of higher and lower esters of saccharose.

The determination of mono- and diesters of fatty acids with saccharose by paper chromatography has been described in an earlier paper[16]. A mixture of the substances or the reaction solution was separated on Whatman No. 1 paper by eluting with the system benzene–*n*-propanol (10:3, v/v). The descending technique was used and for a good separation an atmosphere of 90 % relative moisture in the chamber was very important.

On drying, the paper was washed in a petroleum ether solution of paraffin, m.p. 50° (concentration 10 g/l) and on evaporating the solvent in a drier, the spots were detected by dipping the paper into a water bath at 27° for 2 min. The mono- and diesters emulsified the paraffin and the spots became transparent. The sensitivity of the method was between 0.55 and 1 $\mu$g of mono- and diester.

The higher esters of saccharose showed a much lower emulsifying ability. The monoesters of glucose and fructose behaved similarly. Although those esters could be

identified qualitatively, this method did not ensure a reliable quantitative determination and was inconvenient for other esters. Therefore we tried to develop a new chromatographic method for the determination of higher esters of saccharose than the mono- and diesters.

*Experimental*

*Materials.* The esters of palmitic, stearic and myristic acids with saccharose, used in this experiment, were formed by the reaction of the methyl esters of the fatty acids with saccharose in dimethylformamide[3]. The methyl ester purity was 99–99.5 % (GLC). The saccharose was pure, furnished by Lachema. The esters of saccharose were isolated from the reaction mixture by *n*-butanol, *n*-hexane and acetone extractions.

With ordinary methods[1–3,6] of isolation, *e.g.* by means of solvents, it is not possible to obtain pure esters of saccharose and, as in the previous work, adsorption on a silica gel column was used for isolating pure tri-, tetra-, penta- and hexaesters.

A mixture of esters of saccharose with palmitic acid containing approximately 70 % of tri-, tetra-, penta-, hexa-, hepta- and octa-derivatives, was separated on silica gel Lachema CH (average diameter of pores, 80 Å) by eluting with chloroform and acetone in varying proportions. Fractions of successively lower degree of esterification of saccharose were taken. The method will be described in another paper.

Elementary analyses showed a good agreement with theoretical values. The figures found for triester were:

C = 67.42 %, H = 11.09 % (theoretical C = 68.14 %, H = 10.68 %); for tetraester

C = 69.62 %, H = 11.01 % (theoretical C = 70.43 %, H = 11.04 %); for hexaester

C = 73.30 %, H = 11.10 % (theoretical C = 73.17 %, H = 11.48 %).

The pentaester was not analysed since the amount available was too small for an elementary analysis. The esters were chromatographically pure.

*Procedure.* Portions between 20 and 80 $\mu$g of higher esters in a chloroform solution were applied to 20 F 1 paper (Spezialpapierfabrik, Niederschlag). The mono- and diesters were first separated on silica gel with an eluting mixture of chloroform–acetone (80:20), as they interfered with the reliable determination of triesters and, when present in larger amounts, the spots partially overlapped. Other substances, like saccharose and the remaining solvents, did not interfere.

The paper was eluted by the descending technique with a benzene–*n*-propanol mixture (10:1). Dishes of water were placed in the chromatography chamber, or the whole of the bottom was covered with water to get an optimal atmosphere of approximately 90 % relative moisture at 20°. The elution time was 6–7 h.

The chromatograms were dried first in hot air and then in a drier at 90° for 15 min. For detection purposes the different solubilities of the saccharose esters in various solvents[17] were used. The hydrophobic power of the substances was increased by immersing the paper in a petroleum ether solution of paraffin (m.p. 50°), concentration 5 g/l. The solvent was allowed to evaporate and the chromatogram was again dried in a drier for 10 min at 90°.

The treated chromatogram was dipped into a water bath containing 40 % methanol at 20° ± 1°. To maintain this temperature a Wobsser ultrathermostat (Medingen) was used. In the regions of the higher esters of saccharose with fatty acids

white spots appeared and the surrounding paper became transparent. For quantitative determinations it is of great importance to maintain the prescribed temperature of the bath and the immersion time from 45 to 60 sec. These conditions vary according to the type of chromatographic paper used, for example, with Whatman No. 1 it is necessary either to increase the amount of the bound nonpolar phase by changing the concentration of the paraffin solution from 5 g/l to 10 g/l or to decrease the detection time to 20–30 sec.

The developed paper was dried between two filter papers and the spots were outlined (see Fig. 1). The spacings between the spots increased regularly with increasing amounts of acyl groups in the molecules, as shown by the corresponding $R_F$ values, which for the triester, tetraester, pentaester and hexaester were found to be 0.04, 0.19, 0.37 and 0.54, respectively. On the basis of these regular spacings it was possible to classify the other spots with the $R_F$ values 0.71 and 0.87 as the heptaester and octaester, respectively. The mean difference of $R_F$ values of the spots was 0.17. The $R_F$ values found with the 20 F 1 paper were practically the same as those with Whatman No. 1.

In contrast to the system used for the analyses of mono- and diesters where the $R_F$ values were independent of the type of the fatty acid, the $R_F$ values of the higher fatty acid esters of saccharose differed slightly according to the type of the fatty acid. The $R_F$ values of all esters slightly increased from stearic acid to lauric acid. *E.g.* the $R_F$ values of the saccharose tristearate and trimyristate were 0.02 and 0.08, respectively (Fig. 2). A similar relationship was found also for other esters, the values ranging from 0.02 to 0.04.

The detection of the spots may be repeated on drying.

*Quantitative determination.* The area of the spots was shown to be influenced by

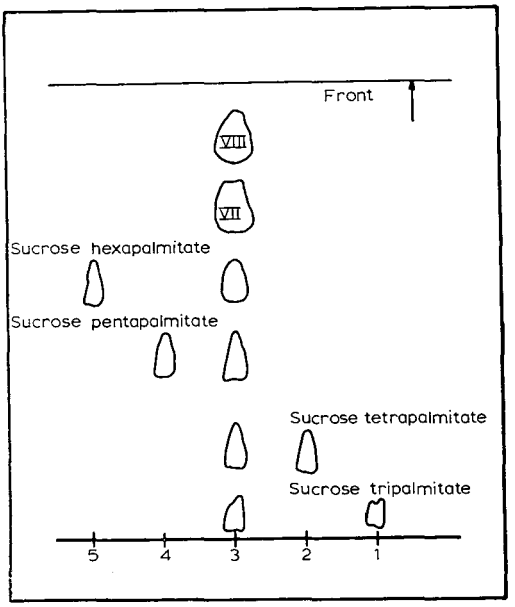


Fig. 1. Paper chromatogram of simple and mixed samples of sucrose esters.

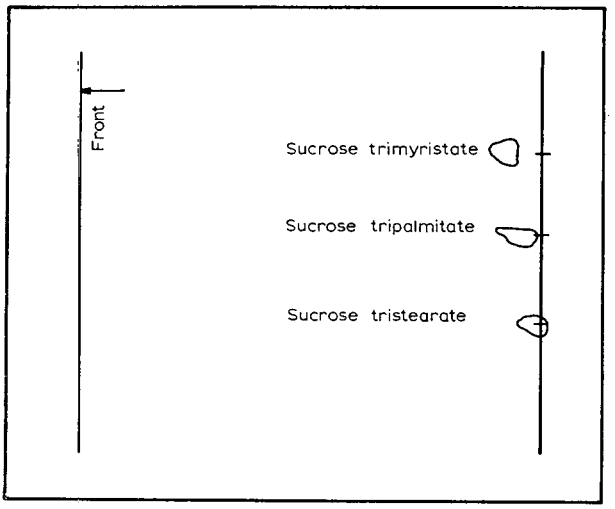


Fig. 2. Paper chromatogram of the triesters of myristic, palmitic and stearic acids with sucrose.

a number of factors, the most important of which are the temperature of the bath, the detection time and the paraffin concentration. For the quantitative determination of unknown samples it is essential to maintain constant conditions, likewise in the case of calibration. The dependence of the area of the spots on the concentration was examined for the tri-, tetra- and pentaesters. The concentrations were between 5 and 70 $\mu$g. It was found that the area of the spot was directly proportional to the concentration. Sets of measurements of 10–20 runs were statistically evaluated and formulated by the equation of the regression line[18].

$$A = b_A \cdot c + a$$

where $A$ is the area of the spot in cm$^2$, $c$ is the concentration of the substance in micrograms, and $a$ and $b_A$ are constants for the straight line in question. The relations for

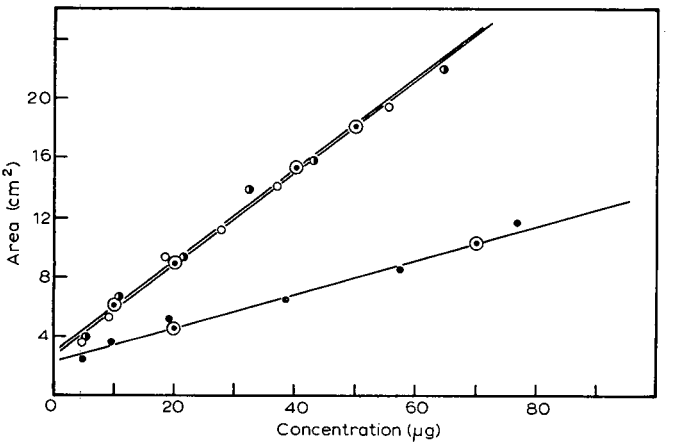


Fig. 3. Plot of area spots *vs.* concentration of sucrose tri-, tetra- and pentapalmitate. (●) Tripalmitate, (○) tetrapalmitate, (◐) pentapalmitate, (⊙) calculated value.

the tri-, tetra- and pentaesters are shown in Fig. 3. The values in the figure are arithmetic means of the measurements made, the encircled points showing the values of the regression line. The corresponding equations were: $A = 0.115 \cdot c + 2.25$ for the triester; $A = 0.307 \cdot c + 2.79$ for the tetraester; and $A = 0.305 \cdot c + 3.01$ for the pentaester.

The sensitivity of the method is fairly high, as we can see from Fig. 1, ranging from 1 to 3 $\mu$g. Thus it is possible to detect with certainty 1–3 % of the substance in the mixture.

The accuracy of the method, assuming the conditions mentioned were strictly observed, is adequate. The error of the determination did not exceed $\pm$ 10 % of the results with concentrations up to 20 $\mu$g, and $\pm$ 5 % with higher concentrations.

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## Biochemistry of sphingolipids. XII. Paper chromatography of dinitrophenyl derivatives of sphingosines and their degradation products

In our previous papers[1–4] we have reported on the thin-layer chromatography of DNP (dinitrophenyl)-derivatives of sphingosines and phytosphingosines on aluminium oxide and silica gel impregnated with sodium tetraborate. This method was widely applied to the separation of long-chain bases prepared from sphingolipids of animal and plant origin.

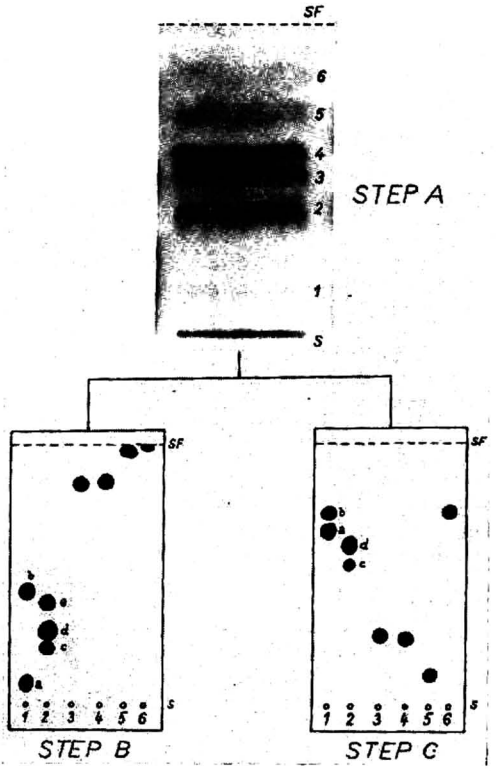


Fig. 1. Schematic representation of the paper chromatographic fractionation of DNP-sphingosine bases and their degradation products. Step A. Preparative chromatography of crude DNP-derivatives on Whatman No. 3 paper impregnated with silica gel. System: petroleum ether (b.p. 60–90°)–diethyl ether (65:35). Detection: U.V. light (254 nm). DNP-derivatives: 1 = phytosphingosines + unidentified degradation products I; 2 = sphingosines + dihydrosphingosines; 3 = O-methyl derivatives I (probably 3-O-methyl-sphingosines); 4 = O-methyl derivatives II (probably 5-O-methyl-$\Delta^3$-sphingosines); 5 = unidentified degradation products II; 6 = 2,4-dinitrophenol. S = start; SF = solvent front. Step B. Separation of DNP-fractions after preparative isolation on Whatman No. 3 silica gel paper impregnated with sodium tetraborate in chloroform. 1a = Phytosphingosines; 1b = unidentified degradation products I; 2c = threo-sphingosines; 2d = erythro-sphingosines; 2e = dihydrosphingosines; 3 = O-methyl derivatives I; 4 = O-methyl-derivatives II; 5 = unidentified degradation products II; 6 = 2,4-dinitrophenol. Step C. Separation of DNP-fractions after preparative isolation on Whatman No. 3 paper impregnated with tetralin in methanol–tetralin–water (90:10:10; upper phase). 1a = $C_{20}$-phytosphingosine + unidentified degradation products I; 1b = $C_{18}$-phytosphingosine; 2c = $C_{18}$-dihydrosphingosine; 2d = $C_{18}$-sphingosine; 3 = O-methyl-derivatives I; 4 = O-methyl-derivatives II; 5 = unidentified degradation products II; 6 = 2,4-dinitrophenol.

During comparative studies on the formation of some degradation products in various hydrolytic procedures of sphingolipids[5], we have found paper chromatography a simple, rapid and valuable technique for the characterization of these substances.

*Experimental*

(*a*) *Hydrolysis of sphingolipids and preparation of DNP-derivatives.* 25 ml of freshly prepared methanol–sulfuric acid reagent (1.25 ml of conc. $H_2SO_4$ diluted to 25 ml with methanol) was added to a sample of 50 mg of human brain sphingolipids (without gangliosides). The mixture was heated for 18 h at 60–70° in a water bath. After removal of fatty acid methyl esters with petroleum ether, the hydrolysate was


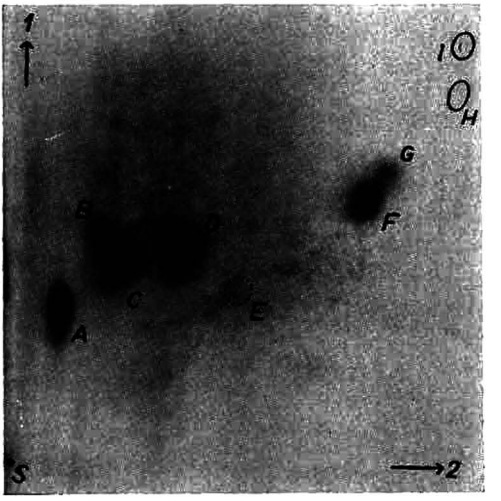


Fig. 2. Preparative chromatography of crude DNP-derivatives on Whatman No. 3 silica gel paper impregnated with sodium tetraborate in chloroform–methanol (100:0.5). Detection: U.V. light (254 nm). DNP-derivatives: 1 = phytosphingosines; 2 = threo-sphingosines; 3 = erythro-sphingosines; 4 = dihydrosphingosines + unidentified degradation products I; 5 = O-methyl-derivatives + unidentified degradation products II + 2,4-dinitrophenol; S = start; SF = solvent front.

Fig. 3. Two-dimensional paper chromatography of DNP-derivatives. 1st dimension: Whatman No. 3 silica gel paper; petroleum ether (b.p. 60–90°)–diethyl ether (65:35). 2nd dimension: chloroform after impregnation with sodium tetraborate. Detection: U.V. light (254 nm). A = Phytosphingosines; B = threo-sphingosines; C = erythro-sphingosines; D = dihydrosphingosines; E = unidentified degradation products I; F = O-methyl-derivatives I; G = O-methyl-derivatives II; H = unidentified degradation products II; I = 2,4-dinitrophenol. S = start.

made alkaline with potassium hydroxide. Sphingosine bases were then extracted with diethyl ether. The ether solution was washed with water, dried over anhydrous sodium sulfate, and evaporated to dryness. The DNP-derivatives were prepared according to KARLSSON[6].

DNP-derivatives of phytosphingosines were prepared from baker's yeast.

(*b*) *Preparative paper chromatography of DNP-derivatives on paper impregnated with silica gel* (*Step A*). The crude DNP-derivatives dissolved in diethyl ether were

spotted as a narrow band along the entire length of the starting line on Whatman No. 3 paper (18 cm × 18 cm) impregnated with silica gel. The chromatogram was developed in petroleum ether (b.p. 60–90°)–diethyl ether (65:35). After drying for a short time in air, the zones were located under ultraviolet light and continuously eluted with methanol.

(*c*) *Chromatography of individual fractions on silica gel paper impregnated with sodium tetraborate* (*Step B*). The eluates of all fractions obtained from preparative chromatography were evaporated at room temperature under nitrogen. After dissolving in a small volume of diethyl ether they were spotted on Whatman No. 3 silica gel paper impregnated with sodium tetraborate. The impregnation was performed by drawing the paper through an aqueous half-saturated solution of sodium tetraborate (22.4 g $Na_2B_4O_7 \cdot 10H_2O$ in 1000 ml water) and drying at 110–120° for 10 min. The chromatograms were run in chloroform or chloroform–methanol (100:0.5).

(*d*) *Chromatography of individual fractions in a reversed-phase system* (*Step C*). The fractions were separated on Whatman No. 3 silica gel paper impregnated with 2.5–5 % solution of tetralin in diethyl ether using methanol–tetralin–water (90:10:10, upper phase) as a solvent system.

Step A (Fig. 1) allows the separation of sphingosines, phytosphingosines and most of the degradation products. Step B is advantageous for the further subfractionation of sphingosines into isomers and differentiation of these from dihydrosphingosines. In addition, phytosphingosines are separated from the degradation products. Step C permits the identification of the homologous DNP-sphingosines, dihydrosphingosines and phytosphingosines.

The preparative chromatographic procedure on silica gel paper with or without impregnation with sodium tetraborate could be used for the quantitative estimation of each type of DNP-derivatives (Fig. 2)[5].

(*e*) *Two-dimensional chromatography*. The combination of the systems described above in a two-dimensional modification would appear to be useful for the rapid qualitative characterization of the constituents of crude DNP-derivatives extracts (Fig. 3).

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## Variations du $R_F$ avec le pH dans la chromatographie sur papier des ptérines

Les solvants employés jusqu'ici pour la chromatographie des ptérines sont très nombreux mais ont été mis au point assez empiriquement. Une catégorie fort utilisée est représentée par des solutions aqueuses de sels divers. Nous avons constaté, au cours d'expériences ayant pour but le choix d'un solvant de ce type, que, pour certaines valeurs de pH, nous obtenions de meilleurs résultats. Cette observation nous a amené à faire varier systématiquement ce facteur, toutes autres choses égales par ailleurs, comme cela a été fait pour les purines et les acides aminés[1,2].

Les composés soumis à la chromatographie possèdent tous le noyau "ptérine" (2-amino-4-oxy-ptéridine); ce sont: ce composé lui-même, l'acide ptérine-6-carboxylique, la leucoptérine, la xanthoptérine, l'isoxanthoptérine, l'érythroptérine et la sépiaptérine. Les cinq premières ptérines sont des produits de synthèse aimablement fournis par Mr le professeur VISCONTINI, les deux dernières proviennent des ailes du papillon *Colias croceus* où elles ont été identifiées précédemment[3,4]. La quantité de substance mise en oeuvre est de l'ordre du $\mu$g. La dissolution des substances est effectuée dans le méthanol–pyridine–eau (4:1:5). Le système tampon employé est obtenu par mélange d'acide acétique et d'ammoniaque 0.1 $N$ en proportions variables ajustées pour obtenir des pH variant de 3 à 11 toutes les demi-unités; il est controlé à nouveau après chromatographie. Il n'est pas effectué d'équilibrage du papier mais le solvant est versé plusieurs heures à l'avance dans les cuves afin de saturer l'atmosphère; les chromatographies sont effectuées simultanément à une température de 22–23°. Le parcours du front est de 150 mm. L'expérience est répétée cinq fois successives et l'étude porte sur la moyenne des résultats obtenus. Les courbes représentant les variations du $R_F$ en fonction du pH sont données sur Fig. 1.

Toutes les courbes présentent au moins un minimum et varient très légèrement aux faibles pH, alors qu'aux pH alcalins nous observons une brusque montée des valeurs de $R_F$. Ceux-ci tendent finalement à ne plus être différenciés suivant les ptérines. L'acide ptérine-6-caboxylique se comporte différemment: de pH 3 à pH 4.5 le $R_F$ augmente rapidement pour tendre à se stabiliser aux pH moyens puis, comme chez les autres substances, augmente de nouveau très vite à pH élevé.

Les variations mineures relevées dans les courbes et qui semblent ne pas provenir d'un phénomène aléatoire sont d'une interprétation difficile. Les inflexions nettes pour l'isoxanthoptérine, la xanthoptérine, la 2-amino-4-oxy-ptéridine paraissent coïncider avec l'emplacement des p$K$[5] quand ceux-ci sont situés dans les domaines moyens du pH.

A chaque ptérine est associée une courbe bien définie; en conséquence l'étude conjointe des courbes d'une ptérine de référence et d'une ptérine connue qui lui est supposée identique fournit un élément d'identification de cette substance, à condition toutefois que la chromatographie de ces deux composés soit effectuée sur le même papier avec au moins deux spots de chacune et un spot du mélange. Nous avons en effet observé de légères variations de $R_F$ d'une feuille de papier à l'autre. Ce critère est nettement supérieur à la simple mesure du $R_F$ dans un solvant donné ou même dans une série limitée de solvants. Ce test peut être associé aux données spectrophotométriques et à l'électrophorèse à pH variables.

D'autre part à certains pH les courbes sont plus ou moins rapprochées les unes

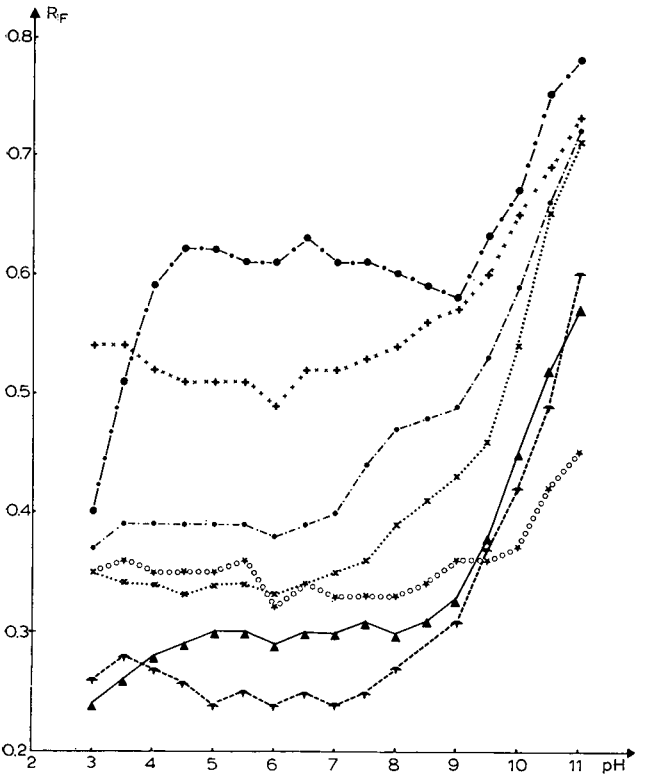


Fig. 1. Variations du $R_F$ de sept ptérines chromatographiées sur papier Whatman No. 1 en fonction du pH du solvant (acétate d'ammonium 0.1 *N*). (●–●–●) Acide ptérine-6-carboxylique; (+ + +) 2-amino-4-oxy-ptéridine; (·–·–·–) xanthoptérine; (○ ○ ○ ○) sépiaptérine; (· · ·) isoxanthoptérine; (– – – –) leucoptérine; (———) érythroptérine.

des autres, donc les ptérines plus ou mois bien séparées, et d'autre part la variation du $R_F$ y est plus ou moins forte. Cette méthode permet par conséquent de choisir le pH auquel un solvant donne la séparation la meilleure et la plus reproductible.

Ces deux applications nous semblent susceptibles d'être dans certains cas utiles à l'étude des composés ptériniques.

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## Preservation of flat acrylamide electrophoresis gels

Workers in the field of starch gel electrophoresis have frequently wished to preserve their original gels even though it is possible to take good photographs of the results. In 1963 DANGERFIELD AND FAULKNER[1] and BAUR[2] independently described techniques for preservation and these were subsequently modified by them[3,4]. We have found that the procedure so described has worked well and has been useful in the classroom as well as in the research laboratory. Nevertheless the procedure appears not to have come into general use. With the advent of acrylamide electrophoresis where flat layers of this material have been used to replace the starch, often in the same equipment, the problem has again arisen and we have found that the DANGERFIELD–BAUR technique is equally valuable. However, as some minor modifications are required to ensure success we are describing the new procedure.

The stained and washed gel is submerged in an aqueous solution containing 10–15% glycerol and about 5% acetic acid; the glycerol was originally added to render the starch transparent but we find a better result is obtained even though the acrylamide is initially clear probably due to the nature of the dialysis membrane used in the procedure. Two sheets of Visking dialysis membrane, about 4 cm longer and wider than the gel, are also submerged in this solution and the whole left for 1–2 h. Five sheets of filter paper, similar in dimensions to the Visking membrane, are laid flat, one membrane sheet is removed, blotted and placed on the paper sheets. The gel is then placed centrally on the membrane leaving about 2 cm of membrane exposed all round the gel. The second membrane sheet is placed on top of the gel, all air bubbles are gently brushed out and a further five sheets of paper placed on top. The whole is clamped along the edges with long-jawed spring or bulldog clips ensuring that the longer dimension is held firm. Diffusion occurs rapidly through the membrane to the surface of the paper and evaporation then takes place; this can be hastened by blowing air from a cold fan over the horizontal sandwich and, by turning it over from time to time, completely even drying results in about 24–48 h. The membrane appears to be available in different thicknesses in different countries and the thinner membrane works more efficiently and rapidly. The dried gel, still held within the membrane sheets, remains as a thin rigid sheet which can be stored indefinitely although it tends to curl when left out in an atmosphere of changing humidity.

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## Electrophoretic separation of the reduction products of the S-sulphonated insulin chains*

Splitting of insulin into its component chains is usually carried out by sulphitolytic cleavage of the —S—S— bonds in the presence of sodium tetrathionate. For recombination studies[1,2] or for experiments on the biological behaviour of the separated chains[3], their S-sulpho derivatives [$A(S\cdot SO_3^-)_4$ and $B(S\cdot SO_3^-)_2$, where A and B indicate respectively glycyl and phenylalanyl chains] are further converted into the reactive sulphydryl form by treatment with an excess of a thiol, usually mercaptoethanol or thioglycolic acid. If the reduction is incomplete, intermediate derivatives containing both $—S\cdot SO_3^-$ and —SH groups are likely to be present in the reaction mixture. From the S-sulpho-A chain one may expect three possible intermediates: $A(S\cdot SO_3^-)_3SH$, $A(S\cdot SO_3^-)_2(SH)_2$ and $A(S\cdot SO_3^-)(SH)_3$; and from the S-sulpho-B chain: $B(S\cdot SO_3^-)(SH)$. Furthermore, the completely reduced forms, $A(SH)_4$ and $B(SH)_2$ will also be present among the reaction products.

The identification of the completely S-sulphonated A and B chains is usually carried out by paper electrophoresis in a 8 *M* urea–3.3 *M* acetic acid buffer, pH 3.2. We have found that the intermediate products can be successfully separated using the same procedure; therefore this reliable and simple tool can be used not only to check the purity of the reduced forms of the chains but also to study the kinetics of the reduction reaction.

The experimental work was carried out starting from $^{125}I$-labelled completely S-sulphonated A and B chains which were prepared from $^{125}I$-insulin according to the procedure given by PRUITT and associates[3].

The incomplete reduction was carried out by dissolving 2.5 mg of each chain in 0.5 ml of a solution of 8 *M* urea and freshly distilled thioglycolic acid ( 1 *M*), keeping the reaction mixture at 25° for 60 min. Electrophoresis was carried out in a small chamber (Elvi model No. 69) in which the paper strip is placed horizontally; the support is Whatman 3MM paper. The buffer was prepared by mixing 800 ml of 10 *M* urea (in deionized water) and 200 ml of glacial acetic acid, to give a final pH 3.2.

Runs were made at a potential gradient of about 7 V/cm and at a current of 1.5 mA. After 16 h running time, the strips were removed, dried at 60° and developed by autoradiography (Ferrania 3N films).

The separation and identification of the reduction products resulting from the reaction between $^{125}I$-labelled completely S-sulphonated A chain and thioglycolic acid was carried out as follows: 25 $\mu$l samples, taken from the reaction mixture, were analyzed by electrophoresis; autoradiography of the paper strips revealed five spots, all but one migrating towards the anode. A distinct tailing effect was observed when the runs were carried out at room temperature.

When electrophoresis was carried out in a cooled chamber at +4°, no tailing was observed and five symmetrical and very well separated spots were found (Fig. 1a). The faster moving component (spot No. 1 in Fig. 1a) is the completely sulphonated A chain. The identity of the remaining spots could be deduced by evaluating the total charge at pH 3.2 of each of the possible components[4].

The A chain contains one terminal amino group (charge + 1 unit), two glutamic

* Work carried out under Euratom contract 048-65-1 BIOI.

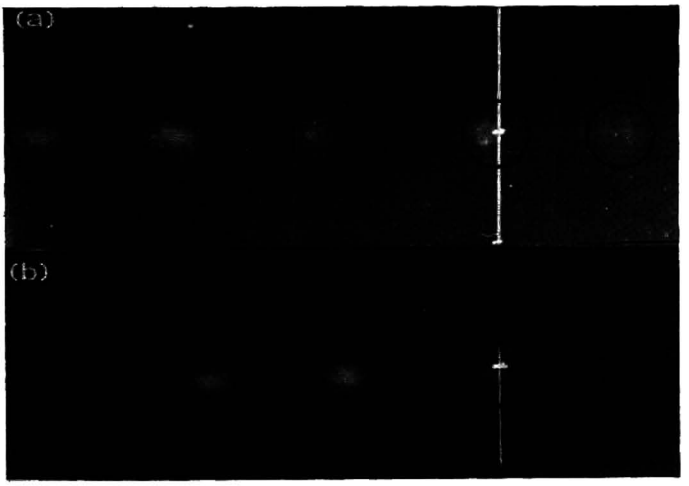


Fig. 1. Electrophoretic patterns (autoradiography) of the reduction products of the S-sulphonated A chain. (a) = Reduction of $A(S \cdot SO_3)_4$ labelled with $^{125}I$; (b) = reduction of $A(^{35}S \cdot SO_3)_4$. For designations see Table I. SP = Starting point.

acid residues ($pK = 4$, charge negligible), one terminal carboxyl group ($pK = 3$, charge $-1/2$ unit) and four S-sulphonated groups (charge of $-4$ units). Therefore the charge of the completely sulphonated A chain at pH 3.2 is evaluated as $-3\,1/2$ units; the reduced components will have lower negative charge values: $(A(S \cdot SO_3^-)_3 \cdot SH = -2\,1/2$; $A(S \cdot SO_3^-)_2(SH)_2 = -1\,1/2$; $A(S \cdot SO_3^-)(SH)_3 = -1/2$; or a positive value: $A(SH)_4 = +1/2$). However the identity of each spot was confirmed by double-tracer experiments. The S-sulpho A chain, labelled with $^{125}I$ and $^{35}S$ was prepared by the sulphitolytic cleavage of $^{125}I$-labelled insulin using $^{35}S$-labelled sodium sulphite. The resulting completely S-sulphonated chains were therefore labelled with $^{35}S$ in the $S \cdot SO_3^-$ group (*i.e.* $^{35}S \cdot SO_3^-$). The double-labelled A chain was reduced with thioglycolic acid and a sample of the reaction mixture was submitted to paper electrophoresis. The $^{125}I$ activity of each spot was directly measured on the paper strip by means of a $\gamma$-ray spectrometer. The strip was then cut in small pieces, each corresponding to a spot; each piece of paper was introduced into a glass vial together with 1 ml of 1 *M* sodium sulphite solution at pH 7; the vials were sealed, heated at 80° in a water bath for 1–2 h, then cooled at +4° and opened. The solution was transferred to a small beaker, diluted to 10 ml, and oxidation to sulphate was carried out with 1 *N* iodine. Sulphate was precipitated as the barium salt and counted in the form of a "thick layer", in a gas-flow counter. Usually no contamination of the samples by $^{125}I$ was found; however, all samples were checked by $\gamma$-ray counting.

From the $^{125}I$ activity measurements the relative amount of A chain in each spot was calculated; taking as a standard of known composition the spot of fully S-sulphonated A chain, the $^{35}S$ activity corresponding to four $-S \cdot SO_3^-$ groups per chain unit was calculated and used to evaluate the number of $-S \cdot SO_3^-$ groups per unit chain in each spot. The results, which are summarized in Table I, show a good agreement with the expected values, confirming the identity of each spot.

No $^{35}S$ activity was found in the spot corresponding to the component moving

TABLE I

NUMBER OF $-S\cdot SO_3^-$ GROUPS CONTAINED PER CHAIN UNIT IN EACH SPOT OF FIG. 1, AS EVALUATED BY DOUBLETRACER ($^{125}I$ AND $^{35}S$) EXPERIMENTS

| *Spot No.** | | 1 | 2 | 3 | 4 | 5 |
|---|---|---|---|---|---|---|
| Composition** | | $A(SSO_3)_4$ | $A(SSO_3)_3SH$ | $A(SSO_3)_2(SH)_2$ | $A(SSO_3)(SH)_3$ | $A(SH)_4$ |
| $-S\cdot SO_3$ groups per chain unit | expected*** | 4 | 3 | 2 | 1 | 0 |
| | found | — | 2.91 | 2.10 | 0.95 | 0.01 |

* The numbers 1–5 refer to Fig. 1a.
** The composition is deduced on the basis of the calculation of the total charge carried by each component (see text).
*** $-S\cdot SO_3$ groups per unit chain as can be expected from the composition given above.

towards the cathode (spot No. 5 in Fig. 1a), which therefore corresponds to the completely reduced form of the A chain ($A(SH)_4$).

The incomplete reduction of the $^{125}I$-labelled and completely sulphonated B chain gave three electrophoretic components as shown in Fig. 2a. Here again the identity of each spot could be deduced by the calculation of the total charge carried by the different components[4].

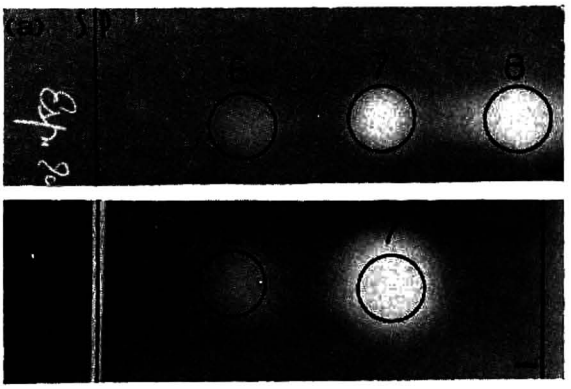

Fig. 2. Electrophoretic patterns (autoradiography) of the reduction products of the S-sulphonated B chain. (a) = Reduction of $B(S\cdot SO_3)_2$ labelled with $^{125}I$; (b) = reduction of $B(^{35}S\cdot SO_3)_2$. SP = Starting point.

The completely S-sulphonated B chain contains one terminal amino group, one lysine residue, one arginine residue, two histidine residues (+5 charge units), one terminal carboxyl group (charge $-1/2$ unit) and two S-sulphonate groups (charge of −2 units). The charge of the S-sulpho B chain is therefore $+2\frac{1}{2}$ units (spot No. 6 in Fig. 2). The reduced components will have higher positive charge values. However, the faster moving spot (spot No. 8 in Fig. 2a) disappeared when the reduction experiment was repeated starting from $^{35}S$-labelled completely S-sulphonated B chain; it therefore corresponds to the completely reduced B chain ($B(SH)_2$) which is unlabelled in the $^{35}S$ experiments.

Using as starting material S-sulphonated B chain labelled with both $^{125}I$ and $^{35}S$, it was confirmed that the intermediate spot of Fig. 2 (spot No. 7) corresponds to a component containing one $—S \cdot SO_3^-$ group per chain unit.

The possibility that oxidative reactions of the —SH groups involving polymerization of the components might occur during the electrophoresis was checked. The relative mobilities of the different components, as evaluated by taking the spots of the completely S-sulphonated chains as a mobility standard, were found to be unchanged when electrophoresis was carried out in a 8 *M* urea, 3.3 *M* acetic acid buffer containing 0.1 *M* thioglycolic acid to prevent oxidation[4].

Further confirmation that oxidation occurs to no appreciable extent during electrophoresis was given by experiments in which the reaction products, formed by separately reducing $^{125}I$-labelled S-sulphonated A and B chains, were mixed and samples of the mixture were analyzed. It was found that all components of the mixture showed a relative mobility very close to that which they showed when the derivatives of the A chain and the B chain were analyzed separately. Furthermore, the relative amount of each component was found to be in excellent agreement with that predicted on the basis of the separate analysis of the original reaction mixtures; it is likely that polymer formation would have resulted in a poor reproducibility. Here again the temperature at which the electrophoresis is carried out was found to be a critical factor. At room temperature tailing effects were observed, whereas at 4° an excellent resolution of the different components was obtained.

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# News

## Apparatus

A new *cellulose-coated sheet for partition thin-layer chromatography and electrophoresis* has been announced by Distillation Products Industries, a division of Eastman Kodak Company.

The new sheet is available in two forms: Eastman Chromagram Sheet 6064 (cellulose without fluorescent indicator) and Eastman Chromagram Sheet 6065 (cellulose with fluorescent indicator).

Features of the new sheet include a sorbent layer of approximately 160 $\mu$ thickness pre-coated on a poly(ethylene terephthalate) base. The sheet's thick coating allows a large sample load to be applied for separation.

Special processing by Kodak results in a highly purified crystalline cellulose essentially free of the gums and resins usually associated with other celluloses. In addition, excellent layer adhesion is obtained without a binder.

This new binderless cellulose sheet offers improved resolution, the result of Kodak's purification, assuring proper crystallinity and proper particle size within the precision applied coating.

Since the sheet contains neither an organic nor an inorganic binder, solvents and visualization reagents used in cellulose chromatography may be applied without restriction. Pre-equilibration is not required and impurity removal by premigration is unnecessary.

In addition to conventional TLC methods including two dimensional, the new Chromagram Sheet may be used for two-dimensional separations by running chromatography in one direction and electrophoresis in the other. When used for TLC, the cellulose Chromagram Sheet may be developed in the Eastman Chromagram Developing Apparatus which enhances reproducibility of results by ensuring repeatable solvent vapor saturation.

In addition to the two new forms announced, the Eastman Chromagram system for TLC includes a silica gel sheet with and without indicator, an alumina sheet without indicator, and new alumina sheet with indicator: Eastman Chromagram Sheet 6063, which has just been introduced.

The Baird & Tatlock Group of Companies, a Division of Derbyshire Stone Ltd., exhibited on Stand No. B6 at LABEX International (International Laboratory Apparatus and Materials Exhibition), which took place at Earl's Court, London, from 13th to 17th March, 1967.

The products of Baird & Tatlock (London) Ltd., Hopkin & Williams Ltd., W. B. Nicolson (Scientific Instruments) Ltd., Optica United Kingdom Ltd., C. F. Palmer (London) Ltd., George T. Gurr Ltd., and L.T.E. Ltd. were shown.

The outstanding new items of Baird & Tatlock (London) Ltd. are the *"Chromafrac" Fraction Collector, Chromapump, Chromatimer* and the *"Dilutronic" Dilutor,* and technical details and photographs of these were made available just prior to the exhibition.

The Baird & Tatlock (B.T.L.) Fraction Collecting System provides a versatile means of dealing with fraction cutting for column chromatography. In particular, the system offers a completely novel method whereby fractions are effectively cut from the inlet side of the column as opposed to the outlet side, which is normally the case with conventional techniques.

The system consists of three basic instruments: the B.T.L. "Chromafrac", the B.T.L. "Chromapump" and the B.T.L. "Chromatimer".

The B.T.L. "Chromafrac" is a rectangular fraction collector with a capacity of 200 tubes held in 10 numbered racks. A stainless steel water bath can be fitted so that the receiver tubes can be maintained at temperatures below ambient by circulation of liquid from an external controller.

The B.T.L. "Chromapump" is essentially a peristaltic pump which can be used to control the rate of eluent through a column and will trigger the dispenser advance mechanism on the B.T.L. "Chromafrac" at intervals corresponding to selected numbers of pump revolutions. This provides a simple method of determining fraction size without recourse to volumetric, gravimetric or drop counting methods with all their inherent faults, and also enables very small fractions to be cut with greatly improved accuracy.

The B.T.L. "Chromatimer" enables fractions to be cut at selected time intervals and when connected to the B.T.L. "Chromafrac", triggers the tube dispenser advance mechanism.

Varian Aerograph demonstrated the first of the gas chromatographs manufactured in the United Kingdom. Specially featured were the *electronic digital integrator*, Model 475, and the *universal analytical preparative unit*, Model 1525.

A new *continuous-monitoring liquid chromatograph*, equipped with a moving-wire transport system and an argon ionization detector, has been introduced by W. G. Pye & Co., Ltd., Cambridge, England. This detector system can be used with almost any type of separation column, it detects all organic compounds that either vapourize or can be pyrolysed at temperatures of 700° or less, and it monitors the eluate from the column continuously and almost instantaneously, without any attention from the operator after injection of the sample.

The instrument can be used for the analysis of most organic materials, including clinical samples, biochemical and pharmaceutical substances, plastics, petroleum products, pesticides, dyestuffs, food products, essential oils, and factory effluents. It is highly sensitive, being capable of detecting many compounds at concentrations of 1 $\mu$g/ml (approximately 1 p.p.m.). When it is used for quantitative estimation, the results are reproducible with a coefficient of variation of 3 %.

The chromatograph was exhibited for the first time at LABEX International (International Laboratory Apparatus and Materials Exhibition) at Earl's Court, London, from March 13th–17th, 1967, and from March 14th–17th at the Effluent and Water Treatment Exhibition in London. It will also be exhibited during 1967 at Mesucora—Paris, Achema—Frankfurt, FEBS—Oslo, IUPAC—Prague, Het Instrument—Utrecht, MAC—Milan, and possibly at the Pittsburgh (U.S.A.) Convention.

AUTOMATIC GAS CHROMATOGRAPH FOR LABORATORY USE

The first automated gas chromatograph for laboratory use was introduced by Barber-Colman at the 1966 Pittsburgh Conference on Analytical Chemistry and Applied Spectroscopy. The instrument is capable of analyzing as many as 72 successive samples before reloading. Samples are loaded into an automatic injector in a matter of minutes. Operating conditions are then selected for isothermal or programmed separations and the start button is pushed. The instrument then operates unattended until all loaded samples have been run and the next batch of samples is ready.

Successive isothermal analyses are performed on the basis of a selected and precisely reproducible time between injections. Control of programmed separations is more sophisticated, but easily understandable and selectable. A gearless electronic programmer provides excellent reproducibility of column temperature programming. Oven cool-down time is minimized by a highly efficient oven damper system. Provisions are made for thorough re-equilibration of column temperatures before the next sample is injected, and injection is synchronized exactly with the start of the next program cycle. The automated system is available in a variety of options, and the automatic injector and other automating modules can be added to existing Series 5000 units.

The basic approach of transferring solutes from a solution to a gauze, then injection into a chromatograph as a concentrated, dried residue, has been authoritatively reported with results indicating an acceptable degree of precision and reliability. An automatic gauze injection system is being used routinely by a Texas hospital, where they also report excellent reproducibility of results.

Time is saved in sample preparation and in each chromatographic separation. Sample concentration is an essential step in many procedures, particularly for pesticides and steroids. In such applications, much time can be saved by evaporation on the gauze.

## New Chemicals

Phase Separations Ltd., 406 New Chester Road, Rock Ferry, Cheshire, Great Britain, have now available a comprehensive selection of high-purity reference and calibration standards suitable for gas and thin-layer chromatography. The lists include hydrocarbons, lipids, mono-, di- and triglycerides, phenols, mercaptans, steroids, etc., and many rare compounds.

A new reagent Bis-Dimethylsilylacetamide (BDSA) has just been made available by Supelco, Inc. of Bellefonte, Pa. BDSA is useful for the preparation of dimethylsilyl ether derivatives of carbohydrates, phenols, sterols, fatty acids, alcohols and other hydroxy compounds. The dimethylsilyl derivatives are more volatile than the corresponding trimethylsilyl derivative and consequently can be analyzed more rapidly by gas chromatography. The use of more volatile derivatives should also permit analysis of compounds which could not be analyzed previously either because of thermal instability or chromatographic limitations. Literature is available.

A series of quantitative mixtures of fatty acids has just been made available by Supelco, Inc., P.O. Box 581, 145 S. Water Street, Bellefonte, Pa. 16823, U.S.A. The mixtures have been prepared so that the composition resembles the natural distribution of fatty acid esters in various natural oils as specified by the AOCS tentative method Ce-1-62. Standards are available for a wide variety of oils including linseed, cottonseed, olive, neatsfoot, palm kernel, safflower, lard, and others.

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## Gas Chromatography

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## 4. TECHNIQUES II

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JOURNAL OF CHROMATOGRAPHY VOL. 28 (1967)

## AUTHOR INDEX

JOURNAL OF CHROMATOGRAPHY VOL. 28 (1967)

# SUBJECT INDEX

# CHROMATOGRAPHIC DATA

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TABLE 1

PC $R_F$ VALUES OF SOME ALIPHATIC POLYAMINES

(J. BORECKÝ, *Mikrochim. Acta*, (1966) 283)

Paper: Whatman No. 3.
Solvents: $S_1$ = Methanol–conc. hydrochloric acid (1:1).
$S_2$ = Ethanol–conc. hydrochloric acid (1:1).
$S_3$ = *n*-Propanol–conc. hydrochloric acid (2:1).
$S_4$ = *n*-Butanol–conc. hydrochloric acid (4:1).
Detection: Ninhydrin with pyridine.

| *Compound* | $R_F$ | | | | *Colour* |
|---|---|---|---|---|---|
| | $S_1$ | $S_2$ | $S_3$ | $S_4$ | |
| Triethylenetetramine | 0.42 | 0.32 | 0.06 | 0.01 | brown-blue |
| Diethylenetriamine | 0.45 | 0.36 | 0.12 | 0.02 | brown-blue |
| Dipropylenetriamine | 0.57 | 0.48 | 0.15 | 0.02 | dark grey-blue |
| Ethylenediamine | 0.51 | 0.44 | 0.20 | 0.05 | grey-blue |
| Trimethylenediamine | 0.58 | 0.49 | 0.23 | 0.06 | brown-violet |
| Tetramethylenediamine | 0.66 | 0.59 | 0.29 | 0.08 | blue-violet |
| Pentamethylenediamine | 0.72 | 0.66 | 0.36 | 0.10 | blue-violet |
| Hexamethylenediamine | 0.76 | 0.73 | 0.42 | 0.13 | dark blue-violet |
| N,N-Diethylethylenediamine | 0.78 | 0.77 | 0.49 | 0.21 | green-brown |
| Methylamine | 0.75 | 0.72 | 0.50 | 0.31 | grey-blue-violet |
| Ethylamine | 0.82 | 0.78 | 0.63 | 0.47 | grey-blue-violet |
| *n*-Propylamine | 0.85 | 0.83 | 0.75 | 0.61 | grey-blue-violet |
| *n*-Butylamine | 0.88 | 0.86 | 0.85 | 0.73 | grey-blue-violet |
| Monoethanolamine | 0.75 | 0.67 | 0.45 | 0.25 | blue |
| 1-Chloro-2-aminoethane | 0.80 | 0.74 | 0.59 | 0.45 | blue-brown |
| Isopropanolamine | 0.82 | 0.73 | 0.55 | 0.35 | blue |
| 3-Aminopropanol | 0.82 | 0.78 | 0.56 | 0.35 | blue |
| N-(2-Hydroxyethyl)-ethylenediamine | 0.64 | 0.60 | 0.26 | 0.08 | brown-grey-blue |

TABLE 2

PC $R_F$ VALUES OF SOME AMINOALCOHOLS

(J. BORECKÝ, *Mikrochim. Acta*, (1966) 284)

Paper: Ederol 208.
Solvent: Ethyl acetate–dimethylformamide (2:1).
Detection: 0.1 % solution of alizarine in ethanol.

| *Compound* | $R_F$ |
|---|---|
| Monoethanolamine | 0.17 |
| Diethanolamine | 0.38 |
| Triethanolamine | 0.67 |
| 3-Aminopropanol | 0.19 |
| Monoisopropanolamine | 0.31 |
| Diisopropanolamine | 0.72 |
| Triisopropanolamine | at front |
| N-Methyldiethanolamine | 0.70 |
| N-(2-Hydroxyethyl)-ethylenediamine | 0.05 |

TABLE 3

TLC $R_F$ VALUES OF SOME LOW MOLECULAR WEIGHT POLYETHYLENE GLYCOLS

(I. A. VAKHTINA, P. A. OKUNEV AND O. G. TARAKANOV, *Zh. Analit. Khim.*, 21 (1966) 631)

Thin layer: Aluminium oxide, loose layer without binder, activity I.
Solvent: Chloroform–ethanol (92:8).
Detection: Iodine vapours.

| *Compound* | $R_F$ |
|---|---|
| Ethylene glycol | 0.11 |
| Diethylene glycol | 0.23 |
| Triethylene glycol | 0.30 |
| Polyethylene glycol mol. wt. 200 | 0.52 |
| Polyethylene glycol mol. wt. 300 | 0.60 |
| Polyethylene glycol mol. wt. 600 | 0.68 |
| Polyethylene glycol mol. wt. 1000 | 0.74 |
| Polyethylene glycol mol. wt. 2000 | 0.80 |

TABLE 4

TLC $R_F$ VALUES OF SOME NITROGEN DERIVATIVES OF CARBONIC ACID

(E. KNAPPE AND I. ROHDEWALD, *Z. Anal. Chem.*, 223 (1966) 176)

Thin layers: $TL_1$ = MN Cellulose powder 300.
$TL_2$ = MN Silica Gel N-HR.

Solvents: $S_1$ = Methanol–3 *N* ammonia (60:75).
$S_2$ = Acetonitrile–petrol ether–carbon tetrachloride–tetrahydrofuran–water–formic acid (80:10:10:10:10:4).

Detection: $D_1$ = *p*-Dimethylaminobenzaldehyde.
$D_2$ = Silver nitrate. The chromatogram is sprayed with a saturated solution of silver nitrate in acetone, then heated to 105° for 15 min and while still hot sprayed with 1 % ammonia solution.
$D_3$ = Ferricyanide with nitroprusside. Equal volumes of 10 % aqueous solutions of potassium ferricyanide, sodium nitroprusside and sodium hydroxide are mixed and diluted three times with water. After 15–20 min the colour of the solution turns to yellow and this solution is used for spraying the chromatogram.
$D_4$ = Sakaguchi reagent. The chromatograms are sprayed with 0.1 % solution of 1-naphthol in 0.1 *N* sodium hydroxide and after several minutes with a solution of 2 ml bromine in 5 % sodium hydroxide.

| *Compound* | $R_F$ | |
|---|---|---|
| | $TL_1S_1$ | $TL_2S_2$ |
| Urea | 0.78 | 0.46 |
| Biuret | 0.68 | 0.57 |
| Guanidine | streak | 0.28 |
| Cyanamide | 0.86 | 0.80 |
| Dicyandiamide | 0.77 | 0.63 |
| Dicyandiamidine | 0.59 | 0.26 |
| Diguanylmelamine | 0.16 | 0.22 |
| Guanylmelamine | 0.30 | 0.22 |
| Melamine | 0.34 | 0.22 |
| Ammeline | 0.57 | 0.22 |
| Ammelide | 0.71 | 0.22 |
| Cyanuric acid | 0.74 | — |
| Benzoguanamine | 0.52 | 0.59 |

TABLE 5

TLC $R_F$ VALUES OF SOME ALKYL- AND ARYL-SUBSTITUTED UREA COMPOUNDS

(E. KNAPPE AND I. ROHDEWALD, *Z. Anal. Chem.*, 217 (1966) 111)

Thin layer: $TL_1$ = MN Silica Gel N-HR.
$TL_2$ = MN Polyamide-Ac.
Solvents: $S_1$ = Carbon tetrachloride–dichloromethane–ethyl acetate–formic acid (70:50:15:10).
$S_2$ = Carbon tetrachloride–ethyl acetate–ethanol (100:5:2).
Detection: *p*-Dimethylaminobenzaldehyde (1% solution in 95 parts of ethanol and 5 parts conc. hydrochloric acid).

| *Compound* | $R_F$ | |
|---|---|---|
| | $TL_1S_1$ | $TL_2S_2$ |
| Urea | 0.10 | 0.10 |
| Methylurea | 0.15 | 0.18 |
| N,N'-Dimethylurea (sym.) | 0.16 | 0.21 |
| Ethylurea | 0.23 | 0.24 |
| N,N-Dimethylurea (asym.) | 0.28 | 0.49 |
| Ethyleneurea | 0.31 | 0.62 |
| Benzylurea | 0.37 | 0.24 |
| Phenylurea | 0.42 | 0.15 |
| *p*-Tolylurea | 0.58 | 0.20 |
| *m*-Tolylurea | 0.59 | 0.20 |
| *o*-Tolylurea | 0.65 | 0.39 |

TABLE 6

PC $R_F$ VALUES OF HIGHER FATTY ACIDS

(C. FAULI AND A. DEL POZO, *Galenica Acta* (*Madrid*), 18 (1965) 11–23; *C.A.*, 64 (1966) 19322a)

Paper: Whatman No. 1.
Impregnation: 5% liquid paraffin in petroleum ether.
Technique: Ascending.
Solvents: $S_1$ = Butanol–acetic acid–water (4:2:1).
$S_2$ = Ethyl acetate–ethanol–water (3:3:3).
$S_3$ = Acetic acid–water (9:1).
$S_4$ = Methanol–benzene–water (7:2:1).
$S_5$ = Petroleum ether–butanol–water (4:4:4).
$S_6$ = Petroleum ether–methanol–water (4:4:4).
$S_7$ = Ethanol–water–conc. ammonia (35:13:2).
$S_8$ = Methanol–acetone (3:1).
$S_9$ = Carbon tetrachloride–methanol–conc. ammonia (81:81:1).
$S_{10}$ = Isopropanol–ethanol–acetic acid–water (8:2.5:4:1.25).
Detection: $Cu(OAc)_2$ and rubeanic acid.

| *Acid* | $R_F \times 100$ | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | $S_1$ | $S_2$ | $S_3$ | $S_4$ | $S_5$ | $S_6$ | $S_7$ | $S_8$ | $S_9$ | $S_{10}$ |
| Caproic | 86 | 94 | 80 | 85 | 88 | 98 | 90 | 90 | 85 | 89 |
| Lauric | 85 | 94 | 66 | 67 | 87 | 97 | 88 | 84 | 77 | 87 |
| Myristic | 84 | 87 | 47 | 60 | 85 | 95 | 87 | 76 | 68 | 85 |
| Palmitic | 81 | 77 | 35 | 41 | 82 | 92 | 85 | 73 | 64 | 82 |
| Stearic | 76 | 65 | 22 | 28 | 82 | 92 | 85 | 69 | 45 | 75 |
| Oleic | 79 | 73 | 32 | 46 | 86 | 96 | 88 | 71 | 52 | 80 |

TABLE 7

TLC $R_F$ VALUES OF GLUCOSE ETHERS

(E. R. RUCKEL AND C. SCHUERCH, *J. Org. Chem.*, 31 (1966) 2233)

Thin layer: Kieselguhr G.
Solvent: Benzene–methanol–ammonium hydroxide (80:20:0.3).

| *Compound* | $R_F$ |
|---|---|
| Methyl 2,3,4-tri-O-methyl-α-D-glucopyranoside | 0.46 |
| Methyl 2,3,4-tri-O-methyl-β-D-glucopyranoside | 0.51 |
| Methyl 2,3,4-tri-O-ethyl-α-D-glucopyranoside | 0.53 |
| Methyl 2,3,4-tri-O-ethyl-β-D-glucopyranoside | 0.57 |
| 1,6-Anhydro-2,3,4-tri-O-methyl-β-D-glucopyranose | 0.58 |

TABLE 8

PC $R_F$ VALUES OF SOME SUGAR ACIDS AND LACTONES

(J. HICKMAN AND G. ASHWELL, *J. Biol. Chem.*, 241 (1966) 1426)

Paper: Whatman No. 1.
Solvents: $S_1$ = Pyridine–ethyl acetate–water (20:72:23).
$S_2$ = Pyridine–ethyl acetate–acetic acid–water (5:5:1:3).
$S_3$ = Ethyl acetate–acetic acid–water (3:1:3).
Detection: Alkaline silver nitrate.

| *Compound* | $R_{Gl}$* | | |
|---|---|---|---|
| | $S_1$ | $S_2$ | $S_3$ |
| Glucuronic acid | 0.00 | 0.42 | 0.46 |
| Galacturonic acid | 0.00 | 0.33 | 0.85 |
| Tagaturonic acid | 0.00 | 0.59 | 0.62 |
| Fructuronic acid | 0.00 | 0.64 | 0.62 |
| Glucuronolactone | | 1.10 | 1.25 |
| Galactonolactone | 1.00 | 1.00 | 1.00 |
| Mannonolactone | 0.85 | 0.91 | 1.12 |
| Gulonolactone | 0.77 | 0.89 | 0.84 |
| Altronolactone | 1.12 | 1.09 | 1.32 |
| Talonolactone | 1.03 | 0.98 | 1.07 |
| Allonolactone | 1.12 | 1.02 | 1.09 |
| Idonolactone | 1.12 | 1.03 | 1.11 |
| Gluconolactone | 1.77 | 1.08 | 1.22 |

* Relative to galactonolactone.

TABLE 9

HIGH-VOLTAGE THIN-LAYER ELECTROPHORETIC MOBILITIES OF AMINO ACIDS
(S. KATZ AND A. LEWIS, *Anal. Biochem.*, 17 (1966) 306)

Thin layer: Cellulose, binder free (Chromedia CC 41).
Electrolytes: $E_1$ = 0.15 *M* formic acid (pH 2.5).
$E_2$ = 0.01 *M* ammonia and 0.0033 *M* acetic acid (pH 10.2).
Voltage: 4.5 kV.
Duration: 16 min.
Detection: Ninhydrin (according to E. D. MOFFAT AND R. I. LYTLE, *Anal. Chem.*, 31 (1959) 926).

| *Amino acid* | *Relative mobility** | |
|---|---|---|
| | $E_1$ | $E_2$ |
| Glycine | 1.10 | 1.5 |
| Alanine | 1.0 | 1.0 |
| α-Amino-*n*-butyric acid | 0.915 | 1.0 |
| Valine | 0.86 | 1.0 |
| Leucine | 0.86 | 1.0 |
| Isoleucine | 0.86 | 1.0 |
| Serine | 0.815 | 2.15 |
| Methionine | 0.76 | 1.6 |
| Phenylalanine | 0.74 | 1.8 |
| Tyrosine | 0.74 | 2.0 |
| Proline | 0.73 | 0.6 |
| Threonine | 0.73 | 3.5 |
| Asparagine | 0.71 | — |
| Glutamine | 0.69 | 1.65 |
| Tryptophan | 0.67 | 1.0 |
| Cystine | 0.66 | 3.9 |
| Cysteine | 0.63 | 3.6 |
| Diiodotyrosine | 0.46 | 2.4 |
| Aspartic acid | 0.62 | 4.4 |
| Glutamic acid | 0.725 | 4.0 |
| Histidine | 1.775 | +2.7** |
| Ornithine | — | −2.7** |
| Lysine | 1.655 | −5.2** |
| Arginine | 1.60 | −4.5** |

* Mobility relative to that of alanine.
** These mobilities are expressed as distance in cm migrated from point of sample application, samples being applied at the centre of the plate.

TABLE 10

PC $R_F$ VALUES OF α- AND ε-PEPTIDES OF LYSINE

(J. D. PADAYATTY AND H. VAN KLEY, *J. Org. Chem.*, 31 (1966) 1934)

Paper: Whatman No. 1.
Solvent: Butan-1-ol–acetic acid–pyridine–water (30:6:20:24).

| *Compound* | $R_F$ |
|---|---|
| α-Cbz-Lys-Bz·HCl | 0.94 |
| α-Cbz-Gly, ε-Cbz-Lys-Bz | 0.93 |
| α-Cbz-Ala, ε-Cbz-Lys-Bz | 0.93 |
| α-Cbz-Phe, ε-Cbz-Lys-Bz | 0.93 |
| α-Cbz-Leu, ε-Cbz-Lys-Bz | 0.93 |
| α-(α-Cbz-Asp-β-Bz), ε-Cbz-Lys-Bz | 0.93 |
| α-Gly-Lys·HOAc | 0.14 |
| α-Ala-Lys·HOAc | 0.18 |
| α-Phe-Lys·HOAc | 0.39 |
| α-Leu-Lys·HOAc | 0.38 |
| α-(α-Asp)-Lys | 0.09 |
| ε-Gly-Lys·HOAc | 0.18 |
| ε-Ala-Lys·HOAc | 0.21 |
| ε-Phe-Lys·HOAc | 0.42 |
| ε-Leu-Lys·HOAc | 0.40 |
| ε-(α-Asp)-Lys | 0.10 |

TABLE 11

TLC $R_F$ VALUES OF PEPTIDES RELATED TO GLUCAGON

(T. A. HYLTON, J. PRESTON AND B. WEINSTEIN, *J. Org. Chem.*, 31 (1966) 3400)

Thin layer: Silica Gel G.
Solvent: Methanol–chloroform (1:9).

| *Compound* | $R_F$ |
|---|---|
| N-Benzyloxycarbonyl-L-tryptophanyl-L-leucine methyl ester | 0.65 |
| N-Benzyloxycarbonyl-L-glutaminyl-L-tryptophanyl-L-leucine methyl ester | 0.47 |
| N-Benzyloxycarbonyl-L-valyl-L-glutaminyl-L-tryptophanyl-L-leucine methyl ester | 0.20 |
| N-Benzyloxycarbonyl-L-phenylalanyl-L-valyl-L-glutaminyl-L-tryptophanyl-L-leucine methyl ester | 0.37 |
| N-Benzyloxycarbonyl-β-*tert.*-butyl-L-aspartyl-L-phenylalanyl-L-valyl-L-glutaminyl-L-tryptophanyl-L-leucine methyl ester | 0.57 |
| N-Benzyloxycarbonyl-L-glutaminyl-β-*tert.*-butyl-L-aspartyl-L-phenylalanyl-L-valyl-L-glutaminyl-L-tryptophanyl-L-leucine methyl ester | 0.20 |
| N-Benzyloxycarbonyl-β-*tert.*-butyl-L-aspartic acid *p*-nitrophenyl ester | 0.84 |
| N-Benzyloxycarbonyl-β-*tert.*-butyl-L-aspartyl-L-phenylalanine methyl ester | 0.85 |

TABLE 12

TLC $R_F$ VALUES OF PEPTIDES RELATED TO GLUCAGON

(A. A. COSTOPANAGIOTIS, J. PRESTON AND B. WEINSTEIN, *J. Org. Chem.*, 31 (1966) 3398)

Thin layer: Baked Silica Gel G.
Solvent: Methanol–chloroform (1:9).

| *Compound* | $R_F$ |
|---|---|
| N-Benzyloxycarbonyl-O-*tert.*-butyl-L-threonine *tert.*-butyl ester | 0.75 |
| O-*tert.*-Butyl-L-threonine *tert.*-butyl ester | 0.85 |
| N-Benzyloxycarbonyl-L-asparaginyl-O-*tert.*-butyl-L-threonine *tert.*-butyl ester | 0.55 |
| L-Asparaginyl-O-*tert.*-butyl-L-threonine *tert.*-butyl ester | 0.16 |
| N-Benzyloxycarbonyl-L-methionyl-L-asparaginyl-O-*tert.*-butyl-L-threonine *tert.*-butyl ester | 0.53 |
| L-Methionyl-L-asparaginyl-O-*tert.*-butyl-L-threonine *tert.*-butyl ester | 0.53 |
| N-Benzyloxycarbonyl-L-methionyl sulfoxide L-asparaginyl-O-*tert.*-butyl-L-threonine *tert.*-butyl ester | 0.25 |

TABLE 13

PC $R_F$ VALUES OF COMPOUNDS RELATED TO ACTH

(W. OELOFSEN AND CH. H. LI, *J. Am. Chem. Soc.*, 88 (1966) 4254)

Paper: Whatman No. 1.
Solvents: $S_1$ = Butan-1-ol–acetic acid–water (4:1:1).
$S_2$ = Butan-2-ol–10% ammonia (85:15).
$S_3$ = Butan-1-ol–pyridine–acetic acid–water (30:20:6:24).
$S_4$ = Butan-1-ol saturated with 0.1% aqueous ammonia.

| *Compound* | $R_F$ | | | |
|---|---|---|---|---|
| | $S_1$ | $S_2$ | $S_3$ | $S_4$ |
| L-Prolinol | 0.42 | 0.55 | 0.56 | 0.34 |
| N-2,4-Dinitrophenylprolinol | 0.87 | 0.83 | 0.89 | — |
| $N^G$-Tosylarginylprolinol | 0.42 | 0.50 | 0.69 | 0.79 |
| $N^\alpha$-2,4-Dinitrophenyl-$N^G$-tosylarginylprolinol | 0.83 | 0.78 | 0.88 | — |
| $N^\alpha$-Carbobenzoxy-$N^G$-tosylarginylprolinol | 0.80 | 0.78 | 0.88 | 0.83 |
| $N^\alpha$-Carbobenzoxy-$N^G$-tosylarginyl-$N^G$-tosylarginylprolinol | 0.85 | 0.80 | 0.89 | 0.90 |
| $N^G$-Tosylarginyl-$N^G$-tosylarginylprolinol | 0.49 | 0.46 | 0.75 | 0.74 |
| $N^\alpha$-Carbobenzoxy-$N^\varepsilon$-*tert.*-butyloxycarbonyllysyl-$N^G$-tosylarginyl-$N^G$-tosylarginylprolinol | 0.88 | 0.86 | 0.91 | 0.91 |
| $N^\varepsilon$-*tert.*-Butyloxycarbonyllysyl-$N^G$-tosylarginyl-$N^G$-tosylarginylprolinol | 0.68 | 0.75 | 0.85 | 0.84 |
| $N^\alpha$-Carbobenzoxy-$N^\varepsilon$-butyloxycarbonyllysyl-$N^\varepsilon$-*tert.*-butyloxycarbonyllysyl-$N^G$-tosylarginyl-$N^G$-tosylarginylprolinol | 0.94 | 0.90 | 0.93 | 0.92 |
| $N^\varepsilon$-*tert.*-Butyloxycarbonyllysyl-$N^\varepsilon$-*tert.*-butyloxycarbonyllysyl-$N^G$-tosylarginyl-$N^G$-tosylarginylprolinol | 0.82 | 0.83 | 0.92 | 0.89 |
| $N^\alpha$-Carbobenzoxy-$N^\varepsilon$-*tert.*-butyloxycarbonyllysylprolylvalyl-glycyl-$N^\varepsilon$-*tert.*-butyloxycarbonyllysyl-$N^\varepsilon$-*tert.*-butyloxycarbonyllysyl-$N^G$-tosylarginyl-$N^G$-tosylarginylprolinol | 0.91 | 0.90 | 0.94 | — |
| $N^\varepsilon$-*tert.*-Butyloxycarbonyllysylprolylvalylglycyl-$N^\varepsilon$-*tert.*-butyloxycarbonyllysyl-$N^\varepsilon$-*tert.*-butyloxycarbonyllysyl-$N^G$-tosylarginyl-$N^G$-tosylarginylprolinol | 0.84 | 0.91 | 0.93 | — |

TABLE 14

TLC $R_F$ VALUES OF SOME SYNTHETIC STEROIDS

(M. S. MOSS AND H. J. RYLANCE, *J. Pharm. Pharmacol.*, 18 (1966) 16)

Thin layer: Silica Gel $GF_{254}$ Merck.
Solvents: $S_1$ = Ethyl acetate.
$S_2$ = Methylene chloride–dioxane–water (2:1:1).
$S_3$ = Plate impregnated by ascending development with 20% formamide in acetone. After drying development with chloroform–ether–water (160:40:1).
$S_4$ = Ether.
Detection: Tetrazolium reagent and then vanillin–perchloric acid reagent.

| *Steroid* | $R_F$ | | | *Detection* | $R_F$ *of steroid acetate in* $S_4$ |
|---|---|---|---|---|---|
| | $S_1$ | $S_2$ | $S_3$ | | |
| Betamethasone | 0.47 | 0.48 | 0.33 | Grey | 0.44 |
| Hydrocortisone | 0.35 | 0.45 | 0.38 | Purple/brown | 0.42 |
| Cortisone | 0.36 | 0.62 | 0.77 | Red/brown | 0.34 |
| Dexamethasone | 0.47 | 0.45 | 0.34 | Grey/purple | 0.45 |
| Fludrocortisone | 0.49 | 0.46 | 0.29 | Purple/brown | 0.55 |
| 6-Methylhydrocortisone | 0.40 | 0.48 | 0.57 | Red/brown | 0.42 |
| 6-Methylprednisolone | 0.38 | 0.41 | 0.45 | Red/brown | 0.37 |
| Prednisolone | 0.30 | 0.38 | 0.25 | Green/grey | 0.40 |
| Prednisone | 0.30 | 0.55 | 0.60 | Grey/purple | 0.29 |
| Triamcinolone | 0.23 | 0.24 | 0.04 | Light brown | 0.22 |

TABLE 15

TLC $R_F$ VALUES OF SOME CARDIAC GLYCOSIDES

(E. J. JOHNSTON AND A. L. JACOBS, *J. Pharm. Sci.*, 55 (1966) 532)

Thin layer: Silica Gel G.
Solvent: Benzene–95% ethanol (7:3).
Detection: Perchloric acid solution and U.V. light (366 mμ).

| *Compound* | $R_F$ | *Fluorescence* |
|---|---|---|
| Acetyldigitoxin | 0.82 | red |
| Digitoxin | 0.72 | red |
| Digoxin | 0.62 | blue |
| Lanatoside A | 0.52 | red |
| Lanatoside B | 0.41 | red |
| Lanatoside C | 0.36 | blue |
| Desacetyllanatoside C | 0.27 | blue |
| Ouabain | 0.09 | yellow-green |

TABLE 16

TLC $R_F$ VALUES OF PHENOLIC COMPOUNDS

(H.-I. JOSCHEK AND S. I. MILLER, *J. Am. Chem. Soc.*, 88 (1966) 3276)

Thin layers: $TL_1$ = Silica Gel G7731.
$TL_2$ = Polyamide MN.
$TL_3$ = Cellulose powder 300 $GF_{254}$.

Solvents: $S_1$ = Benzene–methanol–acetic acid (70:8:4).
$S_2$ = Benzene–methanol–acetic acid (70:8:4) (2 developments).
$S_3$ = Benzene–methanol–acetic acid (80:1:1).
$S_4$ = Benzene–methanol–acetic acid (80:1:1) (2 developments).
$S_5$ = Benzene–methanol–acetic acid (80:1:1) (3 developments).
$S_6$ = Benzene–methanol–acetic acid (70:8:4).
$S_7$ = Benzene–methanol–acetic acid (70:8:4) (2 developments).
$S_8$ = Benzene–*n*-butyl alcohol–water (9:1:10) (lower phase).

| *Compound* | $R_F$* | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | $TL_1S_1$ | $TL_1S_2$ | $TL_1S_3$ | $TL_1S_4$ | $TL_1S_5$ | $TL_2S_6$ | $TL_2S_7$ | $TL_3S_8$ |
| Phenol | 6.3 | 8.0 | 2.3 | 5.3 | 6.4 | 6.2 | 8.1 | |
| Hydroquinone | 3.2 | 5.0 | 0.2 | 0.6 | 0.8 | 2.3 | 3.6 | 7.9 |
| Resorcinol | 3.8 | 5.8 | 0.2 | 0.8 | | 2.2 | 3.3 | 7.7 |
| Catechol | 4.8 | 7.0 | 0.6 | 1.8 | 2.2 | 3.5 | 5.2 | 8.2 |
| 1,2,3-Trihydroxybenzene | 2.7 | 4.4 | 0.2 | 0.3 | 0.5 | 1.8 | 2.7 | 7.5 |
| 1,2,4-Trihydroxybenzene | 2.0 | 3.0 | 0.0 | 0.1 | 0.0 | 1.4 | 2.2 | |
| 1,3,5-Trihydroxybenzene | 1.7 | 2.7 | 0.0 | 0.0 | 0.0 | 1.0 | 1.7 | 6.3 |
| 2-Hydroxydiphenyl ether | 7.7 | 9.1 | 6.5 | 8.0 | 9.3 | 8.3 | 9.5 | t |
| 3-Hydroxydiphenyl ether | 6.4 | 8.6 | 4.9 | 5.8 | 6.9 | 7.5 | 8.9 | t |
| 4-Hydroxydiphenyl ether | 5.9 | 8.3 | 4.0 | 5.5 | 6.5 | 7.8 | 9.2 | t |
| *o*-Aminophenol | 2.4 | 4.0 | | | | 4.5 | 6.7 | |
| *m*-Aminophenol | 2.3 | 4.4 | | | | 4.3 | 6.2 | |
| *p*-Aminophenol | 0.9 | 1.2 | | | | 2.3t | 3.8 | |
| *o*-Cresol | 6.2 | 9.0 | 3.8 | 5.5 | 7.6 | 6.3 | 8.7 | |
| *m*-Cresol | 5.9 | 8.8 | 3.0 | 5.0 | 6.5 | 5.7 | 8.5 | |
| *p*-Cresol | 6.0 | 8.6 | 2.6 | 4.6 | 6.1 | 5.8 | 8.5 | |
| 2,3-Dihydroxytoluene | 5.1 | 7.8 | 1.1 | 2.5 | | 4.4 | 6.5 | 7.5 |
| 2,5-Dihydroxytoluene | 3.5 | 6.7 | 0.4 | 1.2 | | 2.7 | 4.2 | 7.6 |
| 2,6-Dihydroxytoluene | 4.4 | | 0.7 | 1.8 | | 2.6 | 4.2 | |
| 3,4-Dihydroxytoluene | 4.6 | 7.8 | 0.7 | 1.7 | | 3.5 | 5.9 | 7.4 |
| 3,5-Dihydroxytoluene | 4.0 | 6.6 | 0.3 | 0.8 | | 2.3 | 4.2 | 7.4 |
| 2,2′-Dihydroxybiphenyl | 5.8 | 8.6 | 1.4 | 3.4 | 4.3 | 5.6 | 7.9 | 6.9 |
| 2,4′-Dihydroxybiphenyl | 5.0 | 7.8 | 0.9 | 2.4 | | 3.2 | 5.0 | 5.4 |
| 2,5-Dihydroxybiphenyl | 4.9 | 7.7 | 1.1 | 2.4 | 3.5 | 3.7 | 5.2 | 6.2 |
| 3,3′-Dihydroxybiphenyl | 4.7 | 7.3 | 0.5 | 1.3 | 1.7 | 1.9 | 3.5 | 3.3 |
| 4,4′-Dihydroxybiphenyl | 4.2 | 6.9 | 0.5t | 1.3t | 1.9 | 1.7 | 3.0 | 2.2t |
| 2,3,2′,3′-Tetrahydroxybiphenyl | 3.0t | 5.0t | 0.1 | 0.3 | 0.4 | 1.9 | 3.3 | 6.1 |
| 3,4,3′,4′-Tetrahydroxybiphenyl | 1.8 | 2.8 | 0.0 | 0.0 | | 0.3 | 0.7 | 2.2 |
| 2,5,2′,5′-Tetrahydroxybiphenyl | 1.9 | 2.8 | 0.0 | 0.0 | | 0.4 | 0.9 | 6.7 |
| 2,6,2′,6′-Tetrahydroxybiphenyl | 3.0 | 4.4 | 0.0 | 0.0 | | 1.5 | 3.0 | 4.9 |
| 2,4,2′,4′-Tetrahydroxybiphenyl | 2.2 | 3.0 | 0.0 | 0.0 | | 0.6 | 1.0 | 6.0 |
| 4,4′-Dihydroxystilbene | 4.5 | 6.5 | 0.0 | 0.0 | | 1.9 | 3.4 | 0.0 |
| 4,4′-Dihydroxydiphenylmethane | 4.8 | 6.6 | | 1.3 | 2.0 | 3.6 | 5.8 | t |
| 2,2′-Dihydroxydiphenylmethane | 6.4 | 7.8 | | 3.0 | 5.1 | 5.3 | 7.5 | |

(*continued on p. D 10*)

TABLE 16 (*continued*)

| *Compound* | $R_F$* | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | $TL_1S_1$ | $TL_1S_2$ | $TL_1S_3$ | $TL_1S_4$ | $TL_1S_5$ | $TL_2S_6$ | $TL_2S_7$ | $TL_3S_8$ |
| 4-Hydroxybenzyl alcohol | 3.5 | 4.8 | | 0.5 | 0.7 | 3.7 | 5.7 | |
| *p*-Benzoquinone | | | 5.6 | 7.7 | | | 9.9 | |
| Diphenoquinone | | 9.7 | | | | | 9.9 | |
| Tetrahydroxy-*p*-benzoquinone | | | 0.0 | 0.0 | | | 1.7t | |
| 2,5-Dihydroxy-*p*-benzoquinone | | | 0.0 | 0.2t | | | 4.0t | |
| 2,2′-Dihydroxy-5,5′-dimethylbiphenyl | 6.0 | 8.3 | 2.2 | 3.8 | 5.5 | 6.7 | 8.5 | t |
| 2-Hydroxy-4′,5-dimethyldiphenyl ether | 8.2 | 9.2 | 5.3 | 7.8 | 9.5 | 8.9 | 9.7 | 0.0 |
| 4,4′-Dihydroxy-3,3′-dimethylbiphenyl | 5.6 | 7.0 | 1.3 | 3.0 | | 2.8 | 4.5 | t |
| 4-Hydroxy-2′,3-dimethyldiphenyl ether | | | | 8.5 | | 9.0 | 9.7 | |

* t = tailing.

TABLE 17

TLC $R_F$ VALUES OF PHENOLIC COMPOUNDS

(H.-I. JOSCHEK AND S. I. MILLER, *J. Am. Chem. Soc.*, 88 (1966) 3269)

Thin layers: $TL_1$ = Silica Gel G 7731.
$TL_2$ = Polyamide MN.

Solvents: $S_1$ = Benzene–methanol–acetic acid (70:8:4) (1 development).
$S_2$ = Benzene–methanol–acetic acid (80:1:1) (1 development).
$S_3$ = Benzene–methanol–acetic acid (80:1:1) (2 developments).
$S_4$ = Benzene–methanol–acetic acid (70:8:4) (1 development).
$S_5$ = Benzene–methanol–acetic acid (70:8:4) (2 developments).

| *Compound* | $R_F$ | | | | |
|---|---|---|---|---|---|
| | $TL_1S_1$ | $TL_1S_2$ | $TL_1S_3$ | $TL_2S_4$ | $TL_2S_5$ |
| 2-Bromophenol | 7.0 | 4.8 | | 6.3 | 8.0 |
| 3-Bromophenol | 6.5 | 3.2 | | 5.3 | 7.2 |
| 4-Bromophenol | 6.2 | 2.6 | | 5.4 | 7.3 |
| 2,3-Dihydroxybiphenyl | 5.7 | | 3.9 | 5.3 | 7.7 |
| 2,4-Dihydroxybiphenyl | 4.7 | | 1.9 | 3.0 | 5.2 |
| 2,5-Dihydroxybiphenyl | 4.9 | | 2.6 | 3.5 | 5.3 |
| 2,6-Dihydroxybiphenyl | 6.0 | | 4.9 | 4.7 | 6.7 |
| 3,4-Dihydroxybiphenyl | 5.2 | | 2.5 | 3.7 | 5.8 |
| Diphenylene oxide | 9.2 | 7.8 | 8.9 | 9.9 | 9.9 |

TABLE 18

TLC $R_F$ VALUES OF PHENOLIC COMPOUNDS
(R. R. SCHELINE, *J. Pharm. Pharmacol.*, 18 (1966) 665)

Thin layer: Whatman CC.41 cellulose (for $S_1$).
Macherey & Nagel cellulose (for $S_2$).
Solvents: $S_1$ = Benzene–glacial acetic acid–water (6:7:3).
$S_2$ = 20% aqueous potassium chloride–glacial acetic acid (100:1).
Detection: $D_1$ = Fast Blue B salt.
$D_2$ = Diazotised sulphanilamide.

| *Compound* | $R_F$ | | *Colour with** | |
|---|---|---|---|---|
| | $S_1$ | $S_2$ | $D_1$ | $D_2$ |
| 2-Hydroxybenzoic acid | 0.94 | 0.49 | PYBr | PYBr |
| 3-Hydroxybenzoic acid | 0.35 | 0.45 | O | Y |
| 4-Hydroxybenzoic acid | 0.35 | 0.46 | OBr | YO |
| 2,3-Dihydroxybenzoic acid | 0.35 | 0.42 | WBr | PiT |
| 2,4-Dihydroxybenzoic acid | 0.24 | 0.31 | V | Y |
| 2,5-Dihydroxybenzoic acid | 0.16 | 0.43 | WGr | PT |
| 2,6-Dihydroxybenzoic acid | 0.18 | 0.38 | RBr | Y |
| 3,4-Dihydroxybenzoic acid | 0.06 | 0.34 | WT | PT |
| 3,5-Dihydroxybenzoic acid | 0.02 | 0.33 | RPu | GrY |
| 2,3,4-Trihydroxybenzoic acid | 0.05 | 0.28 | PBr | PBr |
| 3,4,5-Trihydroxybenzoic acid | 0.00 | 0.28 | PBr | GBr |
| 2,4,6-Trihydroxybenzoic acid | 0.02 | 0.21 | Pu | YBr |
| 3-Hydroxy-4-methoxybenzoic acid | 0.56 | 0.33 | PiBr | O |
| 4-Hydroxy-3-methoxybenzoic acid | 0.79 | 0.37 | PiBr | O |
| 3,4-Dihydroxy-5-methoxybenzoic acid | 0.10 | 0.29 | WGr | PiGr |
| 3,5-Dihydroxy-4-methoxybenzoic acid | 0.07 | 0.40 | RPu | BrY |
| 3,5-Dimethoxy-4-hydroxybenzoic acid | 0.75 | 0.31 | PiBr | R |
| Phenol | 0.88 | 0.69 | RO | YO |
| 1,2-Dihydroxybenzene | 0.30 | 0.62 | PiGr | PiGr |
| 1,3-Dihydroxybenzene | 0.11 | 0.56 | RPu | GrY |
| 1,4-Dihydroxybenzene | 0.10 | 0.63 | GrBr | Br |
| 1,2,3-Trihydroxybenzene | 0.04 | 0.55 | PiBr | Br |
| 1,3,5-Trihydroxybenzene | 0.00 | 0.42 | Pu | YBr |
| 2-Methoxyphenol | 0.98 | | V | O |
| 1,2-Dihydroxy-3-methoxybenzene | 0.54 | 0.53 | WGr | PiGr |
| 1,3-Dihydroxy-2-methoxybenzene | 0.43 | 0.63 | V | GrY |
| 2,6-Dimethoxyphenol | 0.96 | 0.54 | Pu | R |

* Abbreviations: Br = brown; G = green; Gr = gray; O = orange; P = pale; Pi = pink; Pu = purple; R = red; T = tan; V = violet; W = white; Y = yellow.

## CHROMATOGRAPHIC DATA VOL. 28 (1967)

## AUTHOR INDEX



## CHROMATOGRAPHIC DATA VOL. 28 (1967)

## SUBJECT INDEX