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CHROM. 3330

DERIVATOGRAPHIC STUDIES OF GAS-CHROMATOGRAPHIC PARTITION LIQUIDS. II

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(First received October 5th, 1967; modified October 31st, 1967)

SUMMARY

Further derivatographic investigations were made for the purpose of elucidating some problems concerning column fillings used in gas chromatography. New data are given for the upper temperature limit of applicability of column fillings. The interactions between support and partition liquid, and partition liquid mixtures were studied.

In an earlier paper[1] we reported a derivatographic investigation[2] of gas-chromatographic supports, partition liquids and fillings. In our present paper we wish to report the results of further derivatographic studies of the above materials. So far only thermal methods have been used to investigate the above systems.

Our experiments were made with a Paulik-Paulik-Erdey Orion-Gyem 676 Type derivatograph, at a heating rate of 3°/min, with samples of about 300 mg. Derivatographic measurements were carried out in air, or in a stream of bomb nitrogen at a flow rate of 20 l/h.

The dependence of the upper temperature limit of usefulness of the column filling on the amount of coating was first studied. After selecting the model (Apiezon L–Chromosorb W, 60–80 mesh) the thermoanalytical curves of the pure components were determined (Fig. 1).

As shown by the figure, the solid support studied is of constant weight practically up to 500°, no transformation being shown even by the DTA curve which reflects enthalpy changes, therefore the support does not undergo any structural change in the temperature range studied. The partition liquid is of constant weight up to 240°. Above this temperature decomposition processes also begin to occur in addition to the increase in vapour pressure, as shown by the exothermic nature of the DTA curve. It must be pointed out that at 500° about 6 % of the partition liquid remains on the support under the given experimental conditions as a product of cracking.

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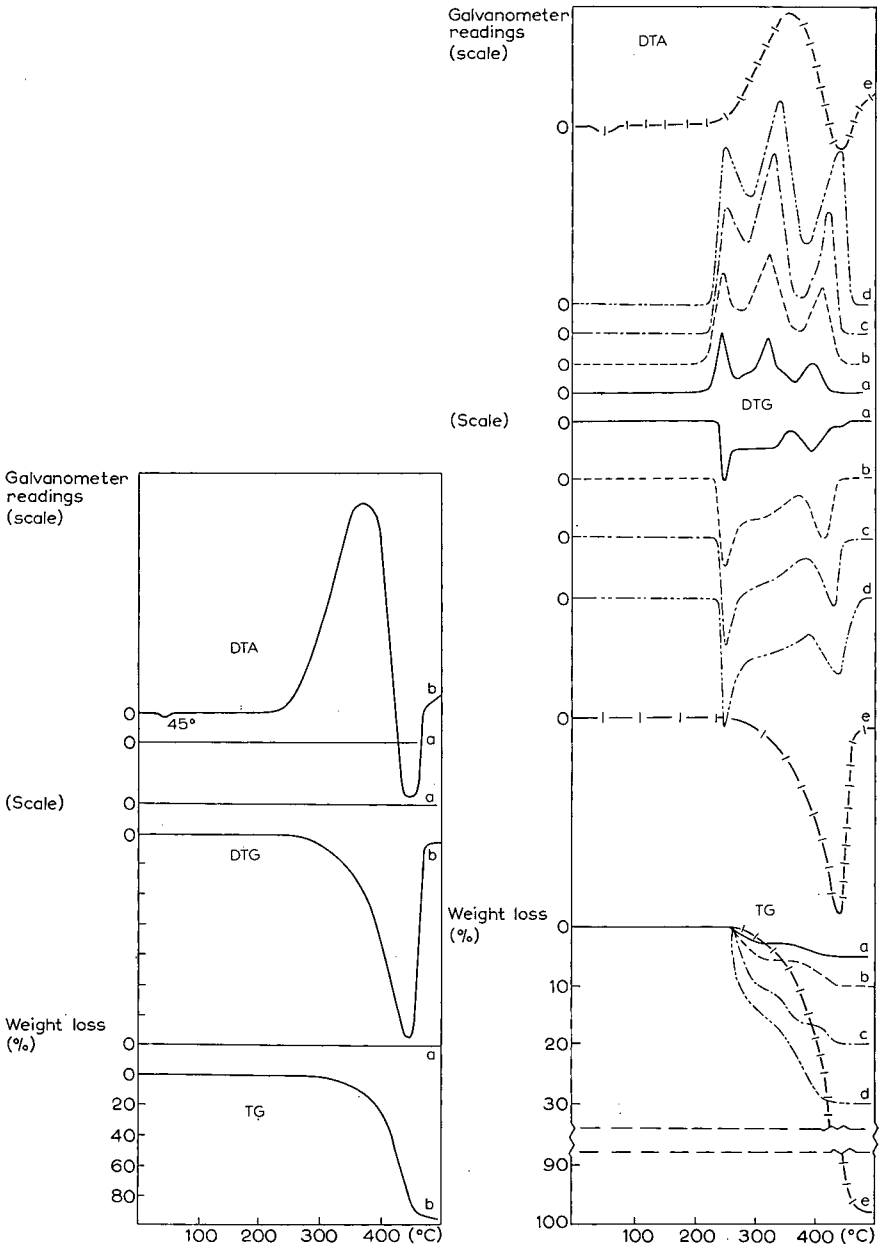


Fig. 1. Derivatograms of Chromosorb W 60–80 mesh (a) and Apiezon L (b). TG = thermogravimetric; DTG = derivative thermogravimetric; DTA = differential thermoanalytical.

Fig. 2. Derivatograms of Apiezon L–Chromosorb W fillings with various percentages of Apiezon coating (a = 5%, b = 10%, c = 20%, d = 30%), and of pure Apiezon L (e). TG = thermogravimetric; DTG = derivative thermogravimetric; DTA = differential thermoanalytical.

This phenomenon must be taken into consideration in each case when one wishes to determine the concentration of coating, and is also of importance in gas chromatography, because, if the column is over-heated the cracking products remaining on the support may make the prolonged use of support impossible.

The derivatograms of column fillings with different concentrations of liquid phase (2–50 %) were then recorded. Fig. 2 shows the thermoanalytical curves (a,b,c,d) of fillings with those concentrations of Apiezon L most frequently used in gas chromatography and also the derivatogram of the pure partition liquid (e).

It was found that for the selected pair of model substances the concentration of the coating has very little effect (5–10°) on the upper temperature limit of applicability of the partition liquid, and the temperature limits found for fillings with different concentrations of coating were practically the same as that found for pure Apiezon L, 240°. The shapes of the derivatograms found for the pure partition liquid and the fillings, however, are rather different in that the thermogravimetric (TG) curve for the fillings has a fraction which suddenly declines while that of the pure partition liquid only smoothly declines. This can be explained by the fact that oxidation processes have an important role in the case of prepared fillings. The absorption of activation energy necessary for oxidation at a given temperature causes the sudden and rapid decomposition of the whole of the partition liquid spread over a large surface.

In order to study other aspects of the interaction between the solid support and the partition liquid, similar fractions (60–80 mesh) of different supports were coated with similar amounts (5 %) of Apiezon L and the thermoanalytical curves of these fillings taken. A few characteristic derivatograms of the many obtained are presented in Fig. 3. The derivatogram of the pure partition liquid (see Fig. 1) is also necessary for the evaluation of the curves.

As shown by Fig. 3 a considerable difference exists between the results obtained with Chromosorb P and Chromosorb P-AW, and those obtained for other carriers. For the two carriers mentioned the upper temperature limit of usefulness was found to be higher by about 40° (280°) than that for all the other carriers studied. This phenomenon is probably due to the differences in the specific surface areas of the supports. Data given by the manufacturer[3] are listed in Table I.

TABLE I

Support	*Specific surface area* (m^2/g)	*Free fall density* (g/cm^3)
Chromosorb P	4.0	0.38
Chromosorb W	1.0	0.18
Chromosorb G	0.5	0.47

Owing to the considerably higher specific surface area of Chromosorb P type supports, the adsorption effect of the support is the one prevailing, which reduces the extent of distillation, and increases the activation energy of oxidation. In the same series of experiments the influence of the pretreatment of Chromosorb W type supports on the upper temperature limit of applicability of partition liquids was also investi-

gated. The same results were obtained for the carrier washed with acid (W-AW) as for that washed with acid and treated with silane (W-AW-ST) and as for untreated carrier.

The derivatographic curves of the pure carriers were also necessary for the evaluation of the series of experiments mentioned above. No very great differences were found between the different types of support studied. The weight of most

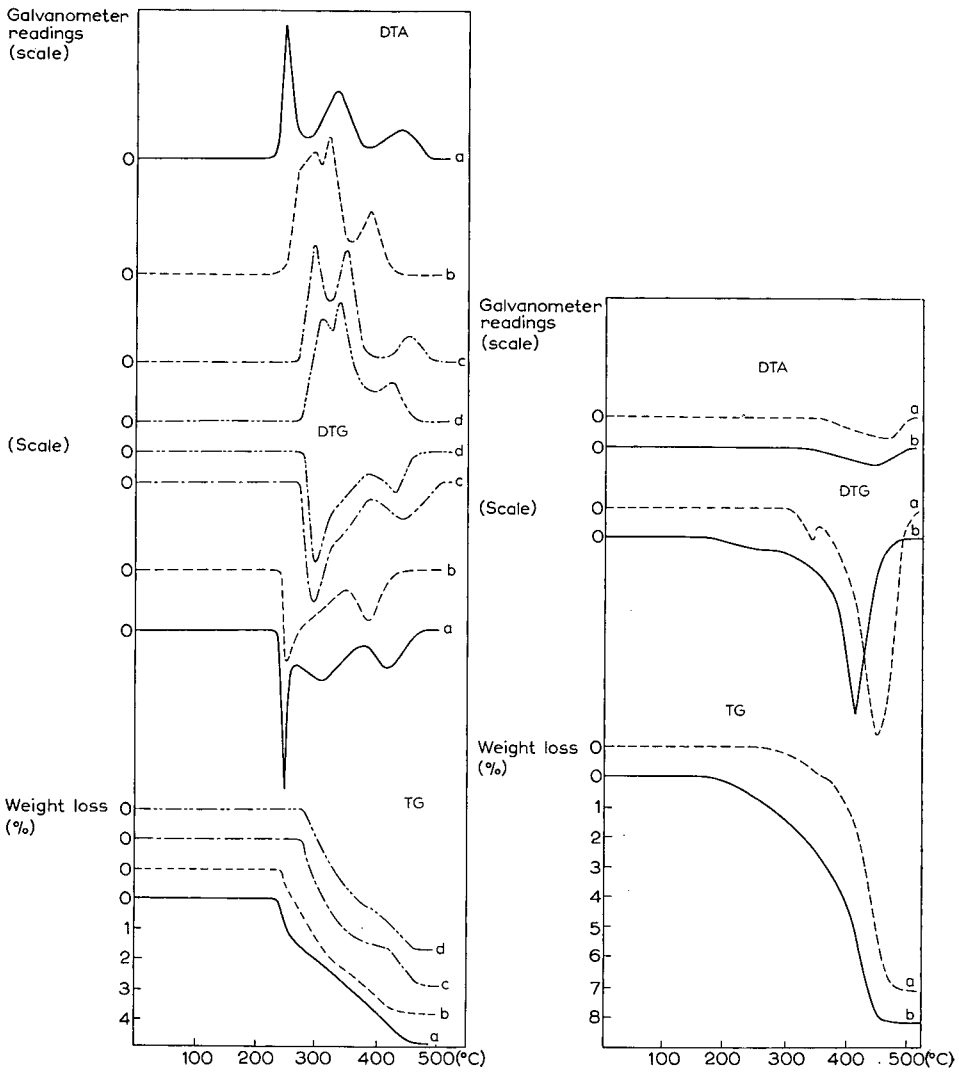


Fig. 3. Derivatograms of Chromosorb W-AW-ST (a), Chromosorb G (b), Chromosorb P-AW (c) and Chromosorb P (d) coated with 5 % Apiezon L. TG = thermogravimetric; DTG = derivative thermogravimetric; DTA = differential thermoanalytical.

Fig. 4. Derivatograms of Chromosorb G coated with 8 % Silicone Oil 550 (a) and of Chromosorb G coated with 7.6 % Silicone Oil 550 + 0.4 % stearic acid (b). TG = thermogravimetric; DTG = derivative thermogravimetric; DTA = differential thermoanalytical.

carriers was found to decrease continuously by 0.3–1.5 % between 20 and 500°. Therefore the heating of the supports before preparing the filling to be used at temperatures (450–500°) higher than the usual seems to be expedient.

The possibility of using the derivatographic method to determine the degree of exhaustion of column fillings is of special importance for studying columns used for preparative purposes. In this case the column is exhausted quickly because of the greater demands made (longer continual use, high flow rate, etc.). Chromosorb G column filling coated with 8 % Silicone Oil 550 was found to lose 0.5 % coating liquid during 6 h use at 170°. The derivatographic method was also used to study the problems concerning the upper temperature limit of applicability of partition liquid mixtures. The derivatograms of fillings prepared with Silicone Oil 550 and Apiezon L, modified with stearic acid (0.4–1.0 %), are presented in Figs. 4 and 5, respectively. It has been found that stearic acid reduces the temperature limit of use of Silicone Oil 550 considerably (by about 30°), and there are also differences in the shape of the curves.

The above phenomenon is even more marked with the partition liquid mixture containing Apiezon L, as shown by Fig. 5. Here the temperature limit was reduced by stearic acid by about 70°.

In future we wish to study the phenomena discussed in our present paper more generally and to interpret them in more detail.

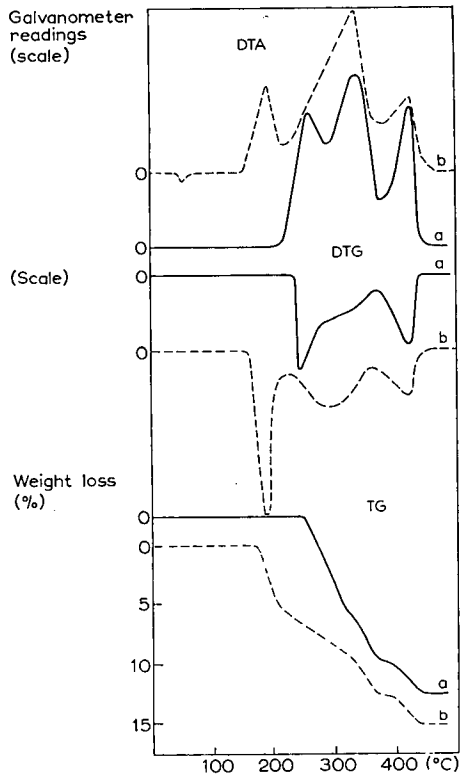


Fig. 5. Derivatograms of Chromosorb P coated with 15 % Apiezon L (a), and of Chromosorb P coated with 14 % Apiezon L + 1 % stearic acid (b). TG = thermogravimetric; DTG = derivative thermogravimetric; DTA = differential thermoanalytical.

ACKNOWLEDGEMENT

We wish to express our thanks to Miss E. SZABÓ for helping us with our experiments and drawing the figures.

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CHROM. 3321

A METHOD FOR THE SEPARATION AND IDENTIFICATION OF BASIC AMINO ACIDS AND HEXOSAMINES*

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(Received November 11th, 1967)

SUMMARY

A method is described for separating and quantifying basic amino acids and hexosamines simultaneously from the column chromatography on Dowex 50W X8 of serum acid hydrolysates. Based on color development with 2,4,6-trinitrobenzene-1-sulfonic acid, differences in the absorption spectra of the trinitrophenylation products of the two types of compounds are characteristic. The ratio of O.D. 355: O.D. 475 provides a quick identification of the type of compound, amino acid or hexosamine.

Spectrophotometric methods based on the reaction of 2,4,6-trinitrobenzene-1-sulfonic acid (TNBS) with primary amino groups have been used for the determination of amino acids, peptides and amines at acid pH[1] and hexosamines at alkaline pH[2]. This paper describes the application of the method at alkaline pH to eluates from the column chromatography of acid hydrolysates of whole serum proteins, a technique which permits the identification and quantitation of the basic amino acids and hexosamines. The trinitrophenylated (TNP)-amino acids and TNP-hexosamines produced by trinitrophenylation at alkaline pH may be differentiated by their absorption spectra in alkaline solutions. The absorption spectra of the TNP-hexosamines change characteristically after acidification of the buffered reaction mixtures[2].

MATERIALS

Standard solutions

D-Glucosamine·HCl (Eastman Organic Chemicals) and D-galactosamine·HCl, L-leucine, L-lysine, L-phenylalanine, L-alanine, L-histidine·HCl, L-tyrosine, and L-tryptophan (Sigma Chemical Company) were used at a concentration of 1.0 m*M*/ml in 0.1 *M* phosphate buffer, pH 7.8.

Reagents for column chromatography

Dowex 50W X8 resin, 200–400 mesh (Sigma Chemical Company) was prepared

* Supported by U.S.P.H.S. Grant GM 11547.

according to the method of MOORE AND STEIN used for the chromatography of basic amino acids[3].

The following buffers were prepared following the technique of MOORE AND STEIN[3]: (a) 0.1 *M* citrate buffer, pH 3.42; (b) 0.1 *M* citrate buffer, pH 5.0; (c) 0.1 *M* phosphate buffer, pH 6.75[4]; (d) 0.2 *M* citrate buffer, pH 6.5.

Reagents for the spectrophotometric assay

Borate buffer. KOH pellets were added to 1.0 *M* H_3BO_3 to give a solution of pH 8.6.

TNBS reagent. 2,4,6-Trinitrobenzene-1-sulfonic acid, 1 mg/ml in distilled water. TNBS supplied by Eastman Organic Chemicals or Sigma Chemical Company was satisfactory.

METHODS

One milliliter aliquots of whole rat serum were precipitated with 10.0 ml 95 % ethanol, washed with 10.0 ml 95 % ethanol and hydrolyzed in 2 ml 3 *N* HCl for 4 h in a 100° paraffin bath. The uncentrifuged acid hydrolysates were evaporated to dryness over calcium carbonate and sodium hydroxide pellets *in vacuo* and redissolved in 1.0 ml 0.1 *M* citrate buffer pH 5.0.

An 0.5 ml aliquot of the redissolved hydrolysate or of a synthetic mixture of amino acids and hexosamines was applied to a 1 × 15 cm Dowex 50W X8 column in 0.1 *M* citrate buffer, pH 3.42. The sample was washed on with 0.1 *M* citrate buffer, pH 5.0, and the column was eluted initially with 25 to 45 ml of this buffer. The column was then eluted with 100 to 150 ml 0.1 *M* phosphate buffer, pH 6.75, followed by 100 to 150 ml 0.2 *M* citrate buffer, pH 6.5. One milliliter fractions were collected into tubes containing 0.11 ml 1.0 *N* HCl. The hexosamines were stabilized in excess hydrochloric acid. The column was eluted at room temperature, but the collection tubes were maintained at 4° in a circulating bath. Later studies showed that refrigeration of the collected fractions was not necessary in the presence of excess hydrochloric acid. Column flow rate was controlled at 2 ml/h by adjusting the height of the buffer reservoir.

For the colorimetric assay, 0.5 ml aliquots of the column fractions were mixed with 0.5 ml distilled water, 3.0 ml 1.0 *M* borate buffer, pH 8.6, and 2.0 ml TNBS reagent. Standards of 0.1 ml of 1.0 m*M* solutions of D-glucosamine·HCl and L-leucine plus 0.9 ml distilled water plus buffer and TNBS were run with each determination. The color was allowed to develop for 15 to 18 h at 15°. Absorption spectra and absorbances at 355 mμ and 475 mμ were read in a Beckman Model DU spectrophotometer. Reaction mixtures were acidified by the addition of 0.1 ml concentrated HCl and the absorption spectra were read again.

RESULTS

Fig. 1 shows a chromatogram of a synthetic mixture of 0.5 mg each of L-leucine, L-phenylalanine, L-lysine, D-galactosamine·HCl and D-glucosamine·HCl. The difference in the relationship of absorbances at 355 mμ and 475 mμ for the amino acids and for the hexosamines clearly distinguished between these two types of compounds.

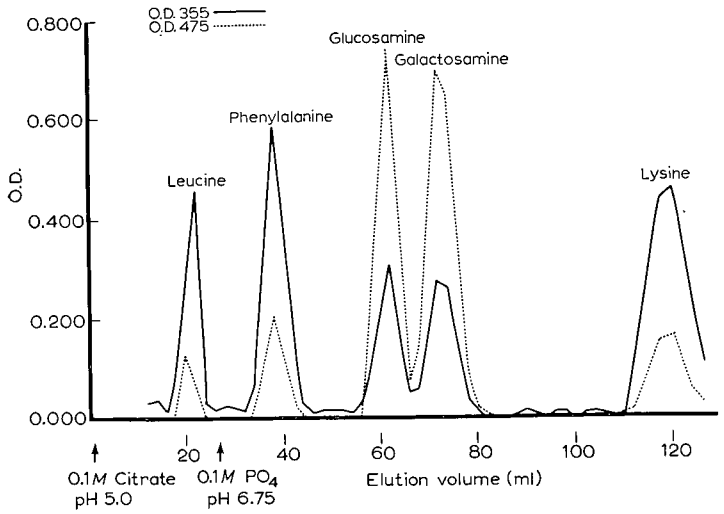


Fig. 1. Chromatogram of a Dowex 50W X8 column of a synthetic mixture of 0.5 mg each of L-leucine, L-phenylalanine, D-glucosamine·HCl, D-galactosamine·HCl and L-lysine. Optical density was read at 355 mμ (———) and 475 mμ (·····).

The ratio of O.D. 355:O.D. 475 for pure amino acids was approximately 2:1 to 4:1; for pure hexosamines, 1:2 to 1:2.5. Contamination of either type of substance with the other brought this ratio closer to 1. Absorbance of the hexosamine chromogen at 355 mμ and 475 mμ was proportional to concentration (Fig. 2a) and the ratio of O.D. 355:O.D. 475 remained constant above hexosamine concentrations of 0.03 m*M* (Fig. 2b).

The elution pattern of a whole serum acid hydrolysate (Fig. 3) contained an initial peak (A) of acidic and neutral amino acids and neutral sugars. Subsequent

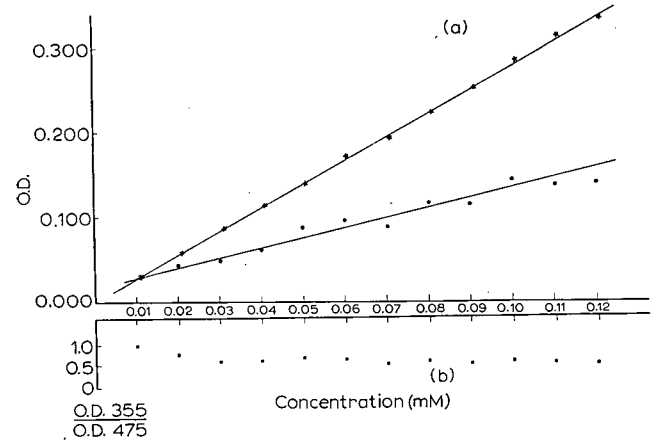


Fig. 2a. Relationship of the absorbances at 355 mμ (●) and 475 mμ (★) for a series of D-glucosamine·HCl concentrations. The series included the range of glucosamine concentrations expected in serum protein acid hydrolysates. Reaction mixtures consisted of D-glucosamine·HCl, 0.01 μmoles to 0.12 μmoles in 1 ml, + 3 ml 1.0 *M* borate buffer, pH 8.6, + 2 ml TNBS reagent.

Fig. 2b. The ratio of O.D. 355:O.D. 475 (■) was 0.5 for the concentrations above 0.03 m*M*.

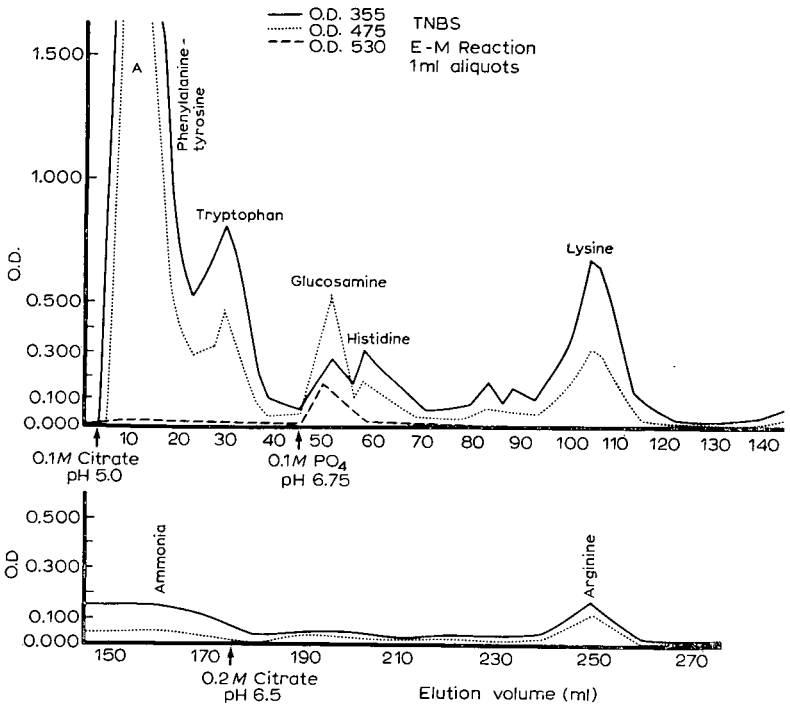


Fig. 3. Separation of the basic amino acids and glucosamine of 0.5 ml of a serum acid hydrolysate on a Dowex 50W X8 column. Optical density was read at 355 mμ (———) and 475 mμ (·····). Elution peak A represented the acidic and neutral amino acids and non-basic sugars. Results of the ELSON AND MORGAN method (------) confirmed the presence of a hexosamine.

peaks were phenylalanine–tyrosine, tryptophan, glucosamine, histidine, lysine, ammonia and arginine identified by comparison of their elution positions with chromatograms of synthetic mixtures of the amino acids and hexosamines. The identification of the glucosamine peak was confirmed by the ELSON AND MORGAN reaction[6]. Addition of glucosamine labeled with tritium or carbon-4 to the serum both before and after acid hydrolysis provided further proof that the hexosamine peak was glucosamine. Elution of the radioactivity from the Dowex 50W X8 column coincided with the elution of hexosamine. Decreasing the elution volume of the initial 0.1 *M* citrate buffer, pH 5.0, allowed a cleaner separation of the hexosamine (Fig. 4). Since the color absorption is proportional to concentration over a wide range, the amino acids and hexosamines may be quantified by reference to the molar extinction coefficients of the trinitrophenylation products of the pure standard solutions.

A comparison of the absorption spectra of the trinitrophenylation products of isolated amino acids and hexosamines differentiated the two types of compounds. Fig. 5 shows the absorption spectra of the reaction mixtures of standard solutions of D-galactosamine·HCl and L-leucine. Absorption maxima of the TNP-galactosamine were at 355 mμ and 475 mμ; absorption maxima of the TNP-leucine were at 355 mμ and 420 mμ. Acidification of the buffered reaction mixtures by the addition of 0.1 ml concentrated HCl had little effect on the absorption spectrum of the TNP-leucine. However, the absorption peak at 475 mμ of the TNP-galactosamine was completely

eliminated and the acidified mixture showed an absorption maximum at 380 mμ. The trinitrophenylation products of L-lysine, L-phenylalanine, L-alanine, L-histidine·HCl, L-tyrosine and L-tryptophan gave absorption curves similar to L-leucine; D-glucosamine·HCl gave an absorption curve similar to D-galactosamine·HCl. The molar extinction coefficients varied among these compounds (Table I).

Spectral analysis was applied to fractions from the Dowex 50W X8 columns to confirm the identification of the isolated substances. Aliquots from the amino acid peaks and from the glucosamine peak demonstrated absorption spectra compa-

TABLE I

MOLAR EXTINCTION COEFFICIENTS FOR THE TRINITROPHENYLATION PRODUCTS OF SOME AMINO ACIDS AND HEXOSAMINES

Reaction mixtures = 0.1 ml of 1.0 m*M* solution of each standard + 0.9 ml distilled water + 3.0 ml 1.0 *M* borate buffer, pH 8.6, + 2.0 ml TNBS reagent.

Compound	*O.D. 355* $\times 10^{-6}$	*O.D. 475* $\times 10^{-6}$	*O.D. 355 : O.D. 475*
D-glucosamine·HCl	1.30	3.12	0.417
D-galactosamine·HCl	1.37	3.57	0.384
L-leucine	1.94	0.49	3.96
L-phenylalanine	1.89	0.87	2.17
L-tyrosine	1.70	0.45	3.78
L-tryptophan	1.58	0.85	1.86
L-histidine·HCl	1.38	0.40	3.46
L-lysine	4.48	1.40	3.20
L-arginine	2.28	1.21	1.88
L-alanine	2.40	0.85	2.82

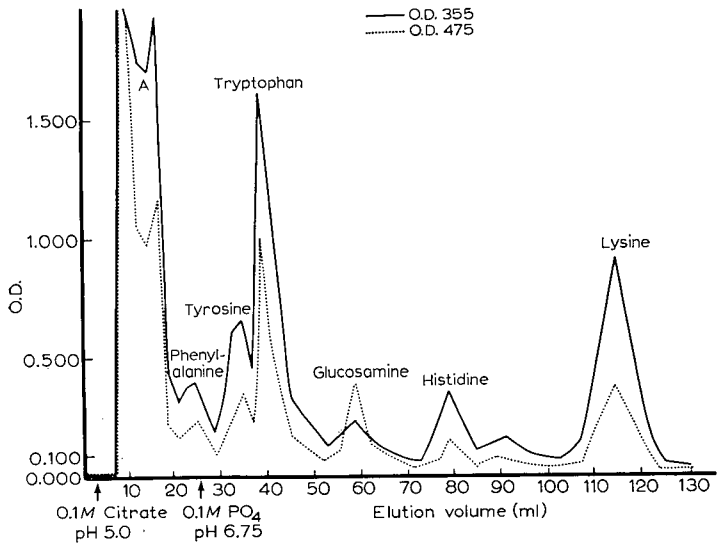


Fig. 4. Chromatogram of a serum acid hydrolysate demonstrating the effect of decreasing the elution volume of the initial 0.1 *M* citrate buffer, pH 5.0, from 47 ml in Fig. 3 to 27 ml. The glucosamine was eluted as a symmetrical peak. O.D. 355 mμ (———), O.D. 475 mμ (·····). Elution peak A represented the acidic and neutral amino acids and non-basic sugars.

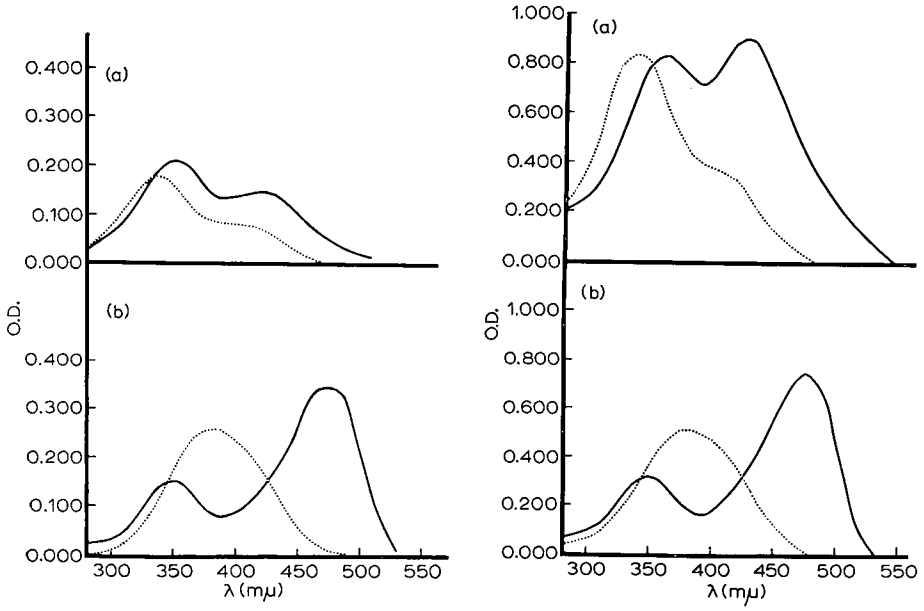


Fig. 5. Absorption spectra of TNP-leucine (a) and TNP-galactosamine (b). The spectra for the buffered alkaline reaction mixtures (———) showed absorption peaks for both compounds at 355 mμ, for the amino acid at 420 mμ and for the hexosamine at 475 mμ. The absorption spectra of the reaction mixtures after the addition of 0.1 ml concentrated HCl (· · · · ·).

Fig. 6. Absorption spectra of fractions from a Dowex 50W X8 column on a serum acid hydrolysate. (a) is a fraction from the phenylalanine-tyrosine peak; (b) is a fraction from the glucosamine peak. (———) and (· · · · ·) denote the spectra of the buffered alkaline reaction mixtures and acidified reaction mixtures respectively.

rable to those obtained with known amino acid and hexosamine solutions (Fig. 6). The addition of concentrated HCl produced a similar elimination of the 475 mμ peak in the column fraction identified as glucosamine.

DISCUSSION

Various procedures have been described for the spectrophotometric assay of amino acids and of hexosamines by separate means. The ninhydrin method[5] does not provide a differentiation between the two types of compounds. The less sensitive and less reliable ELSON AND MORGAN reaction for hexosamines must be used in conjunction with the ninhydrin method to enable a distinction to be made. As shown in Fig. 3, the ELSON AND MORGAN reaction, utilizing twice the test volume of the TNBS method, gave approximately one-third the optical density.

For a complex mixture of amino acids and hexosamines such as a serum protein acid hydrolysate, a method was needed to separate and identify easily the basic amino acid and hexosamine components. Column chromatography on Dowex 50W X8 by the GARDELL method[7] using 0.3 *N* HCl as the solvent did not separate the amino acid and hexosamine components of the serum acid hydrolysates. The TNBS procedure performed on eluates from a GARDELL column showed absorption spectra charac-

teristic of amino acids throughout the entire effluent volume. These spectra were not due to the effect of the 0.3 *N* HCl; hexosamines prepared in this solvent gave absorption curves similar to hexosamines in the 0.1 *M* phosphate buffer, pH 6.75. The GARDELL column method was also slower than the method described in this paper. Both columns were eluted at a flow rate of 2 ml/h; the hexosamines were eluted in 60 to 75 h with the GARDELL method and in 27 to 35 h with the modified MOORE AND STEIN method. Separation of the constituents of a serum acid hydrolysate by paper or thin layer chromatography was likewise unsatisfactory. The hydrolysate was so complex that a solvent system appropriate to separate all components simultaneously was not possible. However, modifying the MOORE AND STEIN method for the chromatography of basic amino acids on Dowex 50W X8 by careful regulation of the elution volume of the initial 0.1 *M* citrate buffer, pH 5.0, gave hexosamine peaks free from contamination by amino acids. Tryptophan not destroyed by acid hydrolysis did not overlap the hexosamine as reported by EASTOE[4] with this adjustment of the initial buffer.

The application of the GALAMBOS AND SHAPIRA TNBS procedure[2] at alkaline pH to the effluent fractions of the Dowex 50W X8 columns provided a simple simultaneous identification and quantitation of the amino acids and hexosamines. The ratios of O.D. 355:O.D. 475 of the absorbances of their trinitrophenylation products were characteristically 2:1 to 4:1 for the amino acids and 1:2 to 1:2.5 for the hexosamines. Additional confirmation of the type of compound, amino acid or hexosamine, was obtained by absorption spectra on the TNBS reaction mixtures. Absorption maxima occurred at 355 mμ for both amino acids and hexosamines, at 420 mμ for amino acids and at 475 mμ for hexosamines.

The advantages of the modified MOORE AND STEIN Dowex 50W X8 column used in conjunction with the TNBS method are obvious. Complex mixtures of amino acids and hexosamines may be separated reproducibly, absorbance is linear with concentration over a wide range, and amino acids and hexosamines may be distinguished and quantified simultaneously.

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CHROM. 3276

THE APPLICATION OF GEL CHROMATOGRAPHY TO THE SEPARATION OF PESTICIDES

PART I. ORGANOPHOSPHORUS PESTICIDES*

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(Received October 30th, 1967)

SUMMARY

This paper reports the use of gel chromatography as a clean-up and separatory technique in the identification of organophosphorus pesticides. Differences in separation on gels swollen in acetone and ethanol are investigated.

INTRODUCTION

Gel chromatography or gel filtration[1,2] has been widely used in the field of protein chemistry[3] but has not, to any extent, been applied to the separation of pesticides. The possibility of separating compounds on the basis of molecular size attracted our attention to this technique as it seemed potentially useful as a clean-up method for separating organophosphorus pesticides (mol.wt. *ca.* 200–350) from common vegetable co-extractives such as chlorophyll (mol.wt. 906) and carotene (mol.wt. 536). This paper describes the separation of organophosphorus pesticides on Sephadex LH-20, a modified dextran gel of lipophilic character.

EXPERIMENTAL

Sephadex LH-20 was allowed to swell for approximately 24 h in the solvents: acetone, tetrahydrofuran or ethanol. The swollen gel slurries were poured into glass chromatographic columns of 2 cm diameter to give a packed bed volume of approximately 75 ml. One millilitre of a mixture of pesticides containing 20 μg of each compound was placed on the top of each column in the corresponding solvent, and eluted at a rate of 1.0 ml/min. Each mixture contained up to five pesticides and always included parathion as an internal standard. Fractions of the eluted solutions were collected and examined for the presence of the component pesticides using gas chromatography[4]. Certain oxidation products which could not be detected on our

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gas chromatographic columns were examined by carrying out a colorimetric phosphorus determination of each fraction.

To evaluate the clean-up potential of these columns several cabbage extracts fortified with pesticides were passed down them. The extracts contained about 60 mg of material, mainly natural pigments, resulting from the extraction of 100 g of cabbage with acetone. Each extract was added to the column dissolved in 1 ml of acetone or

TABLE I

THE SEPARATION OF ORGANO PHOSPHORUS PESTICIDES ON SEPHADEX LH-20

Gel swollen and eluted with acetone			*Gel swollen and eluted with ethanol*	
Pesticide in sequence of elution	*Mol.wt.*	*Elution volume relative to parathion (= 100)*	*Pesticide in sequence of elution*	*Elution volume relative to parathion (= 100)*
Aspon	378	87	Aspon	66
Ethion	384	92	Dimefox	71
Sulfotep	322	93	Phosphamidon	72
Malathion	330	95	Tepp	73
Mecarbam	329	96	Phorate-O-analogue	74
Diazinon	304	98	Sulfotep	75
Demeton-S	258	99	Malathion-O-analogue	76
Pyrimithate	333	99	Bidrin	77
Disulfoton	274	100	Diazinon	77
Parathion	291	100 (Elution vol. 43.5 ml)	Chlorfenvinphos	77
			Demeton-S	79
Fenitrothion	277	101	Mevinphos	79
Phorate	260	103	Thionazin-O-analogue	79
Demeton-O-methyl	230	103	Demeton-S-methyl-sulphoxide*	79
Phorate sulphone	309	104	Trichlorphon	80
Malathion-O-analogue	314	104	Pyrimithate	81
Chlorthion	283	107	Ethion	82
Fenthion	278	108	Phorate sulphoxide	82
Thionazin	248	108	Dichlorvos	83
Thiometon	248	108	Mecarbam	85
Parathion-methyl	249	109	Parathion-O-analogue	86
Fenchlorphos	321	109	Demeton-S-methyl	86
Parathion-O-analogue	275	112	Malathion	86
Chlorfenvinphos	359	114	Disulfoton	87
Bromophos	366	115	Phorate	89
Mevinphos	224	116	Thionazin	89
Phosphamidon	264	117	Demeton-O-methyl	93
Phorate-O-analogue	244	118	Dimethoate	95
Trichlorphon	257	121	Phorate sulphone	98
Dichlorvos	221	122	Demeton-S-methyl-sulphone*	99
Morphothion	285	124	Parathion	100 (Elution vol. 75.5 ml)
Demeton-S-methyl-sulphone*	262	125		
Demeton-S-methyl	230	130	Thiometon	100
Thionazin-O-analogue	232	133	Bromophos	100
Tepp	290	134	Fenchlorphos	101
Bidrin	237	141	Morphothion	106
Phorate sulphoxide	293	147	Fenthion	112
Dimethoate	229	148	Parathion-methyl	114
Dimefox	154	150	Fenitrothion	114
Demeton-S-methyl-sulphoxide*	246	200	Chlorthion	118

* These pesticides were determined by a total phosphorus method.

ห้องสมุด กรมวิทยาศาสตร์

ethanol containing a mixture of pesticides, each at 10 μg. Fractions were collected and examined as above, and the quantity of cabbage coextractive emerging at various stages of the elution was determined by evaporating the portion to dryness to obtain a dry weight of residue.

RESULTS

The pesticide elution sequence, molecular weights and elution volumes relative to parathion on the gel swollen in acetone and ethanol are shown in Table I. The elution sequences in acetone and tetrahydrofuran were identical and the data for the latter solvent have been omitted.

The elution curves for all the compounds, with the exception of trichlorphon, were symmetrical, and recovery from the columns was apparently quantitative, although recovery experiments were only carried out with a few of the pesticides. No significant change in the elution volume of parathion occurred over a period of several weeks on a given column and its band-spread was found to be independent of sample concentration over the range investigated (10–1000 μg/ml), but dependent on flow rate. Fig. 1 shows the separation of parathion and its analogues on the gel swollen in acetone and in ethanol. The higher loading of the oxygen analogue was necessitated by the decreased sensitivity of the gas chromatographic method to this compound[4].

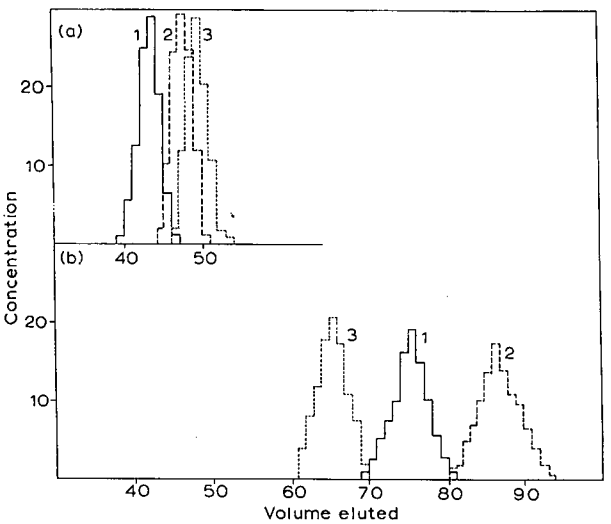


Fig. 1. Separations on a gel swollen in acetone (a) or ethanol (b), elution rate 1 ml/min. 1 = Parathion; 2 = parathion-methyl; 3 = parathion-O-analogue.

The elution volumes of the pesticides added to the cabbage extracts were identical with those obtained using pure pesticides, and did not change despite repeated use of the columns. Table II shows the percentage of cabbage co-extractive material eluting in the various portions of the eluate.

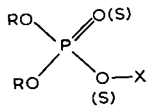
TABLE II

THE SEPARATION OF A CABBAGE EXTRACT ON SEPHADEX LH-20

Gel swollen and eluted with acetone		*Gel swollen and eluted with ethanol*	
Portion of eluate	*% of total coextractive (average of five experiments)*	*Portion of eluate*	*% of total coextractive (average of five experiments)*
0– 35 ml	18	0– 50 ml	24
35– 75 ml	57	50– 85 ml	62
75–100 ml	25	85–150 ml	14

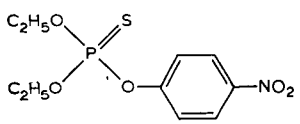
DISCUSSION

Gel chromatography is primarily a means of separating molecules on the basis of molecular size, but departures from "ideal" behaviour can occur; JANSON[5] has recently reviewed this topic. Departures from "ideality" manifest themselves in two ways, compounds either eluting before their "ideal" position as a result of exclusion from the gel or eluting later due to adsorption or electrostatic retardation. In the results shown in Table I the separations on the gel swollen with acetone (also tetrahydrofuran) correspond more closely to "ideality" than is the case with the gel swollen with ethanol. The organophosphorus pesticides are a closely related group of compounds, mainly esters of phosphoric or phosphorothioic acids, having the following general structure:

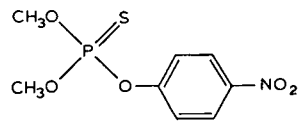


where: R = an alkyl group and X = an organic radical.

Variation in the size of the alkyl group produces the anticipated effect on both columns, *i.e.* the molecule containing the larger alkyl group elutes first. Thus parathion elutes before parathion-methyl (see Fig. 1).



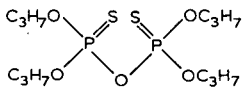
Parathion
R_{va} = 100
R_{ve} = 100



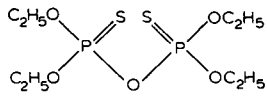
Parathion – methyl
R_{va} = 109
R_{ve} = 114

where R_{va} and R_{ve} are the relative elution volumes in acetone and ethanol, respectively.

Analogous behaviour is shown by the pairs of compounds demeton-S–demeton-S-methyl and disulfoton–thiometon. The sulphur-containing pyrophosphate analogues aspon and sulfotep show similar separation.

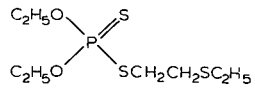


Aspon
R_{va} = 87
R_{ve} = 66

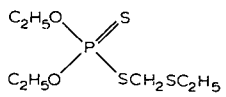


Sulfotep
R_{va} = 93
R_{ve} = 75

Increasing the chain length by $-CH_2-$ in the group X apparently results in a less significant change in molecular dimensions with little separation occurring. Disulfoton and phorate were the only compounds available to us on which this effect could be studied.

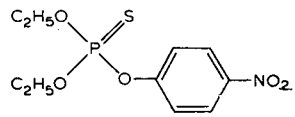


Disulfoton
R_{va} = 100
R_{ve} = 87

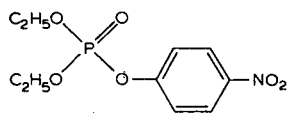


Phorate
R_{va} = 103
R_{ve} = 89

Many pesticides are dependent for their biological activity on conversion of P=S to P=O after application. It would be anticipated that the separation of a mixture of such compounds would result in the larger molecule containing the P=S group eluting first. On the acetone-swollen gel the behaviour is as anticipated but in ethanol the behaviour is anomalous. This may be due to an exclusion process in which the more electrophilic oxygen atom results in a molecule sufficiently polarized to be repelled by the gel in a polar solvent such as ethanol, or is perhaps due to an increase in overall molecular size as a result of solvation induced by hydrogen bonding. Parathion and its oxygen analogue are typical examples (see Fig. 1).

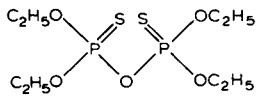


Parathion
R_{va} = 100
R_{ve} = 100

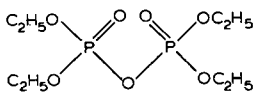


Parathion-O-analogue
R_{va} = 112
R_{ve} = 86

The compounds malathion, phorate, thionazin and thiometon and their oxygen analogues show the same elution pattern as above. The pyrophosphates tepp and sulfotep behave similarly.

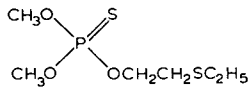


Sulfotep
R_{va} = 93
R_{ve} = 75

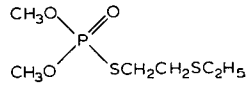


Tepp
R_{va} = 134
R_{ve} = 73

Thiono-thiolo isomerisation of certain pesticides can occur, *e.g.* demeton-O-methyl isomerising to demeton-S-methyl, which results in replacement of P=S by P=O. The elution pattern is as above.



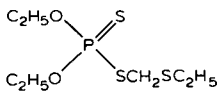
Demeton-O-methyl
$R_{Va} = 103$
$R_{Ve} = 93$



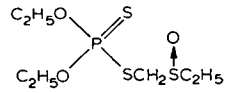
Demeton-S-methyl
$R_{Va} = 130$
$R_{Ve} = 86$

Variations in the structure of the side chain X lead to the great diversity of organophosphorus pesticides. The nature of the side chain also plays an important part in determining the elution characteristics of the compound. In the pesticides studied some noticeable correlations emerge. For example those pesticides containing aromatic side chains, *e.g.* parathion, parathion-methyl, bromophos, chlorthion, fenchlorphos, fenitrothion and fenthion, all elute much later in ethanol than pesticides which lack such aromaticity. This retardation of aromatic molecules by gels swollen in ethanol has been noticed by other workers[6]. Substituents on the aromatic ring do not lead to changes resulting in pronounced separations.

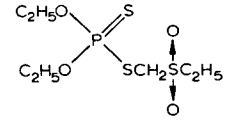
Several pesticides contain side-chains in which an oxidisable sulphur atom is present. Oxidation of such compounds to form the sulphoxide and sulphone is important as such oxidation occurs under natural conditions[7], and the oxidation products are frequently of greater biological activity than the parent compounds. The elution pattern for the phorate oxidation products is shown below; the oxidation products of demeton-S-methyl show a similar inversion of elution order in the two



Phorate
$R_{Va} = 103$
$R_{Ve} = 89$



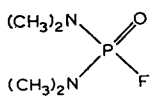
Phorate sulphoxide
$R_{Va} = 147$
$R_{Ve} = 82$



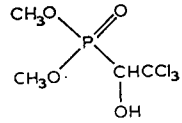
Phorate sulphone
$R_{Va} = 104$
$R_{Ve} = 98$

solvents. The reversal of the elution order is most marked and, if typical, may well provide a means of characterisation which usually requires infra-red spectroscopy[8]. We have used the separations attained on the Sephadex gel to isolate pure samples of the components of oxidised pesticides[4].

Two pesticides which are atypical in structure are dimefox and trichlorphon. The former compound displays a dramatic change in its elution position when the solvent is changed, and the latter was the only pesticide examined which showed marked tailing of its elution peak in all solvents.



Dimefox



Trichlorphon

The results in Table II indicate that Sephadex LH-20 is of little use as a clean-up technique, with much of the pigment material eluting in the range over which pesticides are found to emerge. The constant nature of the elution volume of a particular pesticide on the Sephadex gel might make a separation on this material a useful adjunct to the identification of organophosphorus pesticides at residue levels now based on thin-layer or gas chromatography.

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CHROM. 3318

MEMBRANE CHROMATOGRAPHY OF DYES ON NITROCELLULOSE FILTERS

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(Received November 13th, 1967)

SUMMARY

Various types of nitrocellulose membrane filters were tested as carrier materials for rapid small scale chromatography on strips 3–4 cm long. Model mixtures of 7–8 synthetic dyes were fractionated into their individual components in single chromatographic runs by using the developing systems: ethanol–chloroform (1:1); ethanol–chloroform–acetic acid (20:20:1); or ethanol–ammonia (10:1). The best results were achieved on membranes having mean pore sizes round 0.2–0.4 μm and especially on "Synpor 8 and 9" and "Millipore HA" membranes. Some basic general characteristics of membrane chromatography and its differences from PC and TLC are pointed out and discussed.

Nitrocellulose membrane filters were recently shown to be a convenient material for small scale chromatography and electrophoresis of proteins, as well as for some other substances (see ref. 1). The membranes were used either intact or impregnated with detergents or proteins and separations were done solely in aqueous developing solutions. Under those conditions it was possible to achieve some specific group separations of different types of substances during chromatography[2,3].

The present experiments were undertaken to investigate the applicability of nitrocellulose membranes to the chromatographic resolution of complex mixtures of low molecular weight substances into their individual constituents and also by using appropriate organic solvents. Mixtures of some randomly chosen dyes served as a model system. In addition, we wanted to obtain information as to whether "membrane chromatography" in general (using thin "selfcarrying" standard sheets of chemically homogeneous material with a fine foam-like microstructure and a standardized narrow distribution of pore sizes[4]) could be an alternative to paper chromatography (PC) and thin layer chromatography (TLC[5,6]) (which needs a mechanical support, spreading apparatus and often adhesives for the powdered adsorbents).

At the same time, the experiments served to test nitrocellulose as an adsorbent or carrier, the latter use has not yet drawn wider attention in chromatography.

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

EXPERIMENTAL

Material and methods

Nitrocellulose membranes Synpor (Chemapol, Prague) type 1-10, Sartorius (Membranfiltergesellschaft, Göttingen) type 11001-11011 and Millipore (Millipore s.a., Paris) type VM, VC, HA and RA were used in strips, usually 0.5–1 cm wide and 3–4 cm long. Strips of the same size of acetylcellulose, Oxoid (Oxoid, London), Celotate (Millipore, s.a., Paris), Whatman No. 1 filter paper and thin-layer sheets of silica gel "Siligram" (Kavalier, Votice, ČSSR) and Eastman-Kodak "6060" (London), served as reference adsorbents for a rough comparison of the chromatography of the dyes in the given solvents.

Standard mixtures of 0.1–0.5 % ethanolic solutions of the following dyes were most convenient for our purposes: eosin, orange G, *p*-aminoazobenzene, methyl red, fuchsin, sudan yellow, rhodamine B, methylene blue; with solvents containing ammonia, fuchsin was omitted.

Three solvent systems: 96 % ethanol–chloroform (1:1), 96 % ethanol–chloroform–acetic acid (20:20:1) and 96 % ethanol–25 % ammonia (10:1) were found to be most effective, among a series of other developing systems tested, in separating the given mixture of dyes.

Ascending chromatography was done in 50–100 ml Erlenmayer flasks. The strips were hung on a wire, passing tightly through the cork stopper, and when necessary they could be equilibrated in the vapours of the solvent. The membranes were then immersed in the developing solution placed on the bottom of the flask, by carefully lowering the wire. Samples (volumes of the order of 0.1 μl) were applied by means of a thin capillary or a wick of a thin, hard filter paper[7]. The width of the start streak did not exceed 0.2 mm. The start was usually 0.5 cm above the surface of the solvent. Distances of 1.0 and 1.5 cm led to practically the same results. After chromatography, the strips were laid on a filter paper and their longer sides were covered (about 1 mm from each side) by glass slides to prevent slight deformation in the membranes, which occurs when the strips are dried freely in air. It was often advantageous to treat the dry membranes after chromatography with paraffin oil to make them transparent.

RESULTS AND DISCUSSION

Nitrocellulose membranes proved to be a very good medium for microchromatography of the dyes examined in all three developing systems. Fig. 1 illustrates the formation of distinct spots of the individual dyes in a single run within a very short distance and a short period of time. In some cases, circular or "semicircular[5]" development led to the formation of sharper zones especially in the vicinity of the start. Two-dimensional microchromatography (Fig. 2) in ethanol–chloroform–acetic acid (20:20:1) in the first direction, followed by ethanol–ammonia (10:1) in the second direction, permitted a still better separation of the individual spots.

Equilibration of the strips in the vapours of the solvent for 1–60 min had no practical effect when the system ethanol–ammonia was used. In the other two systems, containing chloroform, a somewhat sharper formation of the spots near to the start was observed after an equilibration for at least 15 minutes. A very sharp

resolution of the dyes was also achieved on membranes which had been impregnated either with Tween 20, Tween 60 or bovine serum prior to chromatography (*cf.* ref. 2). However, the positions and R_F values of some substances were changed (*e.g.* fuchsin and orange G interchanged their positions in the solvent ethanol–chloroform–acetic acid on strips impregnated with Tween 20). The same changed sequence of the two dyes was observed on intact Millipore membranes but not on the Sartorius ones.

Comparison of the results achieved on different membranes showed that the velocity of the advancing solvent front was generally greater on membranes having greater mean pore size, but the separation of the spots was worse.

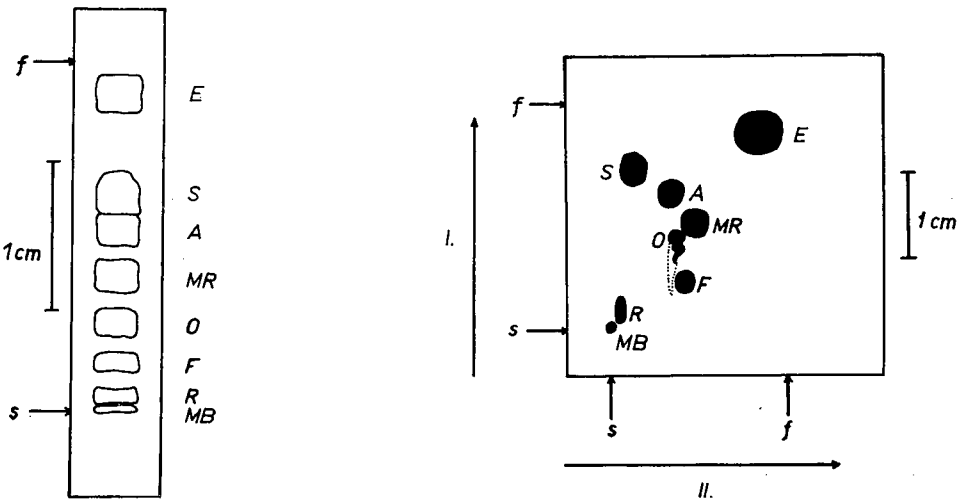


Fig. 1. Microchromatography of dyes on "Synpor 9" membranes. Solvent system: 96% ethanol–chloroform–acetic acid (20:20:1). Time 15 min. Start streak 0.2 mm wide. E = Eosin; O = orange G; A = *p*-aminoazobenzene; MR = methyl red; F = fuchsin; S = sudan yellow; R = rhodamine B; MB = methylene blue; s = start; f = front.

Fig. 2. Two-dimensional chromatography of dyes on "Synpor 8". Direction I: 96% ethanol–chloroform–acetic acid (20:20:1); direction II: 96% ethanol–25% ammonia (10:1). Running time 15 min in both directions, start spot diameter 0.6 mm. For nomenclature of dyes see Fig. 1.

An arbitrary classification of the quality of the chromatographic separation was made using five degrees of assessment; 1 = "very good" (well formed spots, no tailing); 2 = "good" (prolonged spots, slight tailing); 3 = "medium" (still longer spots, longer tailing); 4 = "poor" (partial resolution but no formation of individual spots, enormous tailing); 5 = "no resolution" (various difficulties, irregular development).

The results summarized in Fig. 3 indicate that "Synpor" membranes having mean pore sizes in the range of 0.2–0.4 μm were most suitable for chromatography of the given dyes under given conditions. A similar finding was made for "Millipore" membranes, whereas on "Sartorius" filters the quality of separation seemed to be less dependent on the mean pore sizes. Very good results were achieved with "Synpor 9" in all three solvent systems, together with "Millipore HA" in ethanol–chloroform–acetic acid (20:20:1) and "Sartorius 11002" in ethanol–ammonia (10:1). Comparative runs made in the same solvent systems on paper, the acetyl cellulose membranes

Celotate and Oxoid were generally unsuccessful. Thin layers of silica gel permitted a "medium" separation in the system ethanol–chloroform but the length of the strips was not sufficient to make a better separation possible. This does not mean, of course, that a suitable solvent system could not be found for this carrier medium. However, it was not the aim of this work to investigate in detail the chromatography of dyes on other carriers.

It seems to us that the present results with the dyes as well as former results obtained with proteins and other substances allow some generalizations concerning chromatography on membranes to be made (although the experiments up till now have mostly been done on nitrocellulose filters, and the possibility of separating various other types of substances on this material is still to be investigated). There seem to be several advantages in using membrane filters especially for rapid microscale chromatography. There are also some substantial differences between this type of chromatography, paper chromatography and thin-layer chromatography, which led us to propose the term "membrane chromatography". The following characteristics and practical advantages should be pointed out, to complete the facts already mentioned in the introduction:

Membrane filters have a very uniform microporous structure, the whole material of the strip is chemically homogeneous and its active surface is continuous. This is different from the rough fibrous structure of filter paper used in PC and also from the discontinuous physical and mechanical structure of thin-layers of powdered adsorbents used in TLC. The fine structure of the membranes enables the application of minute samples in very sharp start lines, a fact which has a great positive influence on the quality of the separation (*cf.* Figs. 1 and 2). No mechanical support is necessary as in TLC[6]. Cutting of strips to wanted sizes and handling during all the necessary operations is very convenient, as is their preservation for documentation. The strips can easily be made transparent, *e.g.* with paraffin oil. Membranes of various chemical

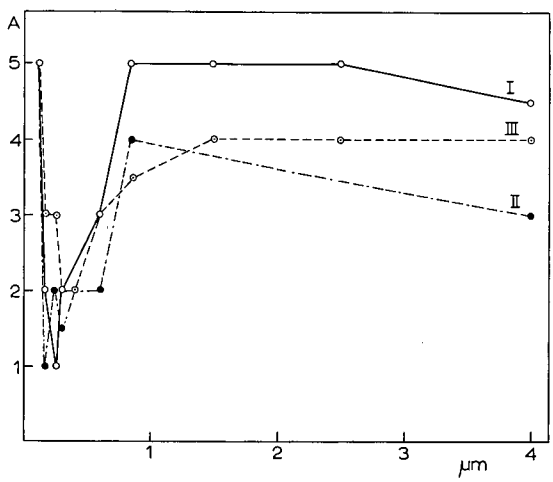


Fig. 3. Quality of the separation depending on mean pore sizes of the Synpor membranes. A = Arbitrary degrees for classifying the quality of separation: (1) very good, (2) good, (3) medium, (4) poor, (5) no separation (*cf.* text); μm = mean pore size. Solvent systems: (I) ethanol–chloroform (1:1); (II) ethanol–chloroform–acetic acid (20:20:1); (III) ethanol–ammonia (10:1).

nature are produced commercially and are available in standard qualities as filtration sheets with given porosity and pore sizes. According to the present experiments no special activation seems to be necessary nor was there a basic need for a long equilibration in the atmosphere of the chamber.

However, the following limitations of nitrocellulose membranes should also be mentioned. Some organic solvents *e.g.* acetone, pyridine, methanol, ethyl acetate, glacial acetic acid, which dissolve nitrocellulose[4] , cannot, of course, be used as developing systems or as solvents for the detecting agents. (However, they are suitable for dissolving those parts of the strips containing the wanted isolated fraction.) Concentrated sulphuric acid, a general detection agent in TLC, also attacks nitrocellulose. Developing systems containing a high proportion of lower alcohols may, after chromatography, cause a slight deformation ("curling") of the membranes during drying in air.

It may be concluded that membrane chromatography seems to offer the possibility of rapid microscale separations of various substances not only in aqueous solutions but also in some organic developing systems in a similar way as in PC and TLC.

ACKNOWLEDGEMENTS

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CHROM. 3306

BIDIMENSIONAL THIN-LAYER CHROMATOGRAPHY OF CARBOHYDRATES ON SILICA GEL IMPREGNATED WITH BORIC ACID

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SUMMARY

The separation of a large number of sugars by means of bidimensional thin-layer chromatography is described.

Factors which influence separation of carbohydrates on silica gel impregnated with boric acid, and the limitations of the monodimensional technique are investigated and discussed.

Many attempts to separate sugar mixtures by thin-layer chromatography have been made since STAHL's first experiments[1,2]. Results obtained with all known adsorbents and with many different types of impregnant and solvent mixtures have proved unsatisfactory[3–21]. Identification of many of the most important sugars was highly dubious.

OVODOV *et al.*[17] have recently stressed that "poor separation of some of the more common sugars and the low capacity of the chromatoplates" are responsible for the lack of general acceptance of thin-layer chromatography in research work on carbohydrates.

The purpose of our work was to explore the limits and possibilities of thin-layer chromatography on silica gel impregnated with boric acid for carbohydrates.

EXPERIMENTAL

Preparation of the chromatoplates

30 g of silica gel, TLC grade, without binder (Fluka DO), was mixed with an 0.03 *M* H_3BO_3 solution (2.1 g of H_3BO_3 in 1 l of distilled water). The suspension obtained was applied in the usual manner to the glass plates (0.3 mm layer). These plates were dried for 24 h at room temperature. They were then activated for one hour at 110°.

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Samples

The following pure sugar samples* were used:

Monosaccharides	*Oligosaccharides*	*α-Methyl derivatives*
Ribose	Sucrose	α-Methyl-D-glucoside
Arabinose	Turanose	α-Methyl-D-mannoside
Xylose	Maltose	
Lyxose	Lactose	
Fucose	Trehalose	
Rhamnose	Raffinose	
Glucose		
Galactose		
Mannose		
Levulose		
Sorbose		
Mannoheptulose		

Monodimensional chromatography

10 mg of each sugar were dissolved in 2 ml distilled water; 1 μl of each solution (= 5 μg of sugar) was applied to the chromatoplate with a micropipette in the usual manner. The chromatoplate was then placed in a tank containing the developing solvent system, the walls being lined with filter paper impregnated with the developing solvent. The plates were so slightly tilted that they were practically upright. The room temperature was kept at 22°.

The following solvent systems were employed:

(1) *n*-Butanol–boric acid, 1 *M* (9:1)
(2) *n*-Butanol–acetone–water (4:5:1)
(3) *n*-Butanol–acetic acid–water (4:1:5)
(4) *n*-Butanol–pyridine–water (8:4:3)
(5) *n*-Butanol–pyridine–acetone–boric acid, 1 *M* (60:3:20:17)
(6) *n*-Butanol–isopropanol–water (3:5:2)
(7) *n*-Butanol–ethanol–water (2:1:1)
(8) *n*-Butanol–ethanol–phosphoric acid, 0.1 *M* (1:10:5)
(9) *n*-Butanol–ethanol–hydrochloric acid, 0.1 *N* (1:10:5)
(10) *n*-Butanol–methanol–water (5:3:1)
(11) *n*-Butanol–ethyl acetate–boric acid, 1 *M* (70:15:15)
(12) *n*-Butanol–ethyl acetate–pyridine–water (2:3:2:3)
(13) *n*-Butanol–ethyl acetate–isopropanol–acetic acid–water (35:100:60:35:30)
(14) *n*-Butanol–ethyl acetate–isopropanol–acetic acid–water (3:10:6:6:3)
(15) *n*-Propanol–water (85:15)
(16) *n*-Propanol–acetic acid–water (4:1:5)
(17) *n*-Propanol–acetic acid–boric acid, 1 *M* (4:1:5)
(18) *n*-Propanol–pyridine–water (5:3:2)
(19) *n*-Propanol–ethyl acetate–water (1:4:2)
(20) Ethyl acetate–acetic acid–water (6:3:2)
(21) Ethyl acetate–pyridine–water (2:1:2)
(22) Ethyl acetate–pyridine–water (3:1:2)

* Nutritional Biochemicals Co., Cleveland, Ohio, U.S.A.

(23) Ethyl acetate–pyridine–water (3:3:2)
(24) Ethyl acetate–pyridine–water (4:3:2)
(25) Ethyl acetate–pyridine–boric acid, 1 *M* (2:1:2)
(26) Ethyl acetate–acetic acid–boric acid, 0.1 *N* (3:1:3)
(27) Ethyl acetate–isopropanol–water (65:22:11)
(28) Ethyl acetate–isopropanol–acetic acid–water (100:60:35:30)
(29) Ethyl acetate–isopropanol–water (100:60:30)
(30) *n*-Butanol–methanol–boric acid, 0.03 *M* (5:3:1)
(31) *n*-Butanol–acetic acid–water (5:4:1)
(32) Ethyl acetate–acetic acid–methanol–water (60:15:15:10)
(33) Isopropanol–ethyl acetate–water (7:1:2)
(34) Chloroform–methanol (6:4)
(35) Isopropanol–water (4:1)
(36) Acetone–water (9:1)
(37) Acetone–water–chloroform–methanol (8:0.5:1:1)
(38) Methanol–chloroform–acetone–ammonia (28 Bè) (5:2:3:2)
(39) Methanol–chloroform–ammonia (28 Bè) (6:4:0.7)
(40) *n*-Butanol–ethyl ether–water (4:5:1)
(41) *n*-Butanol–ethyl acetate–isopropanol–water (200:100:70:35)
(42) *n*-Butanol–ethyl acetate–isopropanol–water (35:100:60:30).

The R_F values relative to each solvent are reported in Table I.

Detection of the spots. After development, the chromatoplate was dried at room temperature for a short time. It was then heated in a 110° oven until the odour of the solvent could no longer be detected (~ 1 h).

TABLE I

R_F VALUES (× 100) OF CARBOHYDRATES WITH EACH SOLVENT SYSTEM LISTED IN THE TEXT

Carbohydrate	*R_F values (× 100)*																
	1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*	*12*	*13*	*14*	*15*	*16*	*17*
Ribose	23	17	42	39	32	33	39	57	53	44	20	42	48	53	36	68	84
Arabinose	21	33	39	44	37	39	40	58	48	46	22	43	43	40	45	68	83
Xylose	28	36	34	41	37	42	41	58	59	48	25	44	50	57	44	72	81
Lyxose	29	36	29	47	34	43	41	57	64	50	26	47	45	48	48	68	83
Fucose	25	49	25	51	36	42	39	59	67	54	24	48	44	46	47	65	83
Rhamnose	33	60	37	52	43	45	43	60	67	64	34	51	54	58	51	69	84
Glucose	20	36	46	52	40	38	41	62	60	55	18	46	37	39	45	68	84
Galactose	18	23	44	47	36	35	40	61	61	51	16	46	33	35	41	68	84
Mannose	26	33	32	51	46	42	45	59	57	47	25	45	54	38	49	70	81
Levulose	19	11	37	34	26	31	34	57	51	37	13	41	38	41	32	69	85
Sorbose	12	10	47	29	17	25	30	54	41	25	10	29	44	40	29	70	81
Mannoheptulose	17	13	30	38	24	33	35	55	57	41	12	38	37	39	38	71	82
Sucrose	15	36	45	56	40	37	40	65	66	55	13	48	28	29	44	69	85
Turanose	15	32	37	44	25	35	31	60	65	47	9	40	25	22	40	63	82
Maltose	11	33	41	49	31	37	35	63	56	48	8	41	26	25	43	68	82
Lactose	7	18	44	47	27	27	31	62	63	42	5	44	17	18	33	67	85
Trehalose	5	36	20	46	27	34	30	68	65	47	7	38	21	22	38	68	83
Raffinose	3	15	26	42	20	26	25	61	62	32	2	41	13	16	32	74	81
α-Methyl-D-glucoside	22	61	29	51	36	43	38	63	64	58	25	48	44	49	49	66	84
α-Methyl-D-mannoside	28	64	36	51	42	46	43	65	66	62	31	52	50	54	51	68	84

The heated chromatoplate was then sprayed with a freshly prepared solution of 20 mg of naphthoresorcinol, 10 ml of ethanol and 0.2 ml of conc. H_2SO_4. After a few minutes at room temperature, well-defined and vivid spots appear against a white background. The white background darkens if the chromatoplates are heated after spraying (as is generally advised in the literature)[3,16,21].

The colours that appear are given in Table II. It should be noted, however, that the colours change slightly depending on the concentration of the sugar.

Bidimensional chromatography

A mixture of 10 mg of each of the 20 sugars was dissolved in 2 ml distilled water. 1 μl of the solution (containing 5 μg of each sugar) was applied with a micropipette to the lower right-hand corner of the chromatoplate about 2.0 cm from each of the edges. The chromatoplate was then placed in the tank as described in the section on monodimensional separations.

The following solvent systems were employed:

a { Run I: Solvent No. 35
  { Run II: Solvent No. 13

b { Run I: Solvent No. 42
  { Run II: Solvent No. 13.

The results obtained with the above pairs of solvents are illustrated in Figs. 1 and 2.

After the first development, the chromatoplate is dried for 1 h in an oven at 40–50°.

18	*19*	*20*	*21*	*22*	*23*	*24*	*25*	*26*	*27*	*28*	*29*	*30*	*31*	*32*	*33*	*34*	*35*	*36*	*37*	*38*	*39*	*40*	*41*	*42*
39	44	43	45	71	48	47	31	13	52	60	22	29	68	54	33	9	35	30	16	19	8	5	12	25
51	51	41	57	73	68	71	51	16	44	65	30	52	63	43	53	28	55	52	36	33	18	3	24	33
57	52	48	49	44	65	62	44	21	57	74	38	40	51	64	48	22	48	46	31	35	19	4	26	39
50	53	47	53	62	63	65	51	24	55	74	41	49	53	59	52	35	50	56	36	37	18	4	26	37
62	58	46	59	67	74	75	54	25	53	74	41	55	51	59	58	49	58	66	37	35	33	3	29	42
64	65	57	62	69	78	79	57	35	63	75	52	62	54	69	63	50	62	70	50	37	34	12	41	57
59	50	35	55	68	74	74	51	8	40	66	26	53	65	44	53	29	61	54	35	19	15	2	25	33
51	48	31	56	70	63	63	43	9	40	65	24	46	60	40	48	17	52	40	22	11	11	2	17	26
55	48	40	53	60	68	66	66	14	45	69	31	53	65	42	56	23	57	52	31	20	16	3	24	36
35	47	33	45	71	40	44	19	6	46	64	16	24	68	43	26	7	33	22	9	12	5	2	8	16
33	56	42	57	64	36	33	11	4	47	66	11	18	65	45	21	6	24	16	8	6	5	1	3	8
33	41	35	26	62	46	44	44	7	43	75	25	27	46	48	33	8	29	24	10	2	5	1	9	16
61	49	26	62	71	84	82	52	6	34	66	23	55	54	34	62	20	70	59	32	9	17	1	19	27
58	59	25	47	68	71	71	49	4	34	68	25	45	31	35	51	20	51	38	19	3	9	0	13	24
58	49	41	56	68	84	82	48	3	28	69	19	52	46	25	62	23	63	53	27	9	14	0	15	21
56	50	15	51	73	67	67	34	3	24	64	12	39	42	21	46	11	52	34	13	2	10	0	6	12
68	63	18	46	70	80	77	42	3	30	75	21	43	26	65	64	25	58	41	19	4	19	11	13	20
62	55	11	39	69	66	62	20	1	23	48	12	26	29	16	52	6	47	25	9	1	7	0	3	8
75	60	52	66	69	84	82	57	29	55	69	45	62	52	62	66	60	67	74	61	45	55	4	37	53
72	63	56	66	69	85	82	59	37	62	71	53	63	54	67	66	62	62	75	61	35	51	10	44	59

TABLE II

COLOURS DEVELOPED BY SPRAYING THE CHROMATOGRAM WITH A NAPHTHORESORCINOL REAGENT

Sugar	*Colour*	*Sugar*	*Colour*
Ribose	Blue-green	Sorbose	Red
Arabinose	Blue-green	Mannoheptulose	Violet
Xylose	Blue-green	Sucrose	Crimson
Lyxose	Blue-green	Turanose	Purple-red
Fucose	Purple-red	Maltose	Gray-violet
Rhamnose	Cherry-red	Lactose	Blue-violet
Glucose	Gray-violet	Trehalose	Blue
Galactose	Gray	Raffinose	Orange-red
Mannose	Violet	α-Methyl-D-mannoside	Blue
Levulose	Dark red	α-Methyl-D-glucoside	Violet

Detection of the spots. Detection of the spots was effected in the same manner as for the monodimensional chromatography.

RESULTS AND DISCUSSION

All 42 solvent systems used with the monodimensional technique were defective in some way. It was consistently observed that the resolution of the carbohydrates was such that only a limited number of sugars could be separated. Even in the best chromatograms, where spots were well-defined and sharp, the relative differences among R_F values were never important enough to permit the identification of more than 6 to 7 sugars at a time.

It was only by resorting to 35 × 20 cm chromatoplates, developed with solvent systems 13 or 14, that we were able to obtain 9 to 10 well-separated sugars. This method, however, was time-consuming, since development took about 12 h.

As far as the shape of the spots was concerned, it was observed that systems containing pyridine produced diffused and tailed spots. Solvents containing methanol or acetone often gave similar results. These same phenomena were accentuated when the solvent mixture combined either methanol and chloroform or methanol and acetone. Some of these solvents did, however, result in good separations, but, as stated above, the shape of the spots was poor.

Developing time with the various solvents was between 2.5 and 5 h; the solvents that permitted a shorter developing time (whose principal components were always methanol or acetone, either combined with water or chloroform, or combined with each other) consistently yielded deformed and diffuse spots.

All the solvents that contained boric acid gave well-shaped spots, but low R_F values and poor separation.

The best results were obtained with solvent systems containing isopropanol, which favours the migration of carbohydrates with a wider spectrum of R_F values. Addition of acetic acid improved separation and sharpened the spots.

The influence of other solvents, such as *n*-butanol, ethyl acetate, ethanol, etc., is more difficult to assess.

The choice of an 0.03 *M* boric acid solution for the impregnation of the silica gel was made on the basis of the fact that lower molarity solutions did not have a good effect on the shape of the spots and higher molarity solutions only reduced the R_F values.

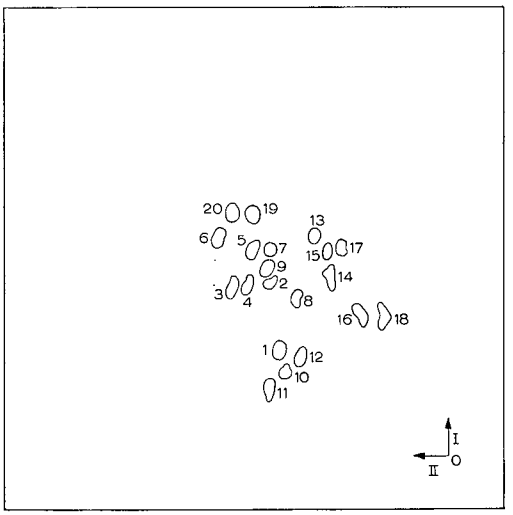


Fig. 1. Bidimensional chromatography of carbohydrates. 1 = Ribose; 2 = arabinose; 3 = xylose; 4 = lyxose; 5 = fucose; 6 = rhamnose; 7 = glucose; 8 = galactose; 9 = mannose; 10 = levulose; 11 = sorbose; 12 = mannoheptulose; 13 = sucrose; 14 = turanose; 15 = maltose; 16 = lactose; 17 = trehalose; 18 = raffinose; 19 = α-methyl-D-glucoside; 20 = α-methyl-D-mannoside. 1st run = isopropanol–water (4:1); 2nd run = *n*-butanol–ethyl acetate–isopropanol–acetic acid–water (35:100:60:35:30). The remarkable separation of monosaccharides and oligosaccharides should be noted.

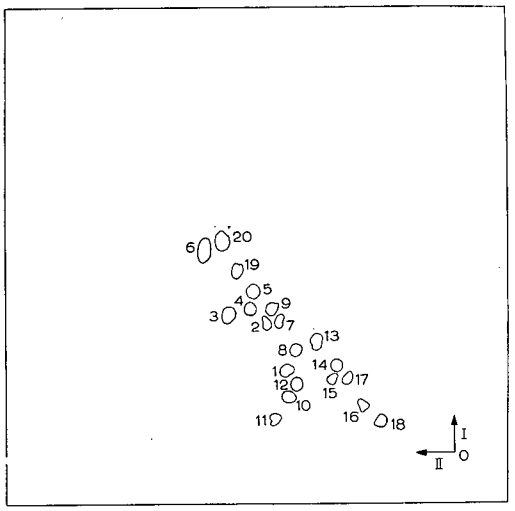


Fig. 2. Bidimensional chromatography of carbohydrates: Same sugars as in Fig. 1. 1st run = *n*-butanol–ethyl acetate–isopropanol–water (35:100:60:30); 2nd run = *n*-butanol–ethyl acetate–isopropanol–acetic acid–water (35:100:60:35:30).

Double development in one direction was also tried[7], but the results did not justify the doubling of the developing time. Modifications of the positions of the spots were obtained, but, very often, the improvement in separation of a specific pair of sugars was counterbalanced by the reduced separation of others.

This method can be successfully applied only to the separation of a few specific mixtures of carbohydrates.

A 30 cm double development run gives better results, but the time needed for development is about 24 h (which cancels the time advantage of the TLC method itself), and the sugars with higher R_F values are excessively diffused.

Continuous flow chromatography[13] was even less satisfactory for the separation of a complex mixture of carbohydrates.

Excellent results were obtained with the bidimensional technique, which, we think, greatly increases the use that can be made of TLC in carbohydrate research. All possible combinations of the best solvents described in Table I were tried.

The best were as shown in Table III.

TABLE III

BEST SOLVENT SYSTEMS FOR BIDIMENSIONAL TLC OF CARBOHYDRATES

Solvent mixture		*Pattern of the spots*
First run	*Second run*	
(a) No. 35	No. 13	see Fig. 1
(b) No. 42	No. 13	see Fig. 2
(c) No. 6	No. 13	similar to (a)
(d) No. 29	No. 13	similar to (b)
(e) No. 41	No. 13	similar to (b)
(f) No. 30	No. 13	similar to (a)

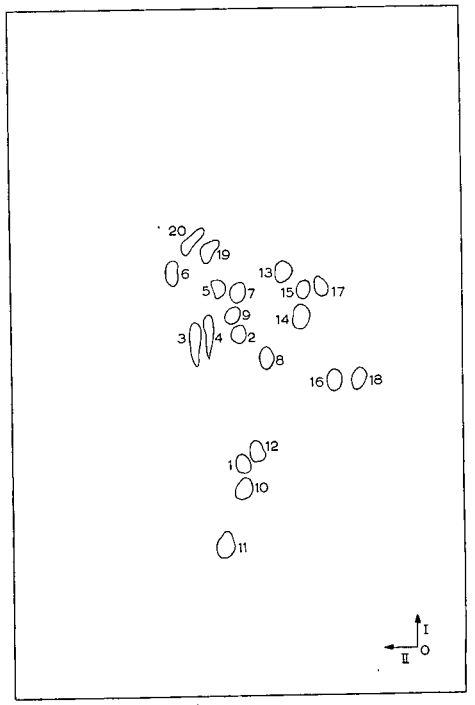
Of all these combinations, the best by far were the first two (also described in the Experimental section).

We observed that if a solvent system containing acetic acid is used in the first development, separation of the spots is impaired, since, under experimental conditions, it is very difficult to completely eliminate the acetic acid which is strongly bound to the silica gel. On the other hand, as seen above, each of the best combinations uses solvent No. 13, which contains acetic acid, for the second run. If solvent No. 13 is substituted by any other solvent system in the second run, the spots obtained are not sharp or well-shaped.

The apparent contradiction implicit in the above statements can probably be explained by the fact that the molecules of acetic acid bound to the silica gel during the first run, occupied a sufficient number of sites to disturb the normal partition on the adsorbent of the solvents of the second run.

Certain sugars, or oligosaccharides, present a special problem in monodimensional thin-layer chromatography: they have very similar mobilities with the majority of the solvents used. When a specific solvent, such as, for example, No. 25, succeeds in separating arabinose from mannose and levulose (one of the most difficult triads to resolve), it cannot separate glucose, sucrose and lyxose from arabinose, nor can it separate raffinose from levulose.

Even our best solvent for a monodimensional run, No. 13, separates the majority of the carbohydrates studied into 4 spots: (1) arabinose, mannose, sorbose, fucose, lyxose, α-methyl-D-glucoside; (2) glucose, levulose, mannoheptulose; (3) sucrose, maltose, turanose; (4) xylose, α-methyl-D-mannoside, ribose.



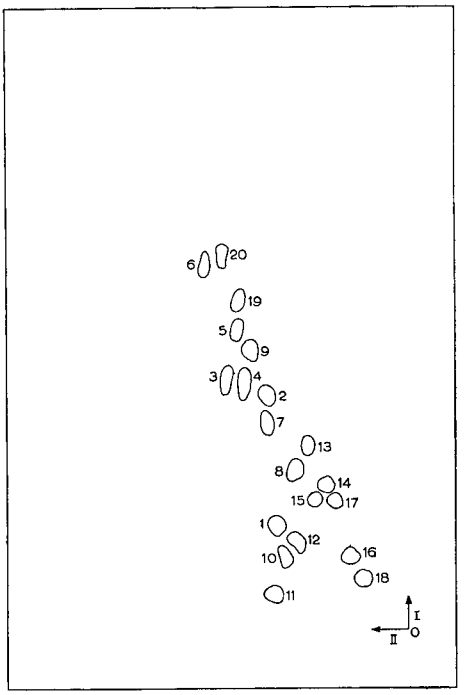


Fig. 3. Bidimensional chromatography of carbohydrates on a 20 × 30 cm chromatoplate: Sugars and solvents same as in Fig. 1.

Fig. 4. Bidimensional chromatography of carbohydrates on a 20 × 30 cm chromatoplate: Sugars and solvents same as in Fig. 2.

For this reason, monodimensional chromatography is too limited to be used as a tool in qualitative analysis involving complex sugar mixtures. Our experience has shown that even the comparison of several chromatoplates developed with different solvents is insufficient for the separation and identification of an unknown complex mixture.

This is obviously not a problem in bidimensional chromatography, which is clearly a more advanced and complete technique. But although the number of sharply separated carbohydrates is much higher than with the monodimensional method, the limitation in this case arises from the fact that slightly larger amounts of each sugar are needed to compensate for the greater diffusion of the spots, and, when a complex sugar mixture is used, the total amount of the substance placed at the origin exceeds the capacity of the silica gel. Different brands of silica gel have slightly different capacities, but the average maximum load on a layer of 250 microns should not exceed 35–50 μg.

Experimentally, we were able to load a 350 micron layer with up to 120 μg of substance, and were still able to obtain good separation of the sugars involved. In fact, we advise the use of a layer thicker than the standard. Commercially prepared chromatoplates are not suitable because the layer is too thin and therefore low in capacity.

A layer thicker than 300–350 microns is not advisable, however, if some of the sugars contained in the mixture are present in a lesser amount than 2–3 μg, because of the difficulties involved in revealing small quantities in thick layers.

It should be noted that quantities of some particular sugars should not exceed specific amounts (4–5 μg for a 350 micron layer) since, even in bidimensional chromatography, they tend to run close together. The sugars with this tendency in the chromatogram illustrated in Fig. 1 are: mannoheptulose–levulose; maltose–trehalose; glucose–arabinose–mannose; rhamnose–α-methyl-D-mannoside.

In short, the quantities of some of the single sugars that can be applied to a chromatoplate, apart from the total capacity of the gel, is a limiting factor that must be added to those mentioned above, when sugars with very similar mobilities are involved.

This limitation can be partially or wholly eliminated by resorting to bidimensional chromatography on a rectangular 30 × 20 cm plate (Figs. 3 and 4).

In this case, the carbohydrates separate much better than with the standard 20 × 20 cm plate. It is necessary, however, for the first run to be the longer of the two. The positions of the sugars, in this case, are similar to the corresponding positions on the standard plate. We strongly advise this modification of the technique whenever the quantities of those sugars that tend to run closely together vary widely in the sample mixture.

For the detection of spots, it is preferable to use the naphthoresorcinol reagent, because the great variety of colours it produces particularly helps in the identification of the carbohydrates (see Table II). This is especially pertinent when two sugars are very close together.

For specific applications, such as the differentiation of aldoses and ketoses, or of the aldohexoses, aldopentoses and ketohexoses, several other reagents have been reported[11,18].

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CHROM. 3301

QUANTITATIVE THIN-LAYER CHROMATOGRAPHY OF NEUTRAL LIPIDS USING SEMI-SPECIFIC COLORIMETRIC AND TITRIMETRIC TECHNIQUES*

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SUMMARY

Quantitative thin-layer chromatography of standard and tissue neutral lipids was accomplished by eluting the separated lipids from the silica gel and utilizing semi-specific colorimetric and titrimetric methods. Reproducibility was good and recoveries of the various neutral lipids were between 94–101 %. Silica gel blank readings were reduced to low values by special washing of the silica gel.

INTRODUCTION

Quantitative thin-layer chromatography of neutral lipids has received considerable attention because of the speed and ease with which small samples can be separated[1–12]. Most of these quantitative methods are based on densitometric or gravimetric techniques[1–8]. Although these are relatively simple and accurate methods, they depend on both the complete conversion of lipid spots to elemental carbon and the precise separation of various lipid groups in order to avoid measuring two or more lipid substances in a given spot.

Additional quantitative methods have been proposed in which the separated lipids are first eluted from the absorbent and subsequently measured by spectroscopic means[9–12]. The use of individual chemical reactions for each group of neutral lipids should make their quantification more specific[7,10,11]. Most reports utilizing elution and spectroscopic techniques have not been published primarily to describe methodology. They therefore have only been concerned with quantifying a single specific neutral lipid[13–15]. Even in the two investigations concerned with quantitative methodology, not all the neutral lipids were studied and the recovery of some lipids was not complete[10,11]. In addition, no information was given regarding the range of various amounts of each neutral lipid that could be quantified. Further, no studies have systematically attempted to compare the thin-layer quantitative methods with silicic acid column chromatography techniques.

* This work was supported by a grant from the United States Public Health Service (AM 08569-03).

The present study delineates a method utilizing previously described semi-specific colorimetric and titrimetric determinations of free and esterified cholesterol, free fatty acids, and triglycerides after they have been separated and eluted from thin-layer chromatoplates. Recoveries of 94–101 % were achieved over a range of 25–200 γ of each neutral lipid. These quantitative analyses by thin-layer chromatography were found to agree closely with silicic acid column chromatography techniques.

MATERIALS AND METHODS

Reagents

The absorbent for thin-layer chromatography was Silica Gel H[a]. Silicic acid (100 mesh) for column chromatography was obtained from Mallinckrodt Chemical Company[b]. The solvents were reagent grade and all except glacial acetic acid and 95 % ethanol were redistilled in glass prior to use[c].

Standards and tissue lipids

Neutral lipid standards were used individually and in various combinations to evaluate their recovery from the chromatoplates. Cholesterol esters from Applied Science Labs, Inc.[d] included cholesteryl myristate, palmitate, stearate, oleate and linoleate. Triolein and tripalmitate were purchased from Eastman Organic Chemicals[e] and palmitic, oleic, linoleic and stearic acids were obtained from Mann Research Labs, Inc.[f]. All standards were found to be at least 98 % pure by thin-layer or gas–liquid chromatography.

A mixture of reference neutral lipids[g] (Model Mixture No. 1) was used to identify the positions of the aforementioned neutral lipid standards or mixed tissue lipids with each chromatographic run. The mixture contained approximately equal weights of cholesterol, cholesterol oleate, triolein, oleic acid and methyl oleate. With the solvent system employed, methyl oleate and triolein were not separated (see Fig. 1).

Mixed tissue lipids were extracted from experimentally produced rabbit skin xanthomas with chloroform–methanol (2:1, v/v), as previously described[16]. Non-lipid impurities were removed by the method of FOLCH, LEES AND SLOANE-STANLEY[17]. The neutral lipids obtained were quantified by the thin-layer chromatography methods described and the results compared with an established quantitative technique utilizing silicic acid column chromatography[18].

Preparative washing of Silica Gel H

250 g of Silica Gel H was placed in a porcelain Buchner filter funnel (Coors No. 4) fitted with two disks of Whatman No. 2 filter paper. The funnel was placed on a 4 l filter flask and the following wash solutions were slowly poured over the absorbent

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[a] Brinkmann Instruments, Inc., Burlingame, Calif.
[b] Mallinckrodt Chemical Company, New York City, N.Y.
[c] Baker & Adamson, General Chemical Division, Morristown, N.J., Merck & Co., Inc., Rahway, N.J.
[d] Applied Science Labs., Inc., State College, Pa.
[e] Eastman Organic Chemicals, Rochester, N.Y.
[f] Mann Research Labs, Inc., New York City, N.Y.
[g] Hormel Foundation, Austin, Minn.

with suction applied: 0.5 l hexane–diethyl ether–glacial acetic acid (90:30:2, v/v/v); 1 l methanol–chloroform–formic acid (2:1:1, v/v/v); 0.5 l diethyl ether; 1 l methanol–chloroform–formic acid (2:1:1, v/v/v); and 2 l of glass-distilled water. Suction was continued until water no longer dripped through the funnel. The washed silica gel was then spread in a pyrex tray lined and covered with aluminum foil and dried at 110° for 48 h. Any large silica gel lumps were broken up after the first 24 h of drying.

Preparation of plates

36 g of washed silica gel was placed in a Waring Blendor (Model No. PB-5A with high and low speed controls) containing 64 ml of glass-distilled water. The resultant slurry was mixed at low speed for 3 min. Mixing for longer periods of time or at higher speed resulted in slurries with thin consistency which prevented optimal spreading on the chromatoplates.

This slurry was usually sufficient to coat five 20 × 20 cm pyrex plates with a 0.25 mm layer of silica gel using the Desaga adjustable spreader. The plates were first air dried and then activated for 1 h at 110° just prior to use.

Chromatographic procedures

Neutral lipid standards or the experimental xanthoma lipids were applied in chloroform solution with a microliter syringe* as narrow streaks, $^3/_4$ in. from the

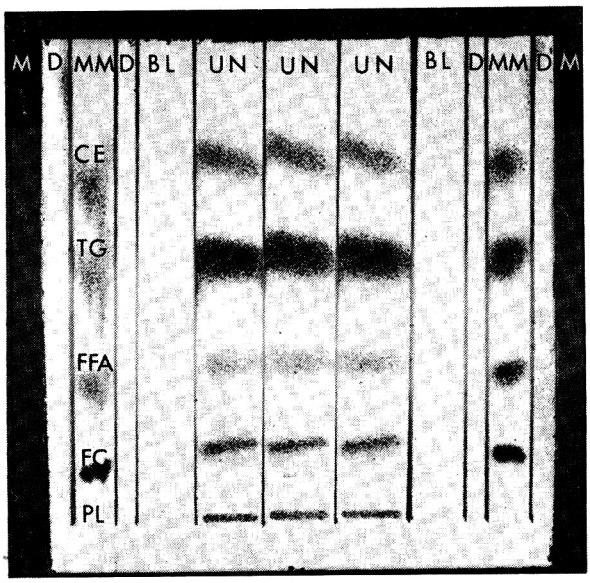


Fig. 1. A picture of the chromatographic separation of experimental rabbit xanthoma (three middle lanes marked "UN") and reference model mixtures (outer $^1/_2$-in. wide lanes marked "MM") shown to display the arrangement of the various lanes discussed in the text. Solvent used was hexane–ether–acetic acid (90:30:2). Detection for this picture was with sulfuric acid spray and charring. Lanes "BL" are blanks, and "D" are divider spaces. "M" is the margin of silica gel scraped from the sides and top of the chromatoplate to allow the teflon spacer of the saturation chamber to fit tightly against it. The streaked samples of rabbit xanthoma and the model mixtures are separated into the major neutral lipids: CE = cholesterol ester; TG = triglyceride; FFA = free fatty acids; and FC = free cholesterol. Phospholipids, PL, are seen in the xanthomas at theorigin.

* Hamilton Co., Inc., Whittier, Calif.

bottom of the plate, on three 1-in. wide lanes in the center of the chromatoplate (Fig. 1, lanes marked "UN"). On either side of the three middle lanes, 1-in. wide lanes served as blanks (Fig. 1, "BL"). Outside the blanks were strips of $^1/_4$, $^1/_2$ and $^1/_4$ in. in width. To the $^1/_2$-in. lanes a sample of the reference neutral lipid model mixture was applied (Fig. 1, "MM") to assist in identifying the positions of the various neutral lipids separated in the central lanes. The $^1/_4$-in. lanes on each side of the model mixture reference lanes were divider spaces (Fig. 1, "D").

The plates were developed in a saturation chamber described by PARKER AND PETERSON[19]. A $^1/_2$-in. wide, 3-sided teflon spacer was placed between the chromatoplate and a similarly sized glass cover plate lined with a piece of filter paper. The whole unit was held together with metal clips. To allow the teflon spacer to fit snugly against the chromatoplate, a $^1/_2$-in. wide margin of silica gel was scraped from the sides and top (Fig. 1, "M").

The saturation chamber was placed in a glass covered tank (27 × 29 × 10 cm) containing 183 ml of hexane–diethyl ether–glacial acetic acid (90:30:2, v/v/v). The solvent was allowed to rise to the top of the adsorbent with an average running time of 30 min.

Detection of chromatographed lipid spots

Since iodine vapors and other indicators such as Rhodamine G and fluorescein interfered with the triglyceride and fatty acid determinations they were not used. Instead, the central lanes, blanks, and inner divider spaces were covered with three layers of paper towels leaving only the outer "MM" and divider spaces exposed. Weights were placed on the edges of the towels and small amounts of concentrated sulfuric acid were sprayed on the uncovered lanes. After removing the towels, the sprayed lanes containing the lipid model mixture were charred with a Fisher microburner to identify the various neutral lipids. Horizontal lines were then drawn across the three center lanes and blanks in positions corresponding to the upper and lower poles of the triglyceride and free fatty acid spots. These areas were scraped individually with a glass slide into appropriate containers after the sulfuric acid sprayed lanes on one side of the plate were carefully wiped off the glass. This prevented sulfuric acid contamination of the scraped silica gel spots of the central lanes from otherwise displaying spurious fatty acid titrations.

The partially scraped chromatoplate was then exposed to iodine vapors to locate the positions of the remaining free and esterified cholesterol spots and to check on the completeness with which the triglyceride and free fatty acid spots were removed. After the iodine had vaporized, the free and esterified cholesterol and their appropriate blanks were scraped into individual receptacles.

Analysis of individual neutral lipids

Cholesterol esters. These compounds (25–200 γ) with their corresponding silica gel blanks were scraped into 20 ml culture tubes (15 × 150 mm). One ml of 1.0 N (5.6 %) KOH in 95 % ethanol (made fresh just prior to use) was added to the tubes which were covered with glass marbles and incubated at 80–85° in a constant temperature water bath for 1 h. The water level in the bath was maintained at the same height as the level of the liquid in the tubes to allow adequate reflux. After cooling, 3 ml of hexane was added, followed by 1.5 ml of distilled water. The tubes were shaken

for 20 sec after capping with polyethylene stoppers (size 0). Appropriate samples (usually 1 or 2 ml) were taken from the hexane layer, dried completely under nitrogen, and quantified by the ferric chloride method of COURCHAINE *et al.*[20], using the directions for total cholesterol determinations. Free cholesterol standards (25–200 γ) were carried through the entire procedure. Results were expressed as the amount of cholesterol in the cholesterol esters.

Free cholesterol. Four ml of glacial acetic acid were added to the samples and blanks, after they were scraped into 20 ml culture tubes, as well as to reagent blanks and standards of free cholesterol (over a range of 25–200 γ). The tubes were mixed on a Vortex Mixer, centrifuged for 10 min at 300 $\times$ *g* and 3 ml of the acid were taken for quantification using the COURCHAINE[20] method for total cholesterol determination.

Free fatty acids. The gel containing these lipids and the corresponding blanks were scraped into 12 ml conical centrifuge tubes. The fatty acids were eluted from the gel with 4 ml ether–methanol (9:1, v/v), followed by two separate 3 ml portions of chloroform. After the addition of each eluent, the tubes were covered with polyethylene stoppers, shaken 20 sec, and centrifuged for 5 min at 300 $\times$ *g*. The supernatants were entirely removed with a volumetric pipette without disturbing the silica gel and combined in 15 ml conical centrifuge tubes (without graduations) and dried under nitrogen. After twice rinsing the sides of the tube with small volumes of hexane, the fatty acids were quantified by the titration method of DOLE[21]. Palmitic acid was used as a standard over the range of 25–200 γ.

Triglycerides. These compounds and their corresponding blanks were scraped into glass funnels specially made by fusing a fritted glass funnel (porosity M, 2 ml capacity, 10 mm diameter disk) to a Kimax funnel. The lipid was eluted into 20 ml culture tubes (15 $\times$ 150 mm) with two portions of chloroform, 2.5 ml, and then two additional portions of diethyl ether (2.0 ml). These eluents were pushed through the silica gel in the funnel by nitrogen delivered through a rubber stopper perforated in the center by glass tubing. The combined eluents were dried under nitrogen and the sides of the tubes were rinsed twice with small volumes of ether. All samples, reagent and silica gel blanks, and standards (25–125 γ) where hydrolyzed by adding 1 ml of 0.4 % KOH in 95 % ethanol (made just prior to use), covering the tubes with glass marbles and heating for 30 min at 70–80° in a constant temperature bath[22]. The water level in the bath was kept at the same height as the level of liquid in the tubes for adequate reflux. After hydrolysis, 0.5 ml of 0.2 *N* H_2SO_4 was added and the samples stirred on a Vortex Mixer and placed in a boiling water bath for 15 min to remove the alcohol. Glycerol was then analyzed[22,23].

The optical density or titrimetric determinations obtained from the appropriate silica gel blanks were always subtracted from the readings derived from each lipid spot.

RESULTS

Quantification of neutral lipid standards is displayed in Figs. 2–5. For each there is a linear relationship between amounts of lipid used and optical density (or in the case of free fatty acids, μmoles of NaOH). This applies when the standards are placed either directly in test tubes (solid line) or are chromatographed (dotted line) and then quantified. The exact optical density readings are given in the inset tables accompanying each figure.

The silica gel blanks referred to Figs. 2–5 represent the optical density or titrimetic readings greater than that of the reagent blank readings. The optical density of silica gel blanks for free and esterified cholesterol average 0.003 ± 0.002 (Figs. 2, 3) which is the equivalent of 2–3 γ of cholesterol, while the triglyceride blanks average 0.030 ± 0.007 (Fig. 4) which represents 4–5 γ of glycerol. Similarly, the free fatty acid silica gel blanks correspond to no more than 5 γ of free fatty acid.

Neutral lipids extracted from experimentally induced cutaneous xanthoma were quantified by thin-layer chromatography in order to test the method on a mixture of unknown tissue lipids. The results obtained were compared with analysis of the lipids from another portion of the same extract after separation by silicic acid

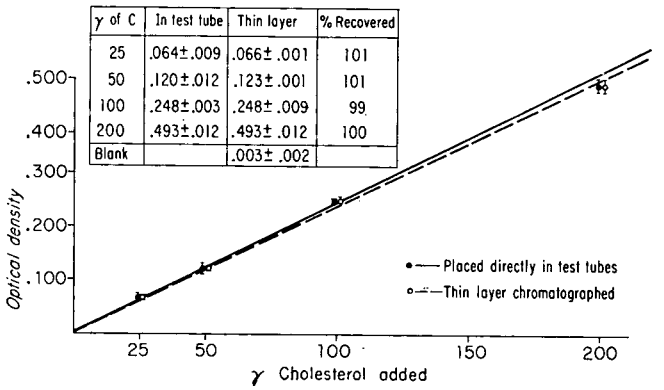


Fig. 2. When optical density readings shown in the table were plotted against increasing concentrations of free cholesterol standard, a straight line was obtained whether the colorimetric reaction was performed on the standard placed directly in test tubes (solid lines) or chromatographed (dashed line). The bars at each point represent one standard deviation and the points represent the mean of four runs. The two lines were almost superimposable reflecting the recoveries of 99–101% shown in Table I. The silica gel blank optical density reading (mean and standard deviation from 20 lanes) was small, but has been deducted from the chromatographed standards' readings before the values in the table and graph were used.

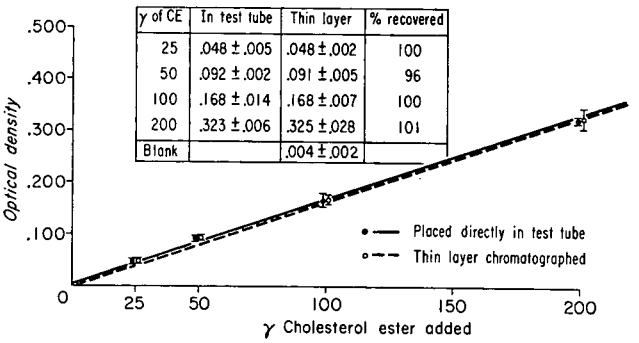


Fig. 3. A mixture of approximately equal amounts of cholesterol myristate, palmitate, stearate, oleate and linoleate was used to display satisfactory recovery of cholesterol esters. On the graph the points were the average of four runs and the bars represent one standard deviation. The linear relationship between concentration of the cholesterol ester standards added and optical density readings were plotted according to the figures given in the table. The silica gel blank optical density reading was small and the mean (20 determinations) has been deducted from the chromatographed standards' readings before inclusion in the table or graph.

column chromatography. Table I summarizes these results. As can be seen, there is close agreement between the two methods.

TABLE I

COMPARISON OF THE QUANTITATIVE THIN-LAYER CHROMATOGRAPHY METHOD TO THAT OF SILICIC ACID COLUMN CHROMATOGRAPHY[a]

Method of chromatography	*Free cholesterol (mg/ml of xanthoma lipid sample)*	*Cholesterol ester (mg/ml of xanthoma lipid sample) (as free cholesterol)*	*FFA (μg/ml of xanthoma lipid sample)*	*TG (mg/ml of xanthoma lipid sample) (as glycerol)*
Thin-layer	0.917 ± 0.030	0.539 ± 0.049	152.0 ± 12.4	15.19 ± 0.30
Column	0.886 ± 0.044	0.573 ± 0.011	161.0 ± 12.2	15.62 ± 0.26
% Recovery[b]	103%	94%	94%	97%

[a] Results of one experiment with each lipid determination run in quadruplicate.

[b] Amount of lipid recovered by thin-layer chromatography when compared with recovery of lipid from the same sample after silicic acid column chromatography.

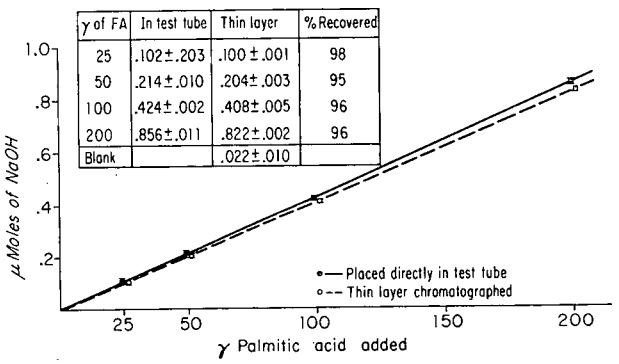


Fig. 4. Recovery of free palmitic acid from chromatoplates was depicted in this graph and table representing the average and one standard deviation of four runs. Between 96–98% recovery was achieved when 25–200 γ of free fatty acid was analyzed. The silica gel blank was the average of 20 determinations.

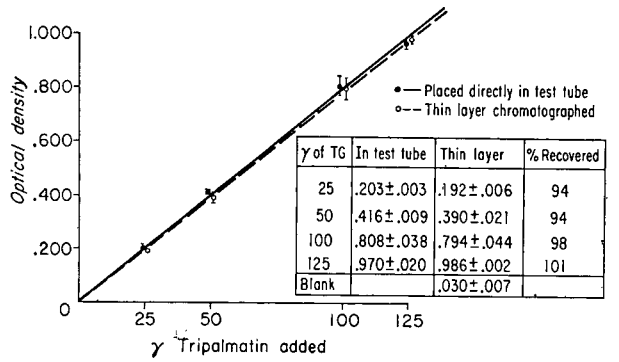


Fig. 5. Tripalmitin recovery from thin-layer plates was 94–101% when 25–125 γ of the standard was analyzed. The graph and table represent the mean and one standard deviation of six runs. The silica gel blank was an average of 20 determinations.

DISCUSSION

Quantitative thin-layer chromatography is particularly useful in dealing with small samples of lipid where laborious biochemical and chromatographic separatory methods are best avoided. The present method provides a means for precise quantification of 25–200 μg of each major group of neutral lipid. The results of this thin-layer method are comparable to those obtained by silicic acid column chromatography.

Elution of the separated neutral lipids avoids the need for special densitometry equipment. Subsequent quantification utilizing semi-specific colorimetric and titrimetric techniques assures measurement of a given lipid even when two or more lipids may reside in a given spot.

Complete elution of the various neutral lipids was achieved with a variety of solvents and methods. Free cholesterol was removed from the gel with glacial acetic acid, while esterified cholesterol was first hydrolyzed and the resulting free cholesterol was extracted with hexane. It was necessary to saponify the cholesterol esters prior to using COURCHAINE's method since cholesterol stearate was not completely soluble in the glacial acetic acid. Elution of free fatty acids required sequential shaking with mixtures of ether, methanol and chloroform. Triglycerides seemed particularly difficult to elute and required the use of simply constructed funnels to hold the silica gel samples while chloroform and ether were allowed to trickle through the funnel. Using this method, recoveries of 94–101 % were achieved. When similar eluents were shaken with the same triglyceride standards on silica gel in tubes, recoveries of only 85–92 % were achieved. These latter recovery figures are similar to those reported by GLOSTER AND FLETCHER[11].

Care must be given to the saponification procedures used for cholesterol esters and triglycerides. Complete hydrolysis depended on maintaining the original volume of the KOH–ethanol solution added to the tubes. If evaporation occurred, hydrolysis was incomplete and results were erratic. To prevent evaporation, long tubes were used (15 × 150 mm culture tubes) and the depth of the water bath was maintained at the same height as the top of the KOH–ethanol solution to allow maximal reflux within the containers.

This method has proved of value to date in accurately analyzing neutral lipids obtained from rabbit and human sera, xanthoma and blood vessels.

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CHROM. 3296

SYSTEMATIC IDENTIFICATION OF PSYCHOTROPIC DRUGS BY THIN LAYER CHROMATOGRAPHY. PART II

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SUMMARY

A thin-layer chromatographic technique was applied to ether extracts of blood and urine samples from patients receiving clinical doses of chlorpromazine, thioridazine, chlorprothixene, meprobamate, amitriptyline, nortriptyline, imipramine and desipramine.

Chromatography of the extracts of blood samples from patients receiving clinical doses of chlorpromazine or thioridazine were negative. Similar specimens extracted after hemolysis of the sample showed the presence of unmetabolized drug and metabolites.

In urine extracts, phenothiazine derivatives administered in daily doses lower than 75 mg were not detectable as unchanged drug; only the presence of metabolites was observed.

Urine extracts from patients on a stabilized drug dose of each of the compounds examined showed typical chromatographic patterns. The R_F values of metabolites constantly found in the samples examined are reported.

In order to demonstrate the applicability of the thin-layer chromatographic technique to the isolation and identification of psychotropic drugs and their metabolic products in biological materials, the method previously described[1] was applied to blood and urine extracts obtained from patients receiving clinical doses of some of these drugs.

The object of this study was: to determine whether the unmetabolized drug could be detected in blood and urine samples after administration of clinical doses; to establish the minimum dose that yields a detectable amount of unchanged drug; to ascertain if the metabolic products of a given drug form typical chromatographic patterns which may be used for the identification of the parent drug; and to achieve the isolation of drugs, composing a heterogeneous group of psychotropic agents, in two chromatographic systems on the basis of their different comportment in respect to the Folin–Ciocalteau reagent.

The following drugs, representing three pharmacological classes of psychotropic agents, phenothiazines, nonphenothiazine tranquilizers and stimulants of the central nervous system were investigated: chlorpromazine, thioridazine, chlorprothixene, meprobamate, amitriptyline, nortriptyline, imipramine and desipramine.

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EXPERIMENTAL

Materials

The subjects were, male and female, schizophrenic patients from Cleveland State Hospital. Blood and urine specimens were collected between one and three hours after ingestion of the drug and kept refrigerated until extracted. This was within three hours. Care was taken to ensure that the specimens were not exposed to sunlight. Urine samples provided by patients who were not receiving any psychotropic drug were used as controls. Control blood samples were made up as a pool, from specimens supplied by the Pathology Department of S. Vincent Charity Hospital.

Extraction method

25 ml of urine adjusted to pH 9–10 with 6 *N* sodium hydroxide or 5–7 ml of whole blood to which was added 1 ml of 6 *N* sodium hydroxide were extracted with two 50 ml portions of ethyl ether. The ether extracts were combined and filtered. One portion of ether (40–45 ml) was dried with 3 g of anhydrous sodium sulfate, filtered and the ether evaporated under forced air at room temperature (Extract A).

The second portion of ether was extracted with two 7 ml portions of 6 *N* hydrochloric acid. The acid solutions were combined, made alkaline (pH 9–10) with 6 *N* sodium hydroxide and re-extracted with two 50 ml portions of ether. The ether extracts were combined, dried with sodium sulfate and the ether evaporated under forced air at room temperature (Extract B). The residues from the two extractions were dissolved in a few drops of methanol. Aliquots of the two alcohol solutions were spotted on the chromatoplate.

Chromatographic method

The chromatoplates, chromatographic systems and detecting reagents were as previously described[1]. The chromatographic systems were:

System I: methanol–12 *N* ammonium hydroxide (100:1.5)
System II: cyclohexane–diethylamine–benzene (75:20:15)
System III: acetone
System IV: chloroform–methanol (90:10)
System V: benzene–ethanol–12 *N* ammonium hydroxide (95:15:5).

Plates developed in systems I and V were coated with a mixture of Silica Gel G and water. Systems II, III and IV were used with Silica Gel G plates prepared with 0.1 *N* sodium hydroxide.

Procedure

A spotted plate containing the two extracts (A and B) from the unknown sample was chromatographed in system I and sprayed with the Folin–Ciocalteau reagent. The observation of the resulting color(s) limited the investigation to a group of drugs yielding the same color reaction, while the R_F value obtained for the unknown further reduced its identification within the limits of a few drugs.

A second plate was then spotted with the remaining alcoholic solutions (A and B) and with the standard solutions of those drugs that showed similar chromatographic and chromogenic comportment. The plate was developed in a second chro-

matographic system selected according to the properties shown by the unknown in system I* and then sprayed with a suitable detecting reagent.

RESULTS AND DISCUSSION

Chromatography of phenothiazines

Urine. Unchanged phenothiazines were found in both extracts A and B, or only in extract B, depending on the amount of drug ingested. Urine extracts from patients receiving small amounts of phenothiazine derivatives (from 5 to 75 mg *pro die*) did not yield any unmetabolized drug. The various metabolites present did not form any typical chromatographic pattern.

In patients on a stabilized drug dose (receiving the same medication for at least 6 weeks) the drug metabolism appeared to be qualitatively constant as shown by similar chromatographic patterns obtained from urine extractions repeated at intervals of a week on the same subjects. Variations in the excretion of the unmetabolized drug between subjects who were given the same doses of medication were noted only in a few cases.

The data collected relative to thioridazine and chlorpromazine are shown in Tables I and II, respectively. To overcome R_F value variations due to uncontrollable experimental conditions, the R_F values of the various metabolites are also reported as relative to the distance moved by the parent drug.

TABLE I

R_F VALUES OF THIORIDAZINE AND ITS METABOLITES

	System I		*System II*		*System III*		*System IV*		*System V*	
	R_F	*Rel.* R_F	R_F	*Rel.* R_F	R_F	*Rel.* R_F	R_F	*Rel.* R_F	R_F	*Rel.* R_F
Thioridazine	0.54	1.00	0.62	1.00	0.23	1.00	0.53	1.00	0.73	1.00
Spot 1*	0.37	0.68	0.53	0.85	0.04	0.17	0.39	0.73	0.65	0.89
Spot 2	0.23	0.43	0.17	0.27			0.32	0.60	0.56	0.77
Spot 3			0.06	0.10			0.18	0.34	0.39	0.53
Spot 4									0.27	0.37
Spot 5									0.14	0.19

* The spots are listed in decreasing values in respect to the position of thioridazine.

TABLE II

R_F VALUES OF CHLORPROMAZINE AND ITS METABOLITES

	System I		*System II*		*System III*		*System IV*		*System V*	
	R_F	*Rel.* R_F	R_F	*Rel.* R_F	R_F	*Rel.* R_F	R_F	*Rel.* R_F	R_F	*Rel.* R_F
Chlorpromazine	0.56	1.00	0.65	1.00	0.30	1.00	0.50	1.00	0.73	1.00
Spot 1*	0.42	0.75	0.34	0.52	0.20	0.66	0.27	0.54	0.53	0.73
Spot 2	0.38	0.68	0.22	0.34	0.06	0.20	0.18	0.36	0.30	0.41
Spot 3	0.27	0.48	0.15	0.23			0.08	0.16	0.17	0.23
Spot 4	0.17	0.30	0.05	0.08					0.07	0.10

* The spots are listed in decreasing values in respect to the position of chlorpromazine.

* See Part I, Tables V, VI and VII.

The results reported are relative to metabolites constantly found in urine extracts from patients on a stabilized drug dose varying from 75 mg to 600 mg *pro die* of thioridazine and from 100 mg to 1200 mg *pro die* of chlorpromazine.

The lower limit of detection of unmetabolized drug corresponded to a drug intake of 75 mg *pro die* of thioridazine and 75 mg *pro die* of chlorpromazine.

Fig. 1 reproduces a chromatoplate developed in system V, containing urine extract (B) from a patient receiving 100 mg of thioridazine *pro die* and from a patient receiving 150 mg of chlorpromazine *pro die*.

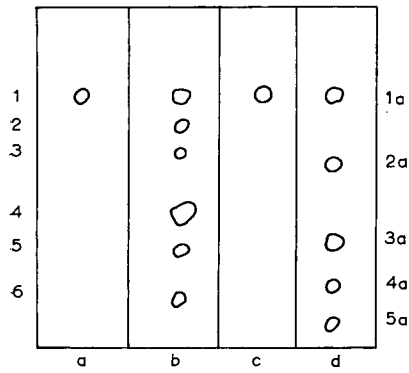


Fig. 1. Thin-layer chromatography of phenothiazines from urine extracts. (a) Thioridazine standard solution; (b) urine extract B from a patient receiving 100 mg *pro die* of thioridazine; (c) chlorpromazine standard solution; (d) urine extract B from a patient receiving 150 mg *pro die* of chlorpromazine. Chromatographic system: benzene–ethanol–12 *N* ammonium hydroxide (95:15:5). Detecting reagent: Folin–Ciocalteau reagent. Colors of the spots: spots 1 and 2 blue-green, 3 and 6 pink, 4 and 5 fuchsia, 1a and 2a fuchsia, 3a and 4a violet, 5a pink.

Blood. With a drug intake of 600 mg of thioridazine or 800 mg of chlorpromazine *pro die*, both the extracts A and B from 5–7 ml of whole blood were negative. Urine specimens collected simultaneously from the same patients and examined using the same procedure confirmed medication intake as shown by the chromatographic patterns obtained. Plates spotted with blood extract B from a patient receiving only chlorpromazine (1200 mg *pro die*) and developed in system IV showed, sporadically, when sprayed with the Folin–Ciocalteau reagent, a reddish-pink spot with an R_F value corresponding to 0.39–0.41. Negative results were also obtained when a pool of 15 ml of whole blood from patients who were given 600 mg of thioridazine *pro die* was extracted according to the procedure described and also on using different extraction procedures and chromatographic methods reported in literature[2,3].

No attempt was made to verify the eventual presence of unmetabolized drug by spectrophotometric studies after elution of the substance from the silica gel, as the object of this investigation was to prove the validity of the thin-layer chromatographic technique and its limits.

Surprisingly positive were, on the other hand, the results obtained when 5 ml of whole blood from patients receiving daily doses of 200–600 mg of thioridazine or 200–800 mg of chlorpromazine were hemolyzed by shaking the sample with 20 ml of distilled water, extracted and the extracts chromatographed according to the procedure described. Unchanged drug was found in 8 of the 10 samples examined in extract B, while the presence of metabolites was constantly observed on all the plates.

The few experiments conducted on these lines did not permit any interpretation of the nature of the reactions involved. However, the possibility of detecting phenothiazine blood levels after hemolysis of the sample appears fairly promising. No reference was found in the literature on this particular subject which is still under investigation in this laboratory.

Chromatography of nonphenothiazine tranquilizers

Chlorprothixene. This drug reacts with the Folin–Ciocalteau reagent at room temperature yielding a bluish color. In the group of psychotropic agents previously investigated, other compounds (clopenthixol, etryptamine, iproniazid, isocarboxazid, nialamide, phenelzine and rescinnamine) have similar chromogenic behavior. Their R_F values in system I are clearly distinct from the R_F value of chlorprothixene, with the exception of clopenthixol and nialamide. The R_F values of these three drugs in system I are: chlorprothixene 0.61; clopenthixol 0.62; nialamide 0.58. To differentiate chlorprothixene from clopenthixol and nialamide system II or system V were used as a second chromatographic system. A good resolution of the three compounds was achieved (Table III).

TABLE III

R_F VALUES OF CHLORPROTHIXENE, CLOPENTHIXOL AND NIALAMIDE IN SYSTEMS II AND V

	Chlor-prothixene	*Clo-penthixol*	*Ni-alamide*
System II	0.66	0.15	0
System V	0.75	0.36	0.07

The plate developed in the second system was sprayed with Mandelin reagent. Chlorprothixene and its metabolites show characteristic brilliant orange or green fluorescences. Table IV shows the R_F values of chlorprothixene and its metabolites in the five chromatographic systems used. Fig. 2 illustrates the chromatographic

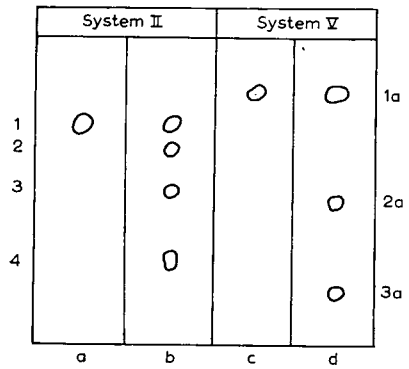


Fig. 2. Thin-layer chromatography of urine extract containing chlorprothixene. (a) and (c) chlorprothixene standard solution. (b) and (d) urine extracts A. System II: cyclohexane–diethylamine–benzene (75:20:15). System V: benzene–ethanol–12 *N* ammonium hydroxide (95:15:5). Detecting reagent: Mandelin reagent. Colors of the spots: spots 1 and 1a orange with brilliant green (orange) fluorescence; spots 2, 3, 4 and 3a orange with brilliant orange fluorescence; spot 2a orange with brilliant green fluorescence. In parentheses the outside fluorescence of the spot.

TABLE IV

R_F VALUES OF CHLORPROTHIXENE AND ITS METABOLITES

	System I		*System II*		*System III*		*System IV*		*System V*	
	R_F	*Rel.* R_F	R_F	*Rel.* R_F	R_F	*Rel.* R_F	R_F	*Rel.* R_F	R_F	*Rel.* R_F
Chlorprothixene	0.61	1.00	0.66	1.00	0.37	1.00	0.60	1.00	0.75	1.00
Spot 1	0.31	0.51	0.59	0.89	0.21	0.57	0.47	0.78	0.44	0.59
Spot 2			0.47	0.71	0.06	0.16	0.31	0.52	0.17	0.23
Spot 3			0.26	0.39			0.20	0.33		

pattern obtained from a urine extract (A) from a patient receiving 150 mg *pro die* of the drug. The plates were developed in systems II and V. Colors and fluorescences reported are those formed by means of the Mandelin reagent. Urine and blood extracts from patients receiving daily doses lower than 50 mg showed only the presence of metabolites.

Meprobamate. Blood and urine samples from patients receiving daily doses of 400–1200 mg of the drug were extracted according to the procedure described. The specimens, after extraction, were re-extracted with two 50 ml portions of ethyl acetate. The ethyl acetate layers were separated, collected, filtered and the ethyl acetate evaporated under forced air at room temperature. The residue was taken up in methanol. The ethyl acetate extracts were found to contain the hydroxy-meprobamate[4], which is not extracted by the ether.

In system I, the drug and its metabolite show very close R_F values, 0.71 and 0.68 respectively, corresponding to the R_F values of the relative standard solutions. The best separation between the hydroxy-meprobamate and the parent drug was obtained in system IV. This system also differentiates the meprobamate from drugs showing the same color reaction with the Folin–Ciocalteau reagent and having similar R_F values in system I: chlordiazepoxide (0.66), benactizine (0.68), diazepam (0.72), tybamate (0.74), buclizine (0.76). In system IV the R_F values of these compounds are clearly distinct in respect to the R_F value of meprobamate, with the exception of chlordiazepoxide (meprobamate 0.42; chlordiazepoxide 0.49). To identify the meprobamate, the plate developed in system IV was sprayed with furfural reagent[5]. Chlordiazepoxide showed a bluish color, while meprobamate and hydroxy-meprobamate form violet colors with characteristic brilliant red fluorescences.

Table V reports the data collected for hydroxy-meprobamate and the parent drug. No specimens were available from patients receiving doses of meprobamate lower than 400 mg *pro die*.

TABLE V

R_F VALUES OF MEPROBAMATE AND HYDROXY-MEPROBAMATE

	System I		*System II*		*System III*		*System IV*		*System V*	
	R_F	*Rel.* R_F	R_F	*Rel.* R_F	R_F	*Rel.* R_F	R_F	*Rel.* R_F	R_F	*Rel.* R_F
Meprobamate	0.71	1.00	0.03	1.00	0.76	1.00	0.42	1.00	0.08	1.00
Hydroxy-meprobamate	0.69	0.97	0	0	0.65	0.85	0.17	0.40	0	0

TABLE VI

R_F VALUES OF AMITRIPTYLINE, NORTRIPTYLINE AND THEIR METABOLITES

	System I				*System II*				*System IV*				*System V*			
	Amitriptyline		*Nortriptyline*		*Amitriptyline*		*Nortriptyline*		*Amitriptyline*		*Nortriptyline*		*Amitriptyline*		*Nortriptyline*	
	R_F	*Rel.* R_F	R_F	*Rel.* R_F	R_F	*Rel.* R_F	R_F	*Rel.* R_F	R_F	*Rel.* R_F	R_F	*Rel.* R_F	R_F	*Rel.* R_F	R_F	*Rel.* R_F
Unchanged drug	0.55	1.00	0.31	1.00	0.71	1.00	0.50	1.00	0.46	1.00	0.19	1.00	0.76	1.00	0.51	1.00
Spot 1	0.31	0.56			0.50	0.70	0.28	0.56	0.19	0.41	0.08	0.42	0.51	0.67	0.25	0.49
Spot 2					0.28	0.39			0.08	0.17			0.25	0.33		

TABLE VII

R_F VALUES OF IMIPRAMINE, DESIPRAMINE AND THEIR METABOLITES

	System I				*System II*				*System IV*				*System V*			
	Imipramine		*Desipramine*		*Imipramine*		*Desipramine*		*Imipramine*		*Desipramine*		*Imipramine*		*Desipramine*	
	R_F	*Rel.* R_F	R_F	*Rel.* R_F	R_F	*Rel.* R_F	R_F	*Rel.* R_F	R_F	*Rel.* R_F	R_F	*Rel.* R_F	R_F	*Rel.* R_F	R_F	*Rel.* R_F
Unchanged drug	0.49	1.00	0.23	1.00	0.65	1.00	0.42	1.00	0.38	1.00	0.12	1.00	0.65	1.00	0.34	1.00
Spot 1	0.41	0.84			0.48	0.74	0.14	0.33	0.12	0.32	0.05	0.42	0.34	0.52	0.09	0.26
Spot 2	0.31	0.63			0.42	0.65	0.06	0.14	0.05	0.13			0.09	0.14		
Spot 3	0.23	0.47			0.14	0.21										
Spot 4					0.06	0.09										

Chromatography of stimulants

Parallel experiments were conducted on two pairs of commonly used antidepressant agents: amitriptyline and its monomethyl analog nortriptyline, and imipramine and its monomethyl analog desipramine.

It appeared advisable to ascertain whether the drug and its monomethyl analog form similar chromatographic patterns and if demethylation reactions take place during the metabolism of the dimethyl compound, yielding metabolites corresponding chromatographically to the respective monomethyl analog.

Amitriptyline–nortriptyline. According to HUCKER[6], demethylation and hydroxylation are the major metabolic reactions of amitriptyline. In a study concerning the excretion of nortriptyline, AMUNDSON AND MANTHEY[7] attempted to identify one of the spots, obtained after chromatographic separation of urine extracts, as 10-hydroxy-nortriptyline. On the bases of these observations, urine and blood extracts from patients receiving daily doses of 25–75 mg of amitriptyline or nortriptyline were spotted on the same plate and chromatographed in the five systems used.

The results of the experiments are shown in Table VI. In systems I, II, IV and V the major metabolite of amitriptyline corresponded to the position of unmetabolized nortriptyline and relative standard solution. Only the presence of the unchanged drugs was observed in system III for the two compounds (amitriptyline R_F 0.26; nortriptyline R_F 0.06). In systems II, IV and V a second metabolite was found to be common to the two drugs.

The spots were localized by means of the Folin–Ciocalteau reagent and by heating the plate at 100° for 10 min. The metabolites and the parent drugs form bluish colored spots. The lower limit of detection of unmetabolized drug corresponded to a daily administration of 25 mg of amitriptyline or nortriptyline.

Imipramine–desipramine. The chromatographic behavior of these two drugs was found to be similar to that of the two cycloheptadiene derivatives described. In system III only the unchanged drugs were detected (imipramine R_F 0.19; desipramine R_F 0.03). In this system a spot corresponding to the position of desipramine was observed irregularly in urine extracts of imipramine. Metabolites of imipramine were found in systems I, II, IV, V. The major spot corresponded consistently to the position of desipramine.

Metabolites of desipramine, found in systems II, IV and V corresponded to analogous spots separated from urine extracts of the dimethyl compound.

Table VII reports the R_F values of imipramine and desipramine and their metabolites. Extracts from specimens corresponding to daily doses lower than 25 mg of the two dibenz-azepine derivatives showed only the presence of metabolites.

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CHROM. 3277

THE USE OF POLYVINYLPYRROLIDONE IN THE THIN LAYER CHROMATOGRAPHIC SEPARATION OF FLAVONOIDS AND RELATED COMPOUNDS

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SUMMARY

A method is described for the separation of phenolic acids and flavonoids by thin layer chromatography using poly-N-vinyl pyrrolidone as adsorbent. The properties and pre-treatment of the adsorbent are described and the R_F values of eighteen naturally occurring phenolic acids and flavonoids in three solvent systems are given.

Paper chromatography has been very widely used in separating and identifying plant phenolics[1–3]. Much of the preliminary work has been done in this field and interest is now turning towards more specific studies, for example, the importance of flavonoids in chemical taxonomy and fungal toxicology, and plant decomposition studies. These entail the handling of large numbers of samples and thin layer chromatography offers a rapid method of identifying the major flavonoids in such samples. Silica gel, cellulose and mixed layers of silica gel and cellulose have been used and more recently various polymers mainly of the nylon type, have been introduced as adsorbents. Such adsorbents are generally referred to in the literature as 'polyamides'[4]. Unfortunately silica gel quenches the characteristic fluorescence of many phenolics although reasonable separations are obtained. Cellulose on the other hand does not affect the fluorescence but poor separations frequently result if a plant extract is used without some form of purification. Polyamide is a difficult material to handle in the form of a thin layer.

Cross linked poly-N-vinyl pyrrolidone (PVP) has been used for a number of years in the clarifying of beer and wines[5] and more recently in chromatographic columns to remove polyphenolic contaminants from plant enzyme preparations[6] and for the purification of anthocyanins[7]. PVP has an adsorption capacity three or more times greater than polyamide, and initial tests showed it to be suitable for the separation of polyhydroxyphenols. It was also found that the ultra-violet fluorescence of flavonoids adsorbed on PVP was frequently considerably greater than if adsorbed on silica gel or cellulose. The range of fluorescent colours produced was also extended. These properties should result in a lower limit of detection and easier recognition of many flavonoids than with previously used adsorbents.

The present work describes the use of PVP in the separation of flavonoids by thin layer chromatography (TLC).

EXPERIMENTAL

Initial investigations into PVP as a TLC adsorbent

Poly-N-vinyl pyrrolidone (PVP) in its cross linked form is insoluble in water, organic solvents, strong acids and alkali. This is in contrast to the straight chain polymer known under various trade names* which is soluble in water and alcohols.

Commercial PVP** was found to consist of particles varying in size from greater than 60 B.S. mesh (250 μ) to less than 300 B.S. mesh (53 μ). The particles swelled slightly in water so that after spreading the plates, drying resulted in the formation of a fine network of cracks throughout the layer. Spots developed on such layers tended to be irregular in shape and the solvent front did not move as a straight line. In an attempt to produce better layers the following modifications were tried.

(*i*) *Organic solvents*

Methanol, acetone, chloroform and ethyl acetate were each used to suspend PVP. Much of the layer was smooth but a few deep and wide cracks inevitably developed which disrupted the separation completely. The layers were also extremely fragile.

(*ii*) *Admixture with Celite 545***

PVP and Celite were mixed 1:1 with water as the suspending medium. A very satisfactory layer was obtained free from cracks. Separation was very poor however, the spots being very large and diffuse, and the characteristic fluorescence of many substances was largely quenched.

(*iii*) *Binding agents*

Two binding agents which have been successfully used with silica gel were investigated. Anhydrous calcium sulphate–PVP (1:7) was spread as a suspension in water. The resulting layer was stronger than with PVP alone but separations were not improved. In a second experiment PVP was slurried with a 2.5 % solution of polyvinyl alcohol in water[8]. The resulting layer was extremely strong, but with aqueous solvents it peeled from the plate.

(*iv*) *Particle fractionation*

A sample of PVP was fractionated using sieves of 60, 100 (149 μ), 170 (88 μ), 240 (66 μ) and 300 B.S. mesh. A sample of each particle size was suspended in water, spread on to a number of plates and these were examined for appearance, rate of solvent travel and the ability to separate a synthetic mixture of flavonoid compounds. The following qualitative observations were made:

(*a*) *Greater than 60 and 60–100 mesh.* The layers were fissured as before with the cracks sharply defined. Solvents travelled extremely rapidly with a straight front, but separated spots were large and diffuse. The dried layer was very powdery making spraying difficult.

(*b*) *100–300 mesh.* These layers had a similar appearance to those above. They were, however, more robust and would withstand being turned face down on to photographic paper when records of fluorescence were required. Solvent travel was slower but the migrating front was straight and the resulting separation reasonably sharp.

* *e.g.* Periston, Plasmosan.
** Polyclar AT: Fine Dyestuffs and Chemicals Ltd., Manchester, 2, Great Britain.
*** Diatomaceous filter-aid: Koch-Light Laboratories Ltd., Colnbrook, Bucks., Great Britain.

(*c*) *Less than 300 mesh.* These layers were very finely reticulated but the fissures themselves appeared to be partly filled with ultra-fine particles. The layer was extremely tough and would withstand vigorous rubbing with the finger. Solvent travel was very slow however, even with volatile solvents, and investigations were therefore not continued.

Preparation of the layer

On the basis of the above work PVP with a particle size of 100–300 mesh was considered suitable for TLC. This fraction also constituted the major part of commercial PVP and further grinding was not necessary. It was found impracticable to remove the less than 300 mesh particles from a large sample by dry sieving, and the following technique was used.

200–500 g of PVP were slurried with water to give a thick paste and washed through a 100 mesh sieve in small portions using a fine but powerful jet of water. The filtrate was then washed through a 300 mesh sieve in a similar manner, allowing the filtrate to run to waste. When all the original material had been treated, the fraction retained on the 300 mesh sieve was washed several times with a large volume of de-ionised water and filtered at the pump. The filter cake was washed with acetone, spread on filter paper and air-dried. The material obtained after this procedure gave uniform layers free from U.V. fluorescent impurities. Layers were prepared using a Shandon 'Unoplan' spreader. 15 g PVP (100–300 mesh) was shaken with 80 ml water; this provided sufficient slurry to coat five 20 × 20 cm plates. As the adsorbent contains no binder it was convenient to prepare the slurry in bulk and store it until required. It was found that plates must be spread with one rapid sweep of the spreader and as soon as possible after pouring the slurry; any delay resulted in water separating from the slurry and seeping out of the bottom of the spreader. As the particle size of the PVP was larger than that commonly used for TLC, a relatively large gap of 500 μ was required in the spreader gate to ensure that plates could be coated quickly and evenly. After each spreading operation, excess water was blotted from the small leading and trailing plates to prevent damage to the other plates. The layers were allowed to air-dry overnight and stored in a cabinet without desiccant. The layers should not be dried by heating at any stage, otherwise large cracks develop.

Solvent systems

All solvents were of analytical reagent grade and were used as received. The following systems were used:

(I) 90 % formic acid;

(II) ethanol–water–concentrated hydrochloric acid (6:3:1, v/v);

(III) acetic acid–water–concentrated hydrochloric acid (30:10:3, v/v) (Forestal solvent).

Detecting agents

(*1*) *Sprays*

(i) Diphenyl-boric acid ethanolamine complex[9], 1 % in ethanol.

(ii) Ferric chloride–potassium ferricyanide, each 1 % aqueous. The reagents were mixed in the proportions 1:1 just prior to spraying.

(*2*) *Radiation source*

U.V. 3660 and 2537 Å. Camag Universal U.V. Lamp Type TL/900.

Photography

Photographs of developed plates were obtained using a Rolleicord Vb camera fitted with a Rolleinar 1 dioptre close-up lens, and a Rollei 1.5 × yellow filter to absorb excessive U.V. radiation. With the Camag light source 30 cm from the plate, exposures of 2 min at *f* 5.6 for Kodak Ektachrome -X (64 ASA) and Kodachrome -X (80 ASA), and 30 sec at *f* 8 for Ilford HP3 (400 ASA) were required, using light of 3660 Å.

Preparation of standards and vegetation extracts

(1) Standards were used as obtained from the suppliers without further purification. Each was dissolved in 90 % ethanol. The concentration was 1 mg/ml.

(2) Flavonoid aglycones were obtained from fresh samples of Rhododendron (*Rhododendron* sp.: whole buds and flowers) and from Bilberry (*Vaccinium myrtillus*: aerial shoots).

The fresh tissue was minced and extracted with cold 80 % methanol for 1 h and centrifuged. The supernatant was extracted twice with petroleum ether to remove chlorophyll, and evaporated to a small bulk at 40° under vacuum. The residue was washed into an evaporating basin and dried *in vacuo* over concentrated sulphuric acid for three days.

To hydrolyse the glycosides present, a suitable weight of the powdered extract was heated with 5 ml *N* hydrochloric acid for 20 min. The extract was filtered into a 10 × 150 mm tube and 1 ml *sec.*-butanol added. 10 % sodium hydroxide was added dropwise with mixing until a permanent cloudiness formed. When the layers had separated more *sec.*-butanol was added to make the volume of the upper layer about 1 ml and the tube shaken again. Samples for spotting were taken directly from the upper layer without separating the two phases, using a wire loop of about 1 μl capacity.

Chromatography

1 μl of each of the standards and 5–15 μl of the sample aglycones were spotted onto 20 × 20 cm plates. Individual standards were run in one direction in each of the solvents I, II and III to obtain R_F values; mixtures of standards and the aglycones were run in two directions using the systems I followed by II and I followed by III. The plates were thoroughly dried in a current of warm air between runs and before spraying.

The plates were developed either in pairs in a small (13 × 27 × 21 cm) glass tank with filter paper saturation or in a Shandon Multiplate tank holding up to 12 plates. In the case of two-dimensional development, a small amount of residue was deposited at the solvent front by the formic acid which interfered with the development in the second direction. This was isolated by scoring two straight lines through the layer just above and below the residue mark prior to the second run.

The use of solvent III resulted in marked solvent demixing. For reasons given below, it was beneficial to extend the development in solvent III when examining the flavonoid content of vegetation. This was achieved as follows.

After running solvent I to within 2 cm of the top of the plate the layer was dried in a current of air and the solvent front isolated by scoring through the layer as

described previously. One μl of a 1 % solution of Bromocresol Green* which travels with the secondary solvent front was spotted in the narrow channel between the upper scored line and the top of the plate and in line with the row of spots separated by solvent I. A sheet 20 cm × 47 cm was cut from standard Whatman No. 4 chromatographic paper and doubled four times to give a pad 20 cm × 3 cm. This was laid over the layer at the end away from the first separation and held in place with three spring clips. The arrangement is shown in Fig. 1. The second development was then carried out, the plates being withdrawn from the tank just before the Bromocresol Green reached the pad.

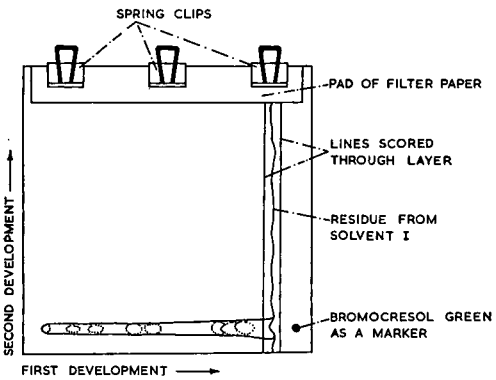


Fig. 1. Diagram of a thin layer plate prepared for extended development in the second direction.

RESULTS AND DISCUSSION

PVP adsorbs phenolic materials very strongly and a very polar solvent is required to move them from the origin. The adsorbent was also found to cause solvent demixing[10] with all solvents tested which contained two or more components. This was true even in the case of solvent I; thus the secondary (β) solvent front was located about 1 cm behind the apparent (α) front after an 18 cm run. The β front of solvent II travelled about 75 % the distance of the α front whilst for solvent III the value was about 50 %. Using solvents I and II, no fluorescent spots were observed in the region between the α and β fronts when running either the standard mixtures or extracts of vegetation. On running the standard mixture with solvent III however, the three coumarins, the glycoside naringin, and chlorogenic, sinapic, ferulic and syringic acids appeared between the α and β fronts. When these compounds were run individually the spots were discrete, but as a mixture, developed first with solvent I and then solvent III, the poor separation given by solvent I combined with the disturbing effect of the β front of solvent III resulted in spreading of the spots to an unacceptable extent. For this reason solvent III was usually used with extended development as described above. Those compounds which travelled ahead of the β front were then resolved on a second plate using solvents I and II. With solvent I a brown streak developed between the origin and the solvent front largely masking the spots present,

* Bromocresol Purple, Alizarin Red S and Sulpho Orange (Tropaeolin O) are also suitable.

TABLE I

R_F VALUES OF VARIOUS FLAVONOIDS AND RELATED COMPOUNDS

		Solvent system		
		I	*II*	*III*
1	Kaempferol	0.31	0.09*	0.46*
2	Quercitin	0.24	0.01*	0.20*
3	Myricetin	0.15	0.00*	0.07*
4	Umbelliferone	0.80	0.86*	0.71
5	Aesculetin	0.77	0.51*	0.57
6	Scopoletin	0.88	0.94*	0.83
7	Delphinidin chloride	0.87	0.06*	0.34*
8	Cyanidin chloride	0.83	0.22*	0.72*
9	Naringin	0.98	0.96*	0.96
10	D-Catechin	Streak 0–0.20	0.00*	0.06*
11	DL-Epicatechin	Streak 0–0.25	0.00*	0.06*
12	Chlorogenic acid	0.84	0.40*	0.57
13	Ellagic acid	0.48	0.07*	0.52*
14	Caffeic acid	0.63	0.21*	0.60*
15	Sinapic acid	0.83	0.74*	0.72
16	Ferulic acid	0.74	0.60*	0.58
17	Gallic acid	0.40	0.07*	0.21*
18	Syringic acid	0.81	0.88*	0.72

* R_F measured to β front.

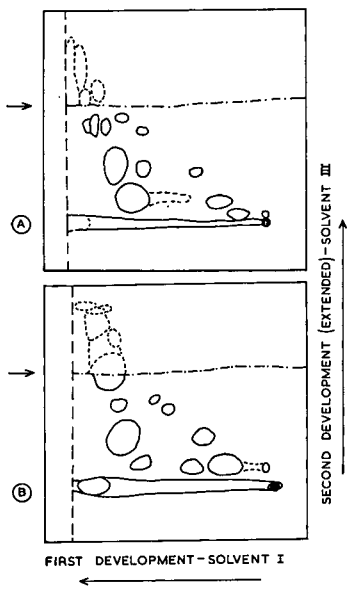


Fig. 2. Tracing of colour photographs taken under U.V. light of separations of flavonoids on PVP. The flavonoid aglycones were prepared as given in the text. Plate A: *Rhododendron* spp. Plate B: *Vaccinium myrtillus*. The small arrows give the position of the secondary (β) solvent front. The vertical broken line on each plate represents the line scored through the adsorbent to isolate the residue deposited by solvent I. The dotted areas above each β front are poorly separated components. Distinct regions can nevertheless be distinguished due to the contrasting fluorescent colours.

the bulk of this material travelling with the solvent front. On development in the second dimension with solvent II or III however, most of the spots were resolved leaving the streak at the origin. This behaviour appears to be analogous to the situation in paper chromatography where a streak develops using 6 % acetic acid but remains at the origin on development in the second direction with butanol–acetic acid–water (4:1:5, organic phase). It was also found that using crude extracts of vegetation the sequence of development of solvent I followed by II or I followed by III could not be reversed. If solvent II or III were used first, a heavy brown deposit was formed which travelled rapidly with the solvent for 3–4 cm and then suddenly stopped. This streak interfered both with the development of spots in the first direction, and subsequent development with solvent I resulted in a very broad brown streak with a disturbing effect on the solvent front. It would appear therefore that the use of formic acid as solvent in the first direction has the advantage of carrying much of the non-phenolic components of crude plant extracts with the solvent front where they can interfere least with the chromatographic separation.

The R_F values of eighteen phenolic compounds in each of the three solvents are given in Table I. Examples of typical separations of the flavonoids in vegetation are shown in Fig. 2.

ACKNOWLEDGEMENT

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CHROM. 3303

ÜBER DEN EINFLUSS DER STRUKTUR BIPOLARER IONENAUSTAUSCHERHARZE (SCHLANGENKÄFIGPOLYELEKTROLYTE) AUF DIE TRENNUNG VON ELEKTROLYT-NICHTELEKTROLYTGEMISCHEN

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(Eingegangen den 13. Oktober 1967; modifiziert den 13. November 1967)

SUMMARY

The influence of the structure of bipolar ion-exchange resins ("snake-cage" polyelectrolytes) on the separation of electrolyte and non-electrolyte mixtures

Twelve "snake-cage" exchange resins were prepared by polymerisation of different amounts of acrylic acid within a strongly basic anion exchanger containing various concentrations of divinylbenzene (DVB).

In addition to the determination of the general characteristic values, the influence of the resin structure on the separation of electrolyte–non-electrolyte mixtures was investigated.

The following mixtures were used as test substances: NaCl–glycerol, NaCl–cane sugar, NaCl–phenol, NaCl–methylene blue and NaCl–acetic acid in aqueous solution.

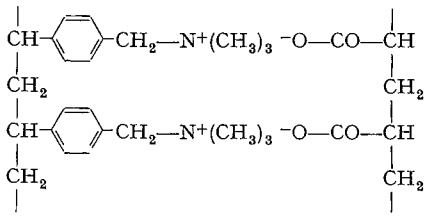
The best separation effects were obtained with anion exchange resins with a low concentration of DVB and by equivalent saturation of the quaternary amino groups in the resin by the carboxylic acid groups of the copolymerised acrylic acid.

The affinity of the "snake-cage" resin in relation to electrolytes, and with it the electrolyte retardation effect, increases with the increase in amount of copolymerised acrylic acid.

EINLEITUNG

Schlangenkäfigpolyelektrolyte stellen Ionenaustauscherharze dar, bei denen ein Polyelektrolyt im Netzwerk eines raumvernetzten Polymeren (Käfig) eingeschlossen ist. Der Käfig kann ein normaler monofunktioneller Anionen- oder Kationenaustauscher sein. Als Polyelektrolyt dient z.B. Polyacrylsäure.

Harze dieser Art wurden zuerst von HATCH, DILLON UND SMITH[1] hergestellt. Durch Polymerisation von Acrylsäure in einem stark basischen Anionenaustauscher auf Basis Styrol/Divinylbenzol mit quarternären Ammoniumgruppen erhielten sie ein Harz, dessen Zusammensetzung schematisch wie folgt wiedergegegeben werden kann.



Die Polyacrylketten sind so in der Harzmatrix verschlungen, dass sie durch kein Lösungsmittel herausgelöst werden können. Schlangenkäfigpolyelektrolyte unterscheiden sich von den übrigen amphoteren Austauschern dadurch, dass die polymeren Gegenionen nicht an der Matrix verankert, sondern relativ beweglich sind. Die Annäherung der polymeren Gegenionen an die Festionen erfolgt bis 10 Å und weniger[1].

Der Ionenaustausch mit den Gegenionen beider Arten von Festionen geht gleichzeitig und reversibel vor sich (Elektrolytadsorption). Der so aufgenommene Elektrolyt (z.B. NaCl) kann aus dem Harz durch Waschen mit destilliertem Wasser wiedergewonnen werden. Nichtelektrolyte werden von Schlangenkäfigharzen in wesentlich geringerem Masse sorbiert. Somit sind Trennungen von Elektrolyt–Nichtelektrolyt-Gemischen möglich, die unter der Bezeichnung Ionen-Verzögerungs-Verfahren (Ion-Retardation) bekannt sind[1,2].

Im vorliegenden Beitrag soll untersucht werden, inwieweit die Elektrolyt–Nichtelektrolyt-Trennung und Ionenverzögerung von der Zusammensetzung und Struktur der Schlangenkäfigharze auf Basis stark basischer Anionenaustauscher und polymerer Acrylsäure beeinflusst werden. Drei stark basische Anionenaustauscher vom Typ Wofatit SB (Vernetzung 2, 4 und 8 % Divinylbenzol (DVB)) dienten als Käfigharz, die zur Polymerisation im Harz benutzte monomere Acrylsäure kam in Form von 2 *N*, 1 *N* und 0.5 *N* Lösungen zum Einsatz. Folgende Elektrolyt–Nichtelektrolyt-Gemische wurden zur Prüfung der Trennwirksamkeit der Harze verwendet:

(50 g Glyzerin + 1 Mol NaCl) pro Liter
(50 g Rohrzucker + 1 Mol NaCl) pro Liter
(25 g Phenol + 1 Mol NaCl) pro Liter
(1 Mol Essigsäure + 1 Mol NaCl) pro Liter
(0.5 g Methylenblau + 1 Mol NaCl) pro Liter

ZUR SYNTHESE UND CHARAKTERISIERUNG DER SCHLANGENKÄFIG-AUSTAUSCHERHARZE

Zur Herstellung der Harze wurden 100 g 2, 4 und 8 % DVB vernetztes Wofatit SB-Harz (Cl^--Form) mit 500 ml einer 2 *N* Acrylsäurelösung versetzt.

Nach zweistündigem Rühren bei Zimmertemperatur erfolgte der Zusatz von 5 ml einer 10 %igen Wasserstoffperoxydlösung. Nach dem Aufheizen setzt bei 90° die Polymerisation ein.

Dies äussert sich in einem sprunghaften Anstieg der Viskosität der Lösung und der Temperatur. Nach 3-stündiger Polymerisation und folgender Abkühlung wird das den Harzkörnern äusserlich anhaftende Polymere mit heissem Wasser entfernt. Die mit 2 *N* Acrylsäure präparierten Harze erhalten die Bezeichnungen:

I/2% DVB
I/4% DVB
I/8% DVB,

die mit 1 *N* und 0.5 *N* Acrylsäurelösung behandelten entsprechend die Bezeichnung II bzw. III.

Unter den beschriebenen Bedingungen gelangte auch eine 1 *N* Methacrylsäurelösung in Gegenwart des Anionenaustauscherharzes zur Polymerisation. Diese Schlangenkäfigharze wurden als:

IV/2% DVB
IV/4% DVB
IV/8% DVB.

bezeichnet.

Sämtliche Harze wurden mit 2 *N* NaOH behandelt und anschliessend neutral gewaschen.

Die in das stark basische Anionenaustauscherharz einpolymerisierte Acrylsäuremenge (Methacrylsäure) wurde nach zwei Methoden bestimmt:

(1) Durch Ermitteln der im Waschwasser noch vorhandenen polymeren Säure.

(2) Bestimmen der Gewichtsdifferenz der Harze vor und nach der Polymerisation.

Beide Methoden liefern bei den Harzen des Typs I bis III annähernd gleiche Ergebnisse.

Eine exakte Methode zur Ionenaustauschkapazitätsbestimmung von Schlangenkäfigharzen wurde ausgearbeitet und basiert auf folgendem:

Durch Variation des Acrylatanteils im stark basischen Anionenaustauscherharz sind drei Möglichkeiten hinsichtlich des Anteiles der jeweiligen Festionen zu unterscheiden:

(1) Die quaternären Stickstoffgruppen des stark basischen Anionenaustauscherharzes sind im Überschuss vorhanden.

(2) Die quarternären Stickstoffgruppen des stark basischen Anionenaustauscherharzes sind vollständig durch Salzbildung mit der polymeren Acrylsäure neutralisiert.

(3) Die Karbonsäuregruppen der einpolymerisierten Acrylatkomponente sind im Überschuss vorhanden.

Versetzt man ein Harz mit einer Verteilung der Festionen gemäss 1. mit einer Salzlösung (z.B. NaCl), so findet ein von den im Überfluss vorhandenen quarternären Stickstoffgruppen ausgehender Anionenaustausch statt. Die durch innere Salzbildung neutralisierten Festionen verursachen gleichzeitig eine reversible Sorption des Natriumchlorids (Na^+ und Cl^-). Behandelt man anschliessend den in der Na^+- Cl^-/Cl^--Form vorliegenden Austauscher mit einem Überschuss an Salzsäure, so werden die Na^+-Ionen der polymeren Acrylatkomponente gegen Protonen ausgetauscht. Der Verbrauch an HCl entspricht der Anzahl der gepaarter Schlangenkäfigfestionen.

Liegt im Harz ein äquivalentes Verhältnis der quarternären Stickstoffgruppen zu den Acrylatgruppen vor, so werden die Natriumchlorid-Ionen gleichzeitig adsorbiert. Der pH-Wert der Lösung bleibt dabei aber konstant. Die Na^+-Ionen der Acrylatkomponente können wieder gegen H^+-Ionen ausgetauscht werden und entsprechen der Anzahl der gepaarter Schlangenkäfigfestionen.

TABELLE I

EIGENSCHAFTSKENNWERTE DER HERGESTELLTEN SCHLANGENKÄFIG-AUSTAUSCHERHARZE

Harz-Bezeichnung	*Anionenaustauschkapazität des Wofatit SB, mval/g wasserfr. Austauscher in der OH^--Form*	*Acrylatanteil in %, bezogen auf wasserfr. Schlangenkäfigharz*	*Mval einpolymerisierte Acrylsäure, bezogen auf 1 g wasserfr. Anionenaustauscherharz*	*Restliche Anionenaustauschkapazität mval/g wasserfr. OH^--Austauscher*	*Kapazität an gepaarten Schlangenkäfigfestionen, mval/g wasserfr. Käfigharz*
I/2 DVB	4.32	26.50	5.0	0.78	3.35
I/4 DVB	3.35	13.18	2.1	2.07	1.17
I/8 DVB	3.23	7.71	1.2	1.10	2.10
II/2 DVB	4.32	17.15	2.9	2.67	1.51
II/4 DVB	3.35	8.62	1.3	2.73	0.60
II/8 DVB	3.23	6.83	1.0	2.69	0.45
III/2 DVB	4.32	12.15	2.0	3.20	1.06
III/4 DVB	3.35	7.89	1.2	2.67	0.58
III/8 DVB	3.23	3.04	0.45	3.00	0.17
IV/2 DVB	4.32			0.21	4.08
IV/4 DVB	3.35			0.17	3.10
IV/8 DVB	3.23			0.07	3.25

Da die Ionenselektivität der sauren und basischen Festionen unterschiedlich ist, ändert sich auch die Sorptionskapazität der Schlangenkäfigharze für verschiedene Elektrolyte.

Bei allen Schlangenkäfigharzen mit polymerer Acrylsäure konnte eine restliche Anionenaustauschkapazität festgestellt werden, obwohl z.B. im Harz 1/2 % DVB ein beträchtlicher Überschuss von Polyacrylsäure vorhanden ist. Wie Tabelle I zeigt, ist auch ein überschüssiger Anteil an Polyacrylsäure vorhanden, der nicht in Form von gepaarten Schlangenkäfigfestionen gebunden ist. Der Grund hierfür liegt vermutlich in einer strukturellen Inhomogenität des stark basischen Anionenaustau-

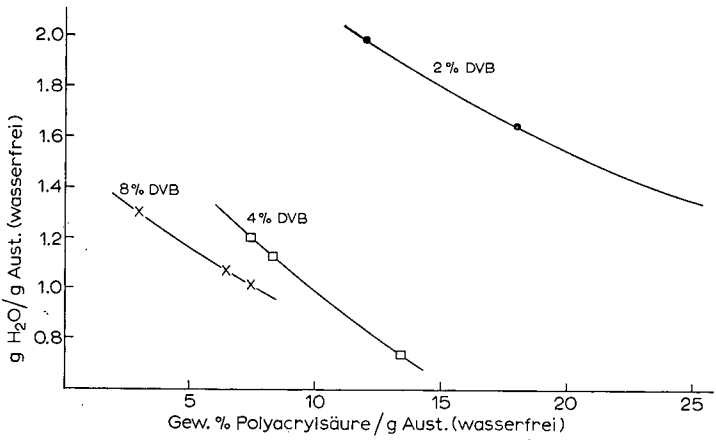


Fig. 1. Quellverhalten der Ionenaustauscher in Abhängigkeit von Polyacrylsäure- und Divinylbenzolgehalt.

scherharzes (unterschiedliche Vernetzung der Matrix) sowie in einer nicht gleichmässigen Verteilung der Acrylatkomponente im Harz.

Zur Bestimmung der Restanionenkapazität wurden 2 g Harz (OH^--Form) bekannten Feuchtigkeitsgehaltes in einem Erlenmeyerkolben eingewogen und mit 100 ml $N/10$ NaCl-Lösung versetzt. Nach 24 Std. lässt sich die vom Austauscher freigesetzte NaOH titrieren. Nach Abtrennen der neutral reagierenden Lösung wird das Harz mit 100 ml $N/10$ HCl-Lösung versetzt und nach 24 Std. der nicht adsorbierte Anteil bestimmt. Aus der vom Harz aufgenommenen HCl-Menge ergibt sich, wie schon oben angeführt, die Kapazität an äquivalenten Schlangenkäfigfestionen.

TABELLE IIa

TRENNUNG DES GEMISCHES GLYCERIN–NaCl

Harz	V_{max} (*Glycerin*) (*ml*)	V_{max} (*NaCl*) (*ml*)	ΔV_{max} (*ml*)
I/2 DVB	90	124	34
II/2 DVB	90	118	28
III/2 DVB	90	88	— 2
IV/2 DVB	84	122	38
I/4 DVB	90	104	
II/4 DVB	90	90	0
III/4 DVB	90	85	— 5
IV/4 DVB	95	120	25
I/8 DVB	90	110	20
II/8 DVB	85	88	3
III/8 DVB	85	85	0
IV/8 DVB	85	104	19

TABELLE IIb

TRENNUNG DES GEMISCHES ROHRZUCKER–NaCl

Harz	V_{max} (*Rohrzucker*) (*ml*)	V_{max} (*NaCl*) (*ml*)	ΔV_{max} (*ml*)
I/2 DVB	86	121	37
II/2 DVB	84	119	35
III/2 DVB	78	110	32
IV/2 DVB	80	140	60
I/4 DVB	78	100	22
II/4 DVB	80	90	10
III/4 DVB	79	88	9
IV/4 DVB	75	127	52
I/8 DVB	87	104	17
II/8 DVB	74	92	18
III/8 DVB	75	90	15
IV/8 DVB	80	118	38

Die Tabelle I zeigt die Eigenschaftskennwerte der hergestellten Schlangen käfigharze.

Die Fig. 1 lässt den Zusammenhang zwischen maximaler Wasseraufnahme (g H_2O/g wasserfreier Austauscher) und Polyacrylsäureanteil (Gew.-%/g wasserfreier Austauscher) bei jeweils konstantem DVB-Vernetzungsgrad erkennen.

TRENNUNG VON ELEKTROLYT–NICHTELEKTROLYTGEMISCHEN

Sämtliche Versuche wurden in einer Kolonne mit einem Durchmesser von 20 mm und 34 cm Länge bei einer Temperatur von 20° durchgeführt. Das Harzvolumen betrug 110 ml, das Volumen der aufgegebenen Lösung 10 ml, die Durchflussrate 5 ml/min.

Für die Trennung Glyzerin/NaCl und Rohrzucker/NaCl geben die Tabelle II und Fig. 2 die entsprechenden ΔV_{max}-Werte der Schlangenkäfigharze bei jeweils konstant gehaltenem DVB-Vernetzungsgrad wieder. Die ΔV_{max}-Werte nehmen mit steigendem Polyacrylsäureanteil im Harz im Sinne einer Elektrolytverzögerung zu, wobei ein Endwert angestrebt wird, der die maximal mögliche Trennung anzeigt.

Eine Trennung im Sinne der Elektrolytverzögerung ist erst von einem bestimmten Gehalt an polymerer Acrylsäure im Austauschergerüst möglich. Ist der Acrylatanteil zu gering und damit die Restanionenaustauschkapazität noch relativ hoch, so resultiert erwartungsgemäss ein Elektrolytausschluss.

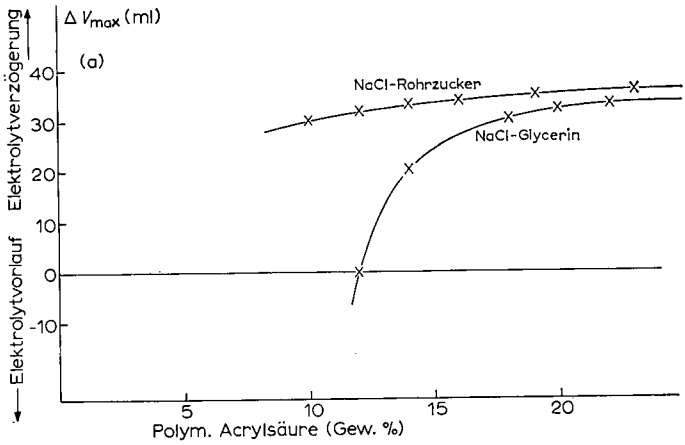
Ein Vergleich der Tabellen I und II zeigt, dass die zur Elution des NaCl erforderlichen Eluatvolumen V_{max} (NaCl) sich im wesentlichen wie die den Käfigharzen entsprechenden Kapazitäten an gepaarten Schlangenkäfigfestionen abstufen. Die Affinität des Harzes zum Elektrolyten und damit der Elektrolytverzögerungseffekt nehmen mit ansteigendem polymeren Acrylsäureanteil zu.:

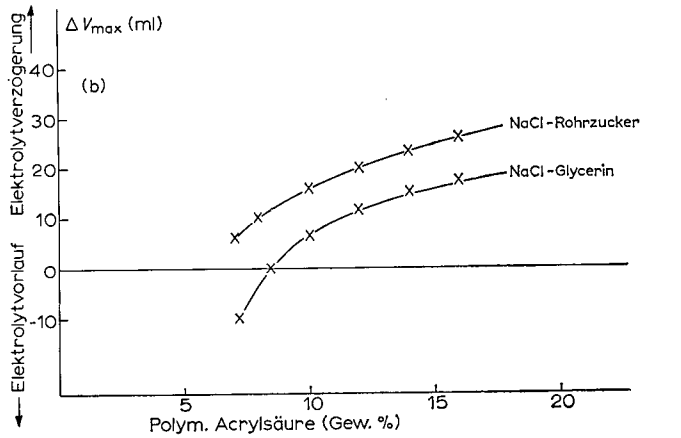
$$\text{Polyacrylat-COO}^- + \text{Me}^+ \longrightarrow \text{Polyacrylat-COO}^-\ \text{Me}^+$$
$$\text{Käfiggerüst-NR}_3^+ + \text{X}^- \longrightarrow \text{Käfiggerüst-NR}_3^+\ \text{X}^-$$
$$\text{MeX} = \text{Elektrolyt.}$$

Es ist anzunehmen, dass die Affinität zum Elektrolyten sowohl von der Intensität der Wechselwirkung zwischen den Polyacrylat-COO^-- und den Käfiggerüst-NR_3^+-Festionen bestimmt wird als auch von der Gegenionen-Selektivität der letzteren. Die Wechselwirkung der unterschiedlich geladenen Festionen hängt u.a. von deren Konzentration im Harz ab (Ladungsdichte) und damit auch von der Grösse des polymeren Acrylsäureanteiles.

Die Affinität der Nichtelektrolyte zum bipolaren Schlangenkäfig-Austauscherharz wird nur gering durch die Harzzusammensetzung beeinflusst, wie aus Tabelle II ersichtlich ist. Die Elutionsmaxima liegen in den Grenzen zwischen 84 und 95 ml für Glyzerin, für Rohrzucker zwischen 75 und 85 ml. Die Schlangenkäfigharze weisen danach eine höhere Affinität zum Glyzerin als zum Rohrzucker auf. Wahrscheinlich resultiert aber diese Abstufung aus einem Siebeffekt auf Grund der unterschiedlichen Molekülgrösse beider Verbindungen.

Negativ verliefen Versuche zur Trennung von Phenol–NaCl; Methylenblau–NaCl und Essigsäure–NaCl. Phenol und Essigsäure werden teilweise irreversibel vom Harz sorbiert.





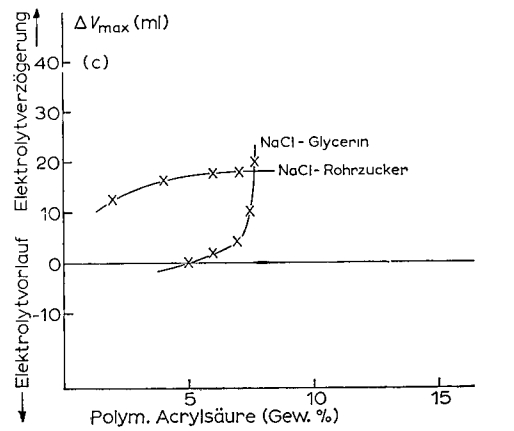


Fig. 2. Abhängigkeit der Differenz zwischen der maximalen Nichtelektrolyt- und Elektrolytkonzentration im Eluatvolumen vom Polyacrylsäureanteil im Ionenaustauscher: (a) bei 2% DVB-Vernetzung; (b) bei 4% DVB-Vernetzung; (c) bei 8% DVB-Vernetzung.

ZUSAMMENFASSUNG

Es wurden 12 Schlangenkäfigaustauscherharze durch Einpolymerisation verschiedener Mengen Acrylsäure in stark basische Anionenaustauscher unterschiedlicher DVB-Vernetzung hergestellt.

Neben der Ermittlung allgemeiner Eigenschaftskennwerte wurde der Einfluss der Harzstruktur auf die Trennung von Elektrolyt–Nichtelektrolyt-Gemischen untersucht.

Als Testsubstanzen dienten dabei die Gemische: NaCl–Glyzerin, NaCl–Rohrzucker, NaCl–Phenol, NaCl–Methylenblau und NaCl–Essigsäure in wässriger Lösung.

Die besten Trenneffekte erfolgen bei niedrigem DVB-Vernetzungsgrad des Anionenaustauscherharzes und bei äquivalenter Absättigung der quarternären Ammoniumgruppen des letzteren durch die Karbonsäuregruppen der in das Harz einpolymerisierten Acrylsäure.

Die Affinität der Schlangenkäfigharze gegenüber Elektrolyten und damit der Elektrolytverzögerungseffekt nehmen mit zunehmendem Anteil an einpolymerisierter Acrylsäure zu.

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CHROM. 3320

USE OF ION-EXCHANGE CHROMATOGRAPHY IN THE SPECTROPHOTOMETRIC ASSAY FOR THE ANTINEOPLASTIC AGENT, HEXAMETHYLMELAMINE, IN BIOLOGICAL FLUIDS

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SUMMARY

A new, simple, rapid, and precise method for the determination of hexamethylmelamine in human whole blood, plasma, and urine has been described. The method is based upon preliminary column chromatography on anion- and cation-exchange resins followed by ultraviolet absorption spectrophotometry.

INTRODUCTION

Hexamethylmelamine (NSC-13875) (HMM) has demonstrated moderate but reproducible antineoplastic activity in animals[1,2]. On the basis of this activity, HMM was evaluated for human toxicity in clinical Phase I trials[3,4], and a satisfactory dosage schedule for further clinical evaluation in Phase II studies was established[3]. Significant regressions of human neoplasms were recorded[3,5] following therapy with HMM. The mode of action of this drug is unknown[2–5].

To investigate the mechanism of action of HMM and to facilitate studies of the absorption, blood levels, and urinary excretion of this drug by patients, it was necessary to develop a method for the quantitative determination of HMM in biological materials. Although it had been suggested that preliminary purification of the HMM present in blood could be achieved by extraction with organic solvents[3], preliminary studies revealed a low, inconsistent recovery of HMM from blood when the suggested method[3] was employed. It was found that chromatography on anion- and cation-exchange resins could be utilized for the purification of HMM in biological samples prior to quantitative estimation by ultraviolet spectrophotometry. The development of this method for the determination of HMM in blood and urine forms the basis of this report.

EXPERIMENTAL

Materials

HMM (2,4,6-tris(dimethylamino)-*s*-triazine) was provided by the Clinical Branch, Collaborative Research, National Cancer Institute, U.S. Public Health

Service. Dowex 1 (Cl^-), 10 % cross-linkage, 200 to 400 mesh, and Dowex 50W (H^+), 12 % cross-linkage, 200 to 400 mesh, were prepared as described by PRICE[6]. The chromatography columns were made by sealing a glass tube of 1.0 cm outside diameter and 30 cm length to the bottom of a 125 ml Erlenmeyer flask. The columns were operated in groups of 4 to 20 under air pressure distributed with a manifold. A Beckman model DU spectrophotometer (Beckman Instruments Inc., Fullerton, Calif., U.S.A. 92634) with matched quartz cells was used for reading the samples.

Ion-exchange chromatography

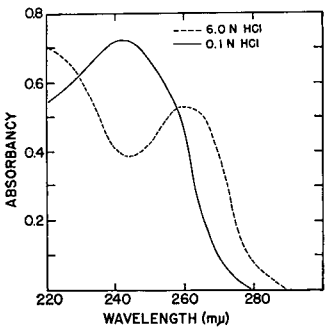
Aqueous solutions, or whole blood, plasma, or urine samples containing known quantities of HMM were acidified with 0.5 ml of 5 *N* HCl and diluted to 25 ml with distilled H_2O. The sample was then applied to a 10-cm resin bed of Dowex 1 (Cl^-) and passed through the column under 1.0 p.s.i. pressure. Then one 25-ml wash of 0.1 *N* HCl was passed through the column and the combined filtrate and wash were pooled. This combined effluent from the Dowex 1 (Cl^-) column was next applied to a 10-cm resin bed of Dowex 50W (H^+) and passed through under a pressure of 0.5 to 1.0 p.s.i. The column of Dowex 50W (H^+) was then washed successively with the following solutions: 50 ml of 0.1 *N* HCl, 100 ml of 0.5 *N* HCl, 100 ml of 1 *N* HCl, 100 ml of 2.4 *N* HCl, 50 ml of 6 *N* HCl, and 100 ml of 6 *N* HCl. Each effluent fraction was collected separately except for the two 6 *N* HCl fractions which were pooled. The pooled 6 *N* HCl effluent was evaporated to dryness *in vacuo*, and distilled H_2O was added and repeatedly evaporated to dryness *in vacuo* several times to remove any remaining HCl. Then 50 ml of 0.1 *N* HCl were added, the residue dissolved, and an aliquot read spectrophotometrically at 242 mμ.

Spectrophotometric determination

Ultraviolet absorption spectra of standard HMM solutions were obtained in 0.1 *N* HCl and 6 *N* HCl (Fig. 1). In the 0.1 *N* HCl solution an absorption maximum was observed at 242 mμ, and in the 6 *N* HCl solution the absorption maximum was at 260 mμ, representing a bathochromic spectral shift for HMM solutions at higher concentrations of HCl. Since the replication of optical density readings was more variable and the peak of maximum absorbance was lower in 6 *N* HCl solutions than in 0.1 *N* HCl solutions, it was elected that all determinations would be made in 0.1 *N* HCl solutions.

Sample preparation

Standard solutions of HMM dissolved in 0.1 *N* HCl were analyzed as described, or were added to whole blood, plasma, or urine samples prior to analysis. Whole blood or plasma samples were prepared by adding standard HMM solutions to 2 ml of whole blood or plasma prior to the addition of 0.5 ml of 5 *N* HCl. Urine samples were prepared by adding standard HMM solutions to 0.25–1.0 % of a 24-h human urine sample, collected in polyethylene bottles containing 20 ml of toluene, prior to the addition of 0.5 ml of 5 *N* HCl. Blanks for whole blood, plasma, or urine were prepared in the above manner except that HMM was omitted from the mixture. These blanks were chromatographed and read against an aqueous solution chromatographed on Dowex 1 (Cl^-) and Dowex 50W (H^+) as described. All analytical samples were compared with a standard curve of HMM prepared in 0.1 *N* HCl (Fig. 2).



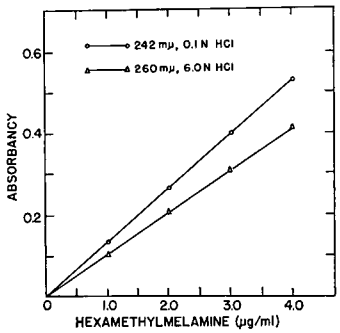
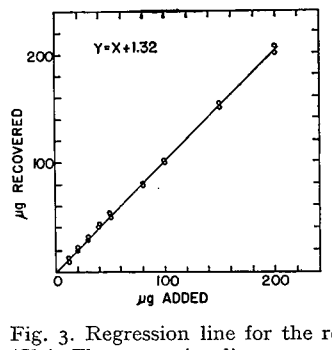


Fig. 1. Ultraviolet absorption spectra of HMM in 0.1 *N* HCl and 6 *N* HCl. A concentration of 5.5 μg/ml of HMM was employed for each solution.

Fig. 2. Standard curves of HMM in 0.1 *N* HCl read at 242 mμ, and in 6 *N* HCl read at 260 mμ. Each curve was calculated by the method of least squares[7] from 50 observations extending over the concentration range of 0.1 μg/ml to 4 μg/ml of HMM. The standard error of the estimate, $s_{y \cdot x}$, for both curves was 0.01 μg/ml.

RESULTS AND DISCUSSION

The ultraviolet absorption spectra of HMM in 0.1 *N* HCl and 6 *N* HCl are presented in Fig. 1. The computed standard curves of HMM in 0.1 *N* HCl and 6 *N* HCl are shown in Fig. 2. The recovery of HMM from Dowex 1 (Cl^-) was quantitative over the range of HMM chromatographed (Fig. 3). When HMM was chromatographed on Dowex 50W (H^+) without prior passage through Dowex 1 (Cl^-), an average of 93.0 % was recovered in the 6 *N* HCl effluent over the range of 10 to 200 μg of HMM. Following chromatography of HMM on Dowex 1 (Cl^-) and Dowex 50W (H^+) the average recovery of HMM was 93.6 % (Fig. 4). Thus, excellent recoveries of HMM



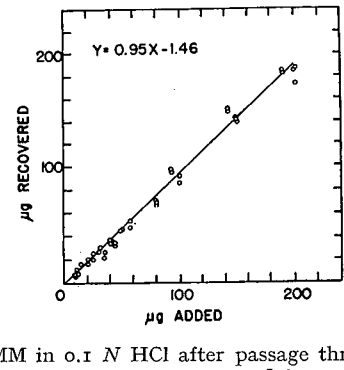


Fig. 3. Regression line for the recovery of HMM in 0.1 *N* HCl after passage through Dowex 1 (Cl^-). The regression line was calculated by the method of least squares[7] from 18 observations extending over the range of 10 to 200 μg of added HMM. The standard error of the estimate, $s_{y \cdot x}$, was computed to be 2.0 μg. The average recovery of HMM was 102 %.

Fig. 4. Regression line for the recovery of HMM in 0.1 *N* HCl after passage through Dowex 1 (Cl^-) and Dowex 50W (H^+). The regression line was calculated by the method of least squares[7] from 41 observations extending over the range of 10 to 200 μg of added HMM. The standard error of the estimate, $s_{y \cdot x}$, was computed to be 6.2 μg. The average recovery of HMM was 93.6 %.

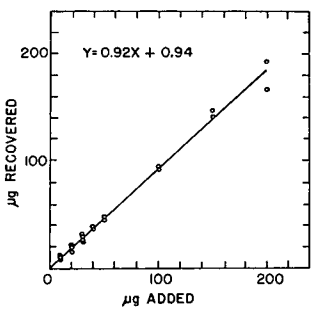
in aqueous solutions could be obtained over a wide range of concentrations with no interference from the blank.

Following the addition of HMM to human plasma samples, excellent recoveries were also observed (Fig. 5). Plasma blank samples containing no added HMM, after being chromatographed on Dowex 1 (Cl^-) and Dowex 50W (H^+), gave optical density readings of about 0.040 when read at 242 mμ with 0.1 N HCl used as the blank. These readings were subtracted from those obtained from samples containing HMM prior to the calculation of recoveries. While the contribution from the plasma blank was small, it appeared that a suitable blank should be analyzed for each sample.

When HMM was added to human whole blood samples and chromatographed as described, average recoveries of 91.7 % of added HMM were obtained over the range of 10 to 200 μg of HMM. The regression line computed for HMM in whole blood was identical to that obtained for HMM in plasma. No preliminary separation of erythrocytes or other formed blood elements was necessary prior to chromatography. Additionally, whole blood blank samples contributed no greater interference than was observed for plasma at 242 mμ.

The recovery of HMM added to human urine samples was comparable to that observed for plasma and whole blood (Fig. 6). However, when urine blank samples containing no added HMM were chromatographed on Dowex 1 (Cl^-) and Dowex 50W (H^+) resins, optical density readings of 0.160 were obtained with 0.25 % of a 24-h urine sample at 242 mμ using 0.1 N HCl as a blank. No suitable way has yet been found to remove these unidentified interfering substances from urine.

The proposed method for the determination of HMM in whole blood, plasma, and urine is rapidly and easily performed. A large number of samples can be analyzed at one time and recoveries of added HMM over a wide range are excellent and reproducible. The contribution of interfering substances to the ultraviolet absorption spectrophotometric determination of HMM in whole blood and plasma is low, but in urine is somewhat greater. However, if appropriate corrections for these interfering



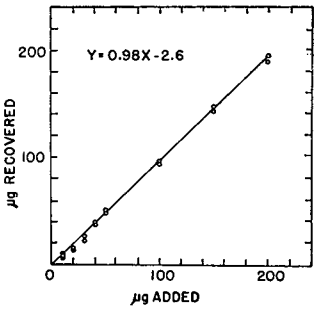


Fig. 5. Regression line for the recovery of HMM added to human plasma after passage through Dowex 1 (Cl^-) and Dowex 50W (H^+). The regression line was calculated by the method of least squares[7] from 22 observations extending over the range of 10 to 200 μg of added HMM. The standard error of the estimate, $s_{y \cdot x}$, was computed to be 5.1 μg. The average recovery of HMM was 93.4 %.

Fig. 6. Regression line for the recovery of HMM added to human urine after passage through Dowex 1 (Cl^-) and Dowex 50W (H^+). The regression line was calculated by the method of least squares[7] from 20 observations extending over the range of 10 to 200 μg of added HMM. The standard error of the estimate, $s_{y \cdot x}$, was computed to be 2.3 μg. The average recovery of HMM was 93.7 %.

materials are obtained, excellent, reproducible recoveries can be obtained. This method should facilitate studies of the absorption, blood levels, and urinary excretion of HMM administered to patients.

ACKNOWLEDGEMENTS

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CHROM. 3319

INORGANIC CHROMATOGRAPHY ON CELLULOSE ACETATE PRE-TREATED PAPER IMPREGNATED WITH 2-THENOYLTRIFLUOROACETONE

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(Received November 13th, 1967)

SUMMARY

The R_F values of inorganic ions on paper pre-treated with 0.25–2.0 % cellulose acetate and impregnated with various concentrations of 2-thenoyltrifluoroacetone (TTA) was studied by using acetate buffer as a developing agent. 1% cellulose acetate pre-treated paper impregnated with 0.2 *M* TTA was found suitable for reversed phase paper chromatography.

The R_F values of inorganic ions were examined on the impregnated paper by using various pH's of the developing agent (0.1 *M* acetate buffer). Separations of Pb–Cu, Co–Cu, Zn–Cu and Fe–Co–Ni were performed in this system.

Reversed phase paper chromatography has been applied to the separation of various radionuclides[1,2]. Most of the results, however, were obtained with liquid ion exchangers as the stationary phase and mineral acids of various concentrations as the mobile phase. In this paper, an attempt was made to use 2-thenoyltrifluoroacetone (TTA) as a substitute for the liquid exchangers. TTA is a chelating agent effective for the solvent extraction of some fission products and the related nuclides[3–17]. Alkaline earth metals were successfully separated with this reagent by reversed phase partition chromatography on a column of Kel-F[18]. The authors have studied the behavior of some fission products and related nuclides on a paper pre-treated with cellulose acetate and impregnated with TTA in benzene. From the results so far obtained, some of these elements could be separated from each other by this technique.

MATERIALS AND METHODS

Toyo filter paper No. 50 (2 × 40 cm) was washed with 10 % hydrochloric acid and distilled water three times alternately, and dried. The paper was extracted with 40–60° petroleum ether, followed by drying at 60° for 1 h. After the pre-treatment of the paper as described later, the treated paper was immersed in a solution of TTA in benzene for 1 h, equilibrated with the developing solution (acetate buffer). Then, it was dried by warm air to remove the excess of benzene.

^{51}Cr, ^{59}Fe, ^{60}Co, ^{65}Zn, ^{85}Sr, ^{90}Y, ^{95}Zr, ^{140}La, ^{144}Ce and ^{137}Cs were used as tracers, to which was added the nitrate of each element as a carrier to make an 0.05 *M*

solution. 0.05 *M* solutions of Al, Cu, Ba and Pb were prepared without adding radioisotopes. For mixtures, the concentration was 0.02 *M* for each element. 0.5 μl of the solution was applied to the paper. Then, paper chromatographic separation was carried out by the descending technique at about 25°, using acetate buffer of various pH's and concentrations as the developing agent. The location of radionuclides on the paper was determined by autoradiography. Ba was detected by spraying a solution of 0.1% (w/v) sodium rhodizonate, a red spot being obtained. Cu was detected by spraying 0.05% (w/v) dithizone in chloroform solution, a brownish red spot being obtained. The spot of Al was detected by spraying a solution of 0.1% (w/v) alizarin-S solution; the spot obtained became purplish red on exposure to the vapour of ammonia and acetic acid. Pb was detected by exposing to hydrogen sulphide, a black spot being obtained.

PRE-TREATMENT OF THE PAPER

Before impregnation of the filter paper with TTA in benzene, pre-treatment of the paper was necessary, in order to make the paper hydrophobic. The pre-treatment method with vaseline or silicone described by BROWN AND KRITCHEVSKY[19] was examined but the migration of the developing agent on the paper impregnated with TTA in benzene yielded irregular fronts. Silanized paper was prepared by placing the paper in a desiccator over dichlorodimethylsilane, allowing it to stand overnight, washing thoroughly and drying at 110°. The pre-treated paper was brittle, and the R_F values observed on this paper were generally low. Acetylation of the paper with acetic anhydride and sulfuric acid[19] caused damage to the paper.

Finally, cellulose acetate pre-treated paper was prepared by the following procedure. The paper was dipped in 0.25–2.0% (w/v) cellulose acetate in dry acetone for 15 min, and dried at 60° for 1 h[20]. Migration of the developing agent on the pre-treated paper impregnated with 0.2 *M* TTA in benzene was uniform and the developed zone was well defined. Therefore, paper pre-treated with cellulose acetate was used throughout the subsequent experiments.

RESULTS AND DISCUSSION

R_F values of the ions at different pH values of developing agent (0.1 M acetate buffer)

Fig. 1 shows the R_F values of ions on paper pre-treated with 1% cellulose acetate in acetone and impregnated with 0.2 *M* TTA in benzene, as a function of the pH of the developing agent (0.1 *M* acetate buffer). Fe(III), Cu(II), and Zr(IV) remain at the origin. The R_F value of Cs is $\sim$0.85 independent of the pH of the buffer, which may be due to non-interaction of Cs with TTA. R_F values of Al, Cr(III), Co(II), Zn, Sr, Y, Ba, Ca, Ce(III) and Pb decrease with increasing pH of the buffer. The relation between R_F and distribution ratio[21] (the ratio of the concentration of the inorganic ion, in the organic and the aqueous phases), obtained from the liquid–liquid extraction between 0.2 *M* TTA in benzene and $\sim 10^{-4}$ *M* Sr, Ce(III), Zn and Co aqueous phase, seemed to show the establishment of reversed phase partition on the paper.

Relation between R_F values and the percentage (w/v) of cellulose acetate in acetone

Fig. 2 shows that the R_F values of Co and Ba on the paper impregnated with 0.2 *M* TTA in benzene are nearly constant over the range from 0.25 to 2.0% (w/v)

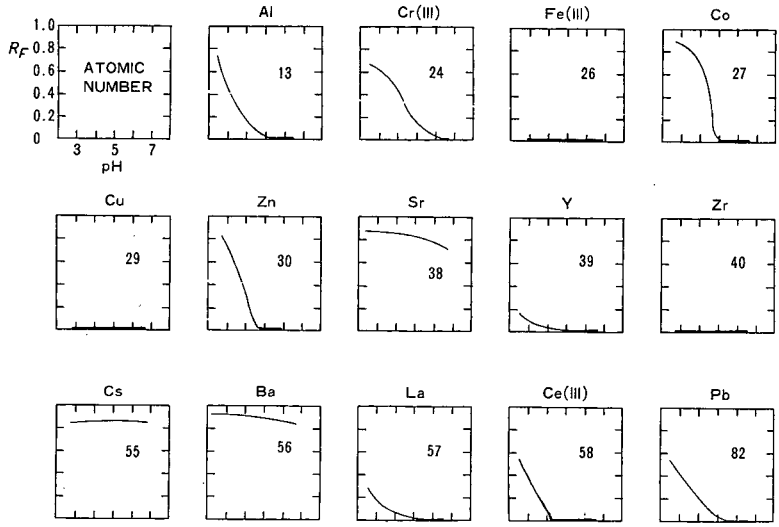


Fig. 1. R_F values of inorganic ions plotted against the pH of the developing agent (0.1 *M* acetate buffer). Paper was pre-treated with 1% cellulose acetate in acetone and impregnated with 0.2 *M* TTA in benzene.

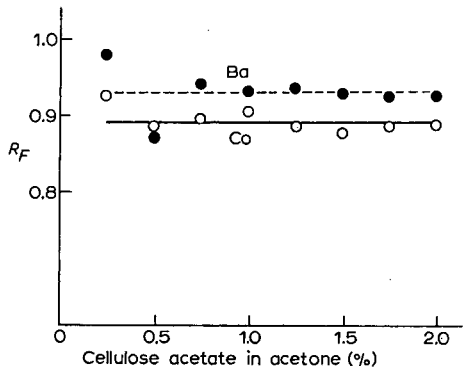


Fig. 2. Relation between R_F values and the percentage (w/v) of cellulose acetate in acetone. Cellulose acetate pre-treated paper was impregnated with 0.2 *M* TTA in benzene. 0.1 *M* acetate buffer (pH 2.5) was used as the developing agent.

cellulose acetate in acetone. The paper pre-treated with >1.5 % solution showed a marked decrease of the rate of migration of developing agent.

Relation between R_F values and the concentration of TTA in benzene

Fig. 3 shows the R_F values of Sr, Pb, Zn, Fe(III), Zr(IV) and Ce(III) as a function of the concentration of TTA in benzene used for the impregnation of the paper, after pre-treatment with 1 % (w/v) cellulose acetate in acetone.

The R_F values of Sr and Ba are both 0.85, independent of the concentration of the TTA in benzene, whereas, those of Pb, Zn, and Al gradually decrease with increasing concentration of TTA and remain constant over the concentration range from 0.15 to 0.4 *M*. Fe(III), Ce(III) and Zr(IV) remain at the origin over the whole pH range.

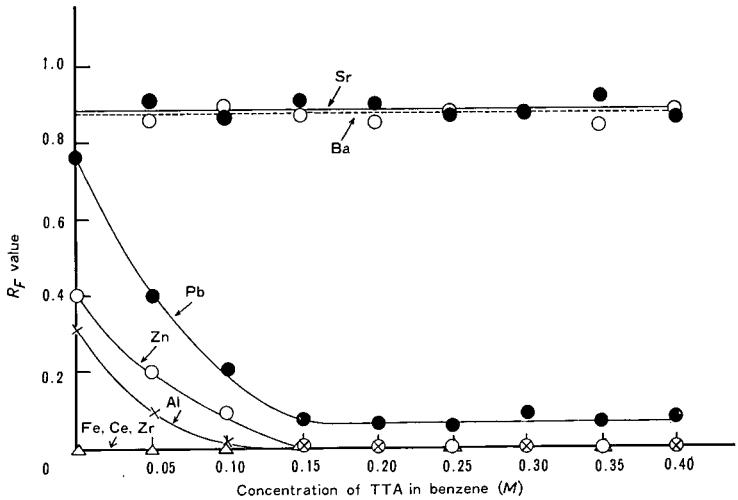


Fig. 3. R_F values for various ions as a function of the concentration of TTA in benzene. Paper was pre-treated with 1% cellulose acetate in acetone and impregnated with various concentrations of TTA in benzene. 0.1 *M* acetate buffer (pH 4.5) was used as the developing agent.

Relation between R_F values and the concentration of the developing agent (acetate buffer)

Fig. 4 shows that R_F values of Ce(III) and Pb on 1% cellulose acetate pre-treated paper impregnated with 0.2 *M* TTA increase with the increase of the concentration of the acetate buffer. The result indicates that interaction exists between these ions and acetate buffer, as described by CERRAI AND GHERSINI[21].

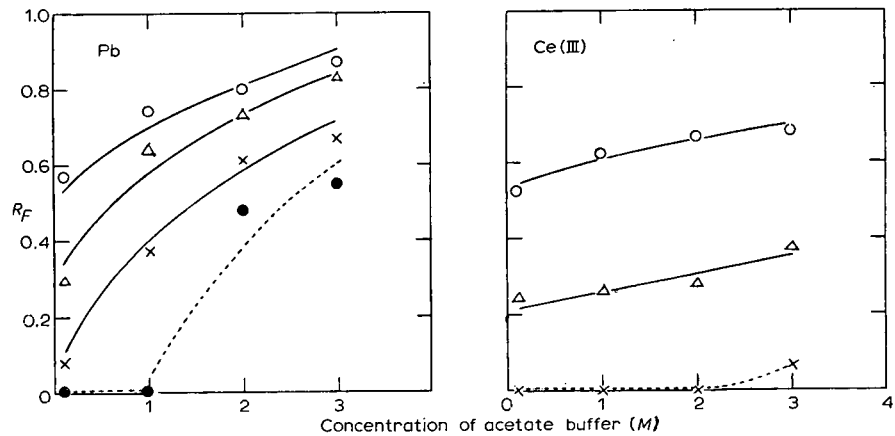


Fig. 4. Relation between R_F values and the concentration of the developing agent (acetate buffer). (—○—) pH of acetate buffer 2.5; (—△—) pH of acetate buffer 3.5; (—×—,---×---) pH of acetate buffer 4.5; (---●---) pH of acetate buffer 5.3. Paper was pre-treated with 1% cellulose acetate in acetone and impregnated with 0.2 *M* TTA in benzene.

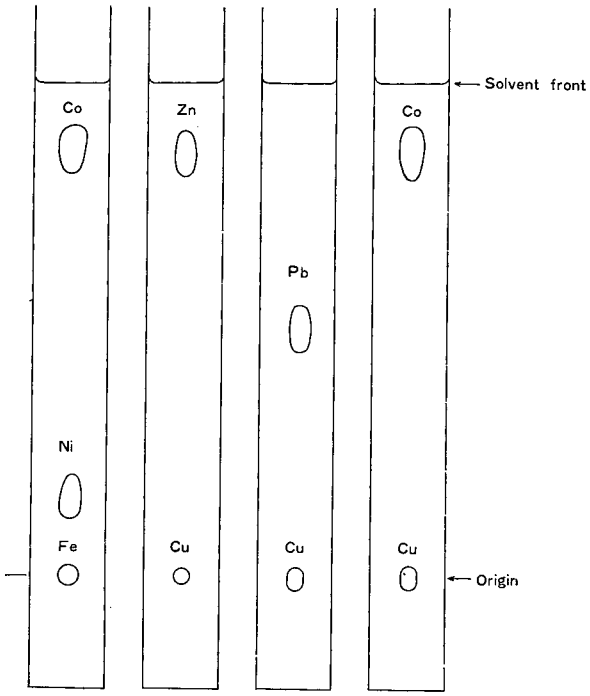


Fig. 5. Separation of some inorganic ion mixtures. Paper was pre-treated with 1 % cellulose acetate in acetone and impregnated with 0.2 *M* TTA in benzene. 0.1 *M* acetate buffer (pH 2.5) was used as the developing agent.

Separation of some inorganic ion mixtures

From Fig. 1, it would be anticipated that mixtures of some inorganic ions can be separated by the 1 % cellulose acetate pre-treated paper impregnated with 0.2 *M* TTA. Fig. 5 shows some typical results of such separations, *viz.* Pb–Cu, Co–Cu, Zn–Cu and Fe–Co–Ni.

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Notes

CHROM. 3326

Gas chromatographic separation of carbonyl fluoride and carbon dioxide

The simultaneous quantitative evaluation of CF_2O–CO_2 mixtures is important in numerous kinetic studies including the oxidation of C_2F_4 and the pyrolysis of CF_2O. The gas chromatographic analysis of CF_2O has been reported by CORDES[1] and BANKS, HASZELDINE AND SUTCLIFFE[2]. Unfortunately neither of the columns used can separate CF_2O–CO_2 mixtures. HEICKLEN and co-workers[3,4] have measured CF_2O in gas mixtures by quantitatively converting the CF_2O to CO_2 on silica gel columns and measuring the CO_2 effluent. The purpose of this paper is to describe a gas chromatographic technique for the simultaneous quantitative determination of both CF_2O and CO_2.

Experimental

Apparatus. An Aerograph model No. 202-B gas chromatograph equipped with a thermal conductivity cell was used for this study. Mixtures were introduced into the gas chromatograph through a gas sampling valve used in conjunction with a 2 ml sample volume. Peak areas were measured with a Disc Integrator (5000 counts per min) which was attached to a Sargent 10 in. recorder.

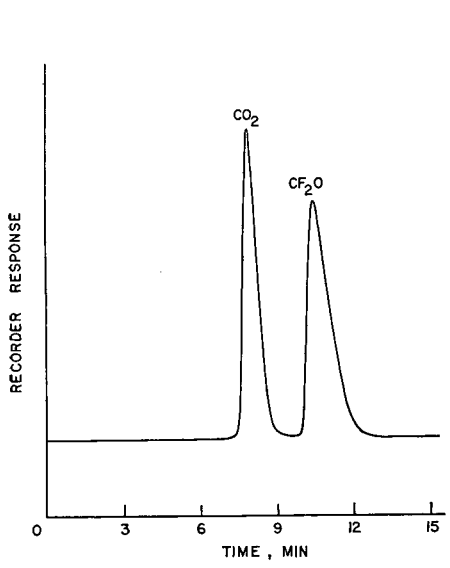


Fig. 1. Typical chromatogram of CO_2–CF_2O mixture.

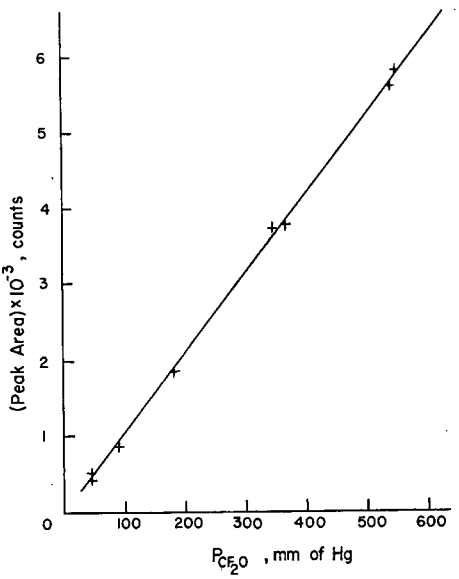


Fig. 2. CF_2O peak area as a function of sample pressure.

Column materials and preparation. A 6 ft. composite column consisting of 2 ft. of 50/80 mesh Porapak (Waters Associate, Inc.) type T followed by 4 ft. of 50/80 mesh Porapak type N was used for the analysis. The column was packed in $^1/_4$ in. O.D. Type 316 stainless steel tubing. Before final installation in the chromatograph, the column was heated to 200° and purged with helium (60 ml/min) for 2 h. Prior to each series of runs the column was conditioned by passing three 250 torr samples of CF_2O through it.

Results and discussion

A typical chromatogram indicating the separation of CF_2O and CO_2 as obtained with the column described above is given in Fig. 1. The operating conditions corresponding to the results given in Fig. 1 are: column temperature 23°; helium flow rate 60 ml/min. A plot of CF_2O peak area as a function of CF_2O pressure in the 2 ml sample volume is shown in Fig. 2. These results indicate that the detector response is linear over an eleven fold increase in CF_2O concentration. The curve given in Fig. 2 approaches the origin as the sample pressure is decreased. This behavior indicated that CF_2O absorption on this column is essentially nonexistent.

Acknowledgement

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CHROM. 3347

Synthetic diamond—A solid adsorbent for corrosive gases

A satisfactory chromatographic system for the analysis of corrosive halogen gases has proven to be an illusive objective. Recent publications[1–5] in this area are indicative of continuing difficulties encountered in the separation of these reactive materials. Primarily chromatographic separations have been concerned with inorganic penta- and hexa-fluorides, chlorine trifluoride and anhydrous hydrofluoric acid. No substrate or support has been recommended for gaseous mixtures containing

elemental fluorine. ROCHEFORT[6] has proposed a chromatographic procedure for fluorine. However, this is achieved by a preliminary reaction with NaCl and chromatographic separation of the resulting chlorine.

Diamond is relatively inert to chemical attack and experiments were initiated to determine its suitability for the chromatographic separation of fluorine and other corrosive halogen gases. Diamond particles with sufficient surface area for chromatographic applications are now available from synthetic sources. Diamond crystallites, prepared by subjecting graphite to explosion developed shock waves, have a particle size of approximately 100 Å and a surface area of 200 to 260 m^2/g. Although the surface functional groups on the shock-formed diamond have not been completely characterized, diamond's infrared spectra show carbonyl and hydroxyl absorptions. The nature and synthesis of shock-formed diamonds have been discussed previously[7].

Experimental

Gas chromatography was carried out utilizing a custom built unit fitted with tubing and valves of nickel and monel. The detector used is a thermal conductivity cell, made by Gow Mac Instrument Company, Madison, N.J., fitted with nickel filaments mounted in a nickel block using polytetrafluoroethylene insulation.

Separations were made with an 18 ft. by 0.125 in. O.D. nickel column packed with a mixture of CaF_2–shock-formed diamond. The packing is prepared by shaking 9 parts CaF_2, 30–70 mesh, with 1 part synthetic diamond. Even though the diamond particles are considerably smaller no physical separation was observed. All separations were performed at ambient temperature. Samples of shock-formed diamond can be obtained from Industrial Chemicals Division, Allied Chemical Corporation, Morris-

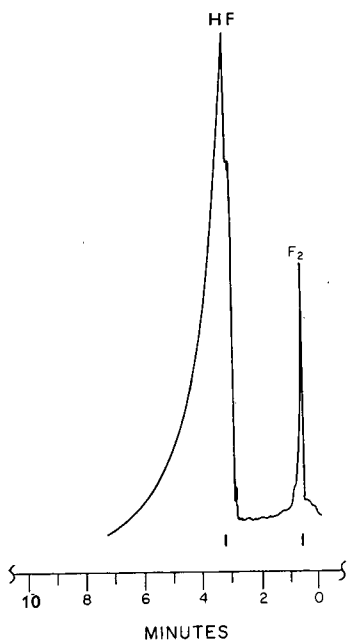


Fig. 1. Separation of HF and F_2. Conditions: Ambient temp., 80 cc/min He, 18 ft. × 1/4 in., 10% diamond–90% CaF_2.

town, N.J. High purity CaF_2 is available from Harshaw Chemical Company, Cleveland, Ohio.

Prior to use, the column and chromatograph were passivated by treatment with ClF_3.

TABLE I

RELATIVE RETENTION ON DIAMOND–CaF_2 COLUMN AT AMBIENT TEMPERATURE

Compound	*Relative retention*
F_2	0.5
HCl	1.0
ClF_3	2.0
HF	2.5

Results and discussion

Columns packed with particles the size of shock-formed diamond inherently require a large applied pressure to maintain a nominal carrier gas flow. Attempts to alleviate this problem by using mixtures of shock-formed diamond with conventional chromatographic supports were unsuccessful. In each case the smaller diamond particles separated causing a severe restriction of the carrier gas flow. However, mixtures of CaF_2 and shock-formed diamond remain homogeneous. Adhesion of the diamond particles is apparently quite strong. We have encountered no difficulty in the operation of 18 ft. columns.

Binary mixtures of anhydrous HF–F_2, HF–HCl and HF–ClF_3 were prepared. Vapor phase samples were injected into the chromatograph and separations effected at ambient temperatures with the helium flow at 80 cc/min. Retention data appear in Table I and a typical chromatogram in Fig. 1. Although the separation of fluorine and air was not achieved at room temperature, the proposed column appears to be adequate for the analysis of anhydrous HF. There was no indication of any reaction between fluorine and the adsorbent.

Supplementary data[8] for the separation of various fluorocarbon derivatives indicate that the diamond columns are similar in behavior to silica gel.

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CHROM. 3329

Separation of biphenyl and the terphenyls by gas–solid chromatography on graphitized carbon black and on Chromosorb coated with caesium chloride

Much attention has been paid to the gas chromatographic separation of the polyphenyls which are important moderator coolants for atomic reactors. A mixture of biphenyl and the polyphenyls has been separated, at higher temperatures, on Chromosorb coated with Apiezon L, Carbowax or silicone oil[1–3]. Eutectic mixtures of potassium and silver nitrates[4] or of K, Na and Li nitrates[5] have also been used. The latter are more stable at higher temperatures. Very good results have been obtained with sorbents modified[6–8] with inorganic salts such as LiCl, CsCl, $CaCl_2$ and K_3PO_4, which gave successful separations of the terphenyls and quaterphenyls up to hexaphenyls. Thus, gas–liquid chromatography has been replaced by chromatography on a modified sorbent surface. Other authors[9–11] have separated the polyphenyls by chromatography on columns packed with a mixture of Bentone 34 and 10% silicone grease or with 3% Apiezon L on Celite.

TABLE I

EXPERIMENTAL CONDITIONS

Instrument type	Carlo Erba C	Perkin Elmer F-11
Detector	FID	FID
Sensitivity	16–256 × 10	10–100 ×
Column dimensions	100 cm; diameter 5 mm	60 cm; diameter 1 mm
Adsorbent	Chromosorb P, 15% CsCl	Graphitized carbon black
Particle size	80–100 mesh	0.075–0.1 mm
Temperature of:		
column	230°, 251°, 270°, 300°	380°, 390°, 400°
sampling point	335°	400°
detector	340°	400°
Carrier gas	Nitrogen	Nitrogen
Flow rate at 25°, ml/min	48.8	1.7
Specific area, m^2/g	—	7.6

In the present paper (*cf.* ref. 12) we have separated a mixture of benzene, biphenyl and all the terphenyls by gas–solid chromatography using graphitized carbon black which proved to be a suitable adsorbent for this analysis. The surface of graphitized carbon black as studied by KISELEV[13], reveals non-specific properties and permits the separation of a mixture of the polyphenyls on a short column.

Experimental

Separation was performed (Table I) with an F 11 gas chromatograph (Perkin-Elmer, Beaconsfield, Great Britain) and with the Carlo Erba instrument, Model C (Milan, Italy). The graphitized carbon black used was a Sterling MT 3100 specimen produced by Cabot (Cambridge, Mass., U.S.A.). The total specific area was determined by adsorption of krypton at the temperature of liquid nitrogen.

The samples were injected with a Hamilton 1 μl microsyringe (Whittier, Calif., U.S.A.). The results of the separation are illustrated in Fig. 1.

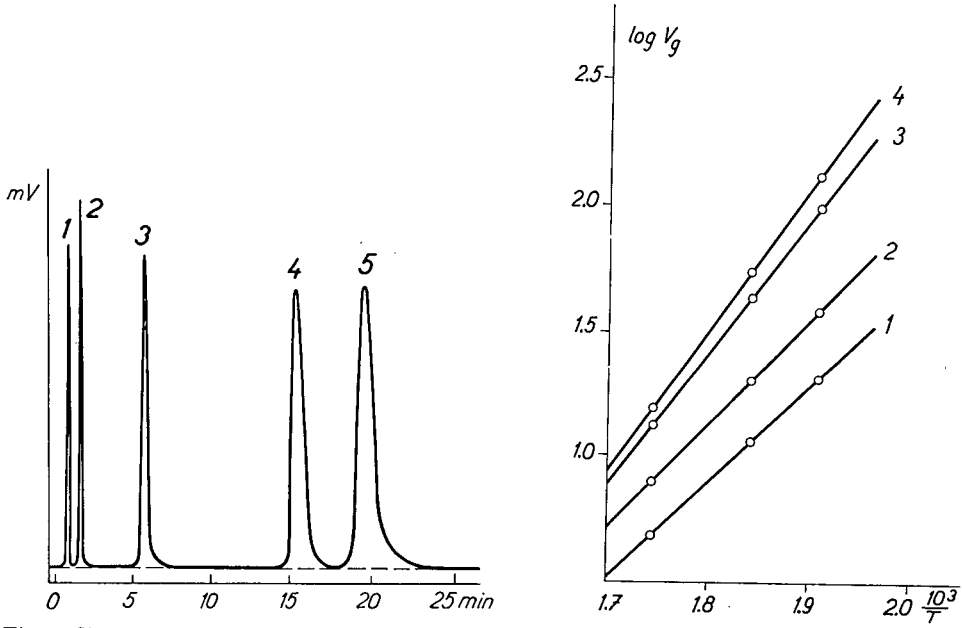


Fig. 1. Chromatogram of the mixture of polyphenyls on caesium chloride modified Chromosorb P. 1 = Solvent; 2 = biphenyl; 3 = *o*-terphenyl; 4 = *m*-terphenyl; 5 = *p*-terphenyl.

Fig. 2. The isosters of biphenyl and the terphenyls on Chromosorb P modified with caesium chloride at temperatures ranging from 230° to 300°. 1 = Biphenyl; 2 = *o*-terphenyl; 3 = *m*-terphenyl; 4 = *p*-terphenyl.

The peaks obtained in the separation on Chromosorb P coated with 10% CsCl at 230° or 251° are not very symmetrical. At the above temperatures, *o*-terphenyl tails and at 270° *m*-terphenyl also tails, but the time of analysis is favourable (Table II).

On the basis of these measurements, the course of the isosters of the components chromatographed was plotted for the column packed with CsCl on Chromosorb P, as log V_g against $1/T$. The plot is shown in Fig. 2.

These data were used for expressing the heats of adsorption of the aromatic hydrocarbons of the biphenyl series. The best separation was achieved at 270° to 300°.

TABLE II

SPECIFIC RETENTION VOLUMES FOR THE COMPONENTS ON CAESIUM CHLORIDE MODIFIED COLUMN PACKING AT VARIOUS TEMPERATURES

Component	V_g				ΔH_a
	230°	*251°*	*270°*	*300°*	
Biphenyl	—	20.9	11.5	4.91	17.8
o-Terphenyl	86.0	38.9	20.6	8.10	19.4
m-Terphenyl	196	99.8	42.8	13.5	24.6
p-Terphenyl	336	133	54.4	15.7	26.3

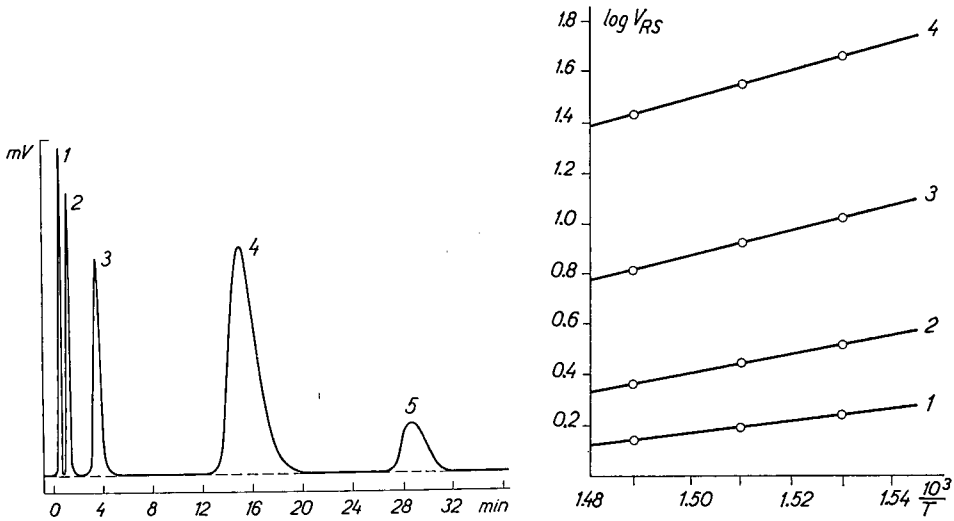


Fig. 3. Chromatogram of the mixture of polyphenyls on graphitized carbon black. 1 = Benzene; 2 = biphenyl; 3 = *o*-terphenyl; 4 = *m*-terphenyl; 5 = *p*-terphenyl.

Fig. 4. The isosters of biphenyl and the terphenyls on graphitized carbon black over the temperature range 380–400°. 1 = Benzene; 2 = biphenyl; 3 = *o*-terphenyl; 4 = *m*-terphenyl.

The separation of biphenyl and the terphenyls on graphitized carbon black is illustrated in Fig. 3. The peaks are symmetrical at 390°, but *meta*- and *para*-terphenyls can be seen to tail. The strong dispersion interaction with graphitized carbon black is reflected in longer retention times even when a comparatively high temperature of the chromatographic column is chosen.

The absolute retention volumes for the individual components at two different temperatures are listed in Table III, which was prepared by performing calculations according to ref. 14.

The isosters of the components are shown in Fig. 4.

Discussion

The order of elution of the components from the column packed with graphitized carbon black can be interpreted on the basis of the structure of these molecules.

TABLE III

ABSOLUTE RETENTION VOLUMES V_{RS} (V_R PER m^2 OF ADSORBENT AREA) OF THE COMPONENTS ON GRAPHITIZED CARBON BLACK AT VARIOUS TEMPERATURES

Component	V_{RS}		$\log V_{RS}$		ΔH_a
	380°	*400°*	*380°*	*400°*	
Benzene	1.74	1.39	0.240	0.1432	10.7
Biphenyl	5.40	2.31	0.513	0.3638	16.6
o-Terphenyl	10.4	6.08	1.016	0.8100	22.8
m-Terphenyl	46.2	26.4	1.665	1.4214	27.0
p-Terphenyl	—	—	—	—	—

The separation is considerably influenced by non-specific interactions of the sorbate with the sorbent, independent of local distribution of electron density over the nuclei of the polyphenyls.

The spherical configuration of the *o*-terphenyl molecule permits less of a dispersion interaction with an edge or plane of the graphitized carbon black surface than does *m*-terphenyl, which interacts to a still lesser degree with the carbon black surface than *p*-terphenyl. The retention volume of *p*-terphenyl, which is relatively large as compared with that of *m*-terphenyl, suggests that the interaction of *p*-terphenyl with the surface of graphitized carbon black occurs between the surface and the flat phenyl nuclei. The separation of the polyphenyls on Chromosorb P coated with 10% CsCl is most advantageous at 270° or at higher temperatures, where isothermal conditions can be maintained. The symmetry of the peaks is improved with increasing temperature and at 300° all of the components in question will be well separated in 4 min, whilst highly symmetrical peaks are obtained.

The heats of adsorption agree well with previous data for benzene. The value of —10.7 kcal/mole measured for benzene by gas chromatography agrees well with —10.3 kcal/mole measured by a static method[14].

A comparison of the heats of adsorption obtained on graphitized carbon black and on Chromosorb P coated with caesium chloride for the terphenyls, shows that the heats of adsorption on graphitized carbon black are higher. This probably depends on the dispersion forces which, for a non-specific adsorbent, are stronger than those encountered with caesium chloride where the principle of separation is not yet known.

The heats of adsorption obtained in the above manner are valuable because they were actually measured, since calorimeters suitable for the determination of these values, are unobtainable on purely constructional grounds. The values obtained are of sufficient accuracy for the usual measurements performed in catalysis and chemical engineering.

Conclusions

Graphitized carbon black can be used for the analytical separation of polyphenyls in a gas–solid system. Molecules having like carbon numbers, *e.g.* the terphenyls, manifest the influence of their geometrical configuration. The heats of adsorption can be used to predict the spatial configuration of such molecules.

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CHROM. 3366

Essential oils and their constituents

Part XLI. Identification of sesquiterpene hydrocarbons in oil of opoponax*

In a paper from this laboratory the occurrence of isomeric bisabolenes in oil of opoponax has been reported[2]. A personal communication, received since, claiming the presence of only α-santalene and α-bisabolene in this oil[3] prompted us to re-examine our samples following column and gas chromatographic purification.

Fig. 1 shows the gas chromatogram of the sesquiterpene fraction as originally recorded employing the silicone nitrile XE-60 column[2]. Four fractions corresponding

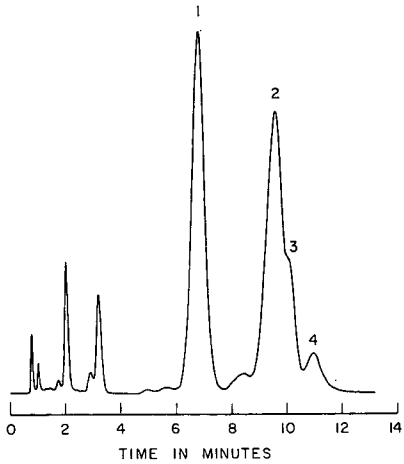


Fig. 1. Gas chromatogram of the sesquiterpene fraction of oil of opoponax.

* For the previous paper, see ref. 1.

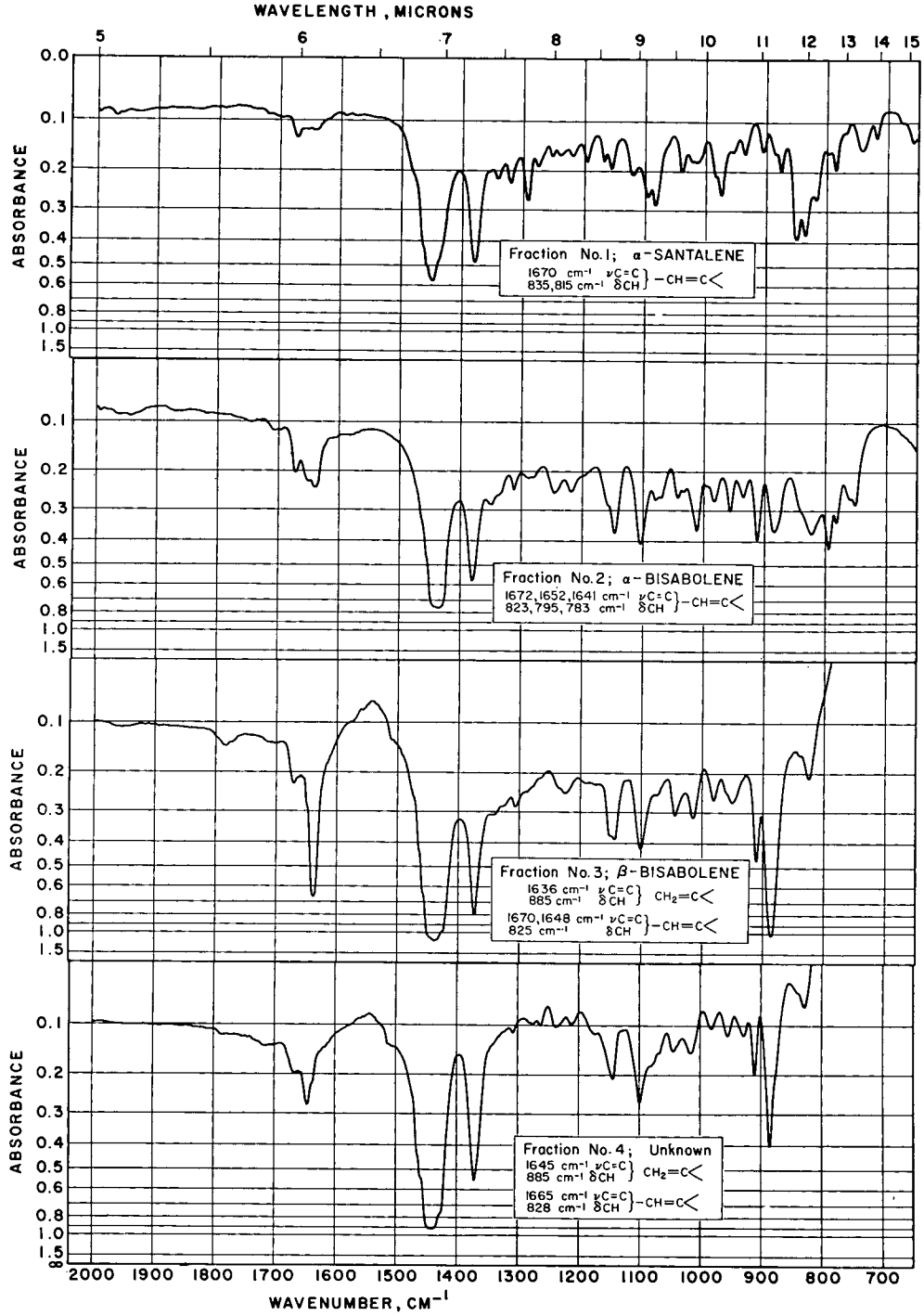


Fig. 2. Infrared spectra of the gas chromatographic fractions. Instrument: Perkin-Elmer 221. Phase: fraction Nos. 1 and 2, liquid film; fraction Nos. 3 and 4, CCl_4 solution.

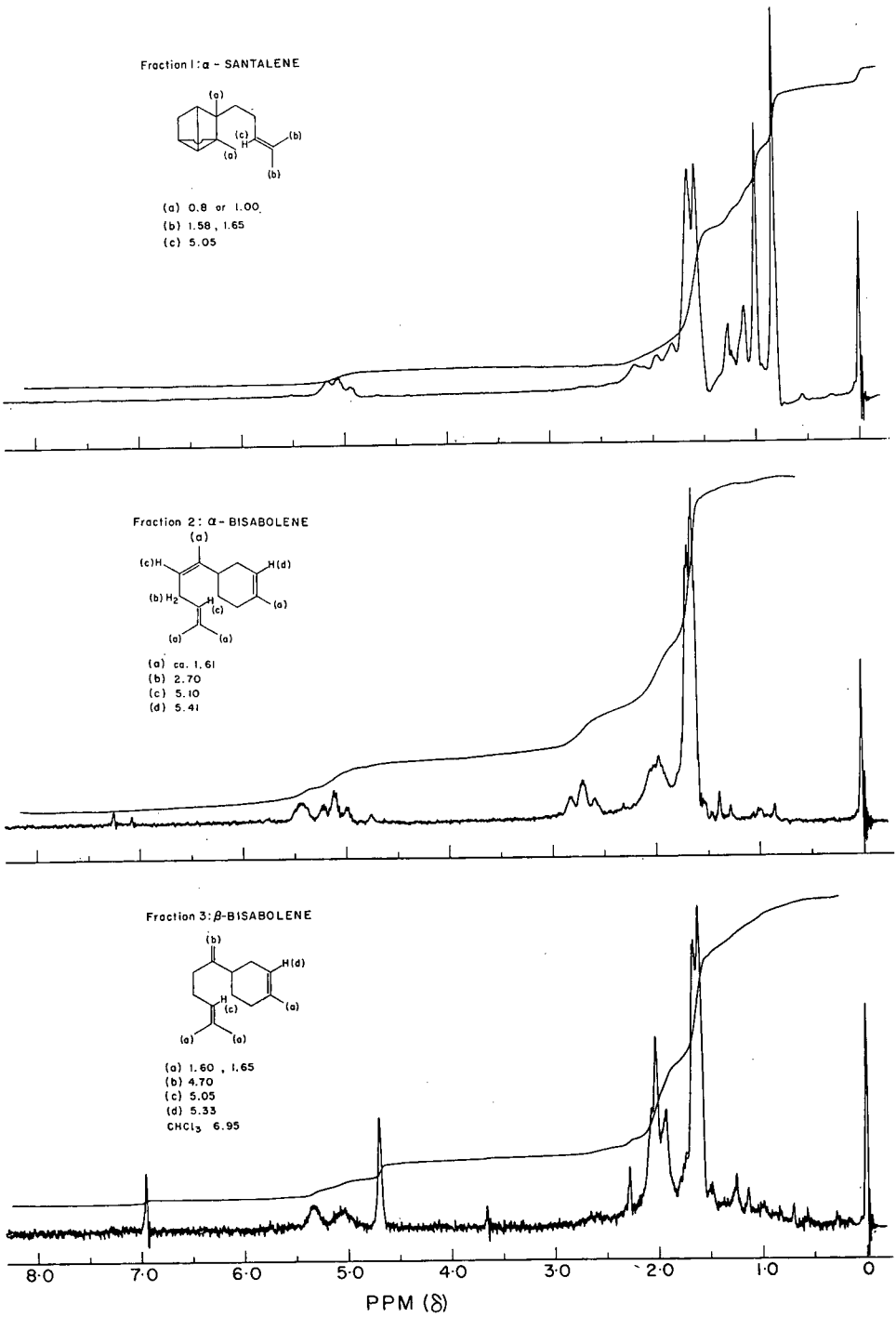


Fig. 3. NMR spectra of the gas chromatographic fractions. Instrument: Varian A-60A. Solvent: $CDCl_3$. Internal Reference: TMS.

to the peaks so numbered were collected. The infrared and NMR spectra of fractions 1 and 2 (Figs. 2 and 3) agreed with those of α-santalene and α-bisabolene. The infrared spectrum of fraction 3 was identical to that reported for β-bisabolene[4]. The NMR spectrum of the fraction was consistent with the structure of β-bisabolene and also agreed with that measured for this sesquiterpene by BATES[5]. Fraction 4 could not be re-examined due to polymerisation during storage. Its infrared spectrum as originally obtained (Fig. 2) was found to be identical with that of regenerated bisabolene[4].

We, therefore, conclude that oil of opoponax contains α-santalene, α-bisabolene and β-bisabolene, as well as some other isomeric bisabolene.

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CHROM. 3344

The gas chromatographic analysis of industrial rubber compositions

A semiquantitative method has been developed for the analysis of industrial rubber compositions using pyrolysis–gas chromatography.

A Perkin Elmer Model 800 chromatograph and the Pyrolysis Accessory of the same firm[1] were used. Twenty-five components were separated by temperature programming and using Apiezon L as stationary phase. Retention data are given in Table I and Fig. 1, where the separation time of isoprene was 2.06 min.

TABLE I

RELATIVE RETENTION DATA WITH RESPECT TO ISOPRENE (2.06 min)

No.	*Compound*	*Relative retention time*
1	Butadiene	0.77
2	Isoprene	1.00
3	a_1	1.61
4	a_2	2.00
5	a_3	2.30
6	a_4	3.31
7	a_5	3.69
8	a_6	4.38
9	a_7	5.00
10	a_8	5.52
11	a_9	5.92
12	a_{10}	6.62
13	a_{11}	7.08
14	Styrene	7.62
15	a_{12}	8.24
16	a_{13}	8.68
17	a_{14}	9.07
18	a_{15}	9.30
19	a_{16}	9.70
20	Methylstyrene	10.1
21	a_{17}	10.6
22	a_{18}	11.1
23	Dipentene	11.4
24	a_{19}	11.8
25	a_{20}	12.5

Experimental

Column packing: Apiezon L, 15 % on Embacel 60–100 mesh
Column length: 183 cm
Column I.D.: 0.32 cm
Temperature programme: 40–120°; 3.3°/min
Injection port temperature: 130°
Pyrolysis temperature: 700°
Carrier gas: argon, with a flow rate of 25 ml/min
Detector: FID.

The method is based on the determination of the relative amounts[2] of five main components: the butene fraction, isoprene, styrene, methylstyrene and dipentene.

For semiquantitative determination peak areas were used; these were calculated from peak height × ½ peak height width. The detector response was taken as unit for all components. The semiquantitative analysis is based on the relative quantity of a single component A_x, which is defined as the ratio of the peak area of one component x to the sum of the peak areas of the five main pyrolytic components. A_B = the butene fraction; A_I = isoprene; A_S = styrene; A_{MS} = methylstyrene and A_D = dipentene. These quantities were calculated from the following equation:

$$A_x = \frac{\text{quantity of component } x}{\text{quantities of butenes + isoprene + styrene + methylstyrene + dipentene}}$$

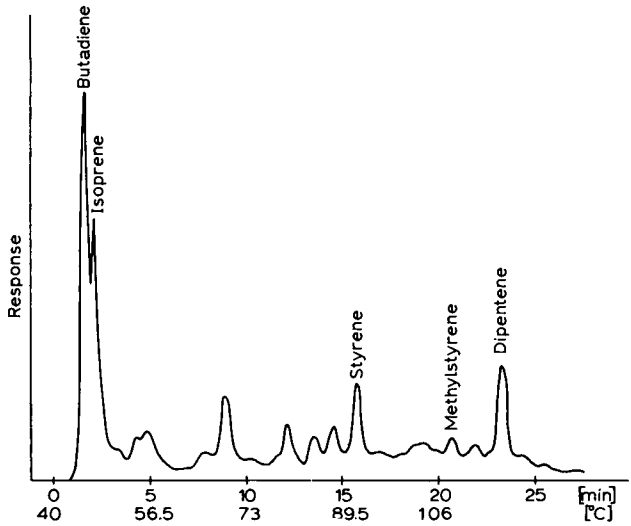


Fig. 1. Pyrogram of the elastomer: RSS-2 25%, SBR-1502 25%, BR-11 50%.

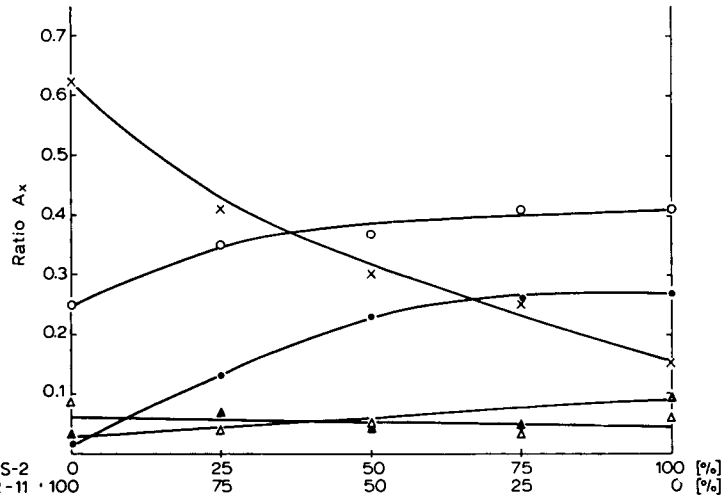


Fig. 2. Standard curves of different compositions of RSS-2 and BR-11. A_B (×); A_I (○); A_S (△); A_{MS} (▲); A_D (●).

Standard curves were plotted from samples of known composition of different stocks of natural rubber RSS-2, 1,4-*cis*-polybutadiene rubber BR-11 (Shell), styrene–butadiene rubber Europrene-1502 (Anic) and high styrene resin KBS-4581 (Chieh, Poland). The standard formulation for these was:

Elastomer	100
ZnO	3
Carbon black: IEP-black (Israel)	48
Antioxidant: ASM-PBN (Bayer)	0.8
Antiozonant: Santoflex IP (Monsanto)	0.7
Accelerator: Vulkacit CZ(Bayer)	1
Stearic acid	3
Paraffin	0.6
Sulphur	2

The samples were extracted with an acetone–chloroform solution 32:68 prior to the pyrolysis. The extraction time was 16 h. The results are collected in Tables II–VI and Figs. 2–6 show the standard curves for different vulcanised rubber stocks.

The method described yielded satisfactory results in the definition of the rubber

TABLE II

COMPONENT RATIOS OF DIFFERENT COMPOSITIONS OF NATURAL AND 1,4-*cis*-POLYBUTADIENE RUBBER

Rubber	*% Composition*				
RSS-2	—	25	50	75	100
BR-11	100	75	50	25	—
Fraction	*Ratios*				
A_B	0.62	0.41	0.30	0.25	0.15
A_I	0.25	0.35	0.37	0.41	0.41
A_S	0.085	0.041	0.049	0.032	0.063
A_{MS}	0.029	0.072	0.047	0.048	0.097
A_D	0.014	0.13	0.23	0.26	0.27

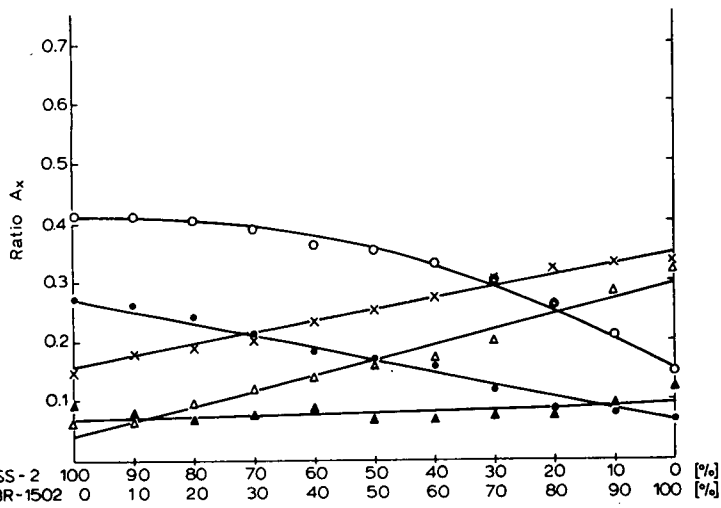


Fig. 3. Standard curves of different compositions of RSS-2 and SBR-1502. A_B (×); A_I (○); A_S (△); A_{MS} (▲); A_D (●).

TABLE III

COMPONENT RATIOS OF DIFFERENT COMPOSITIONS OF NATURAL AND STYRENE–BUTADIENE RUBBER

Rubber	% *Composition*										
RSS-2	100	90	80	70	60	50	40	30	20	10	—
SBR-1502	—	10	20	30	40	50	60	70	80	90	100
Fraction	*Ratio*										
A_B	0.15	0.18	0.19	0.20	0.23	0.25	0.27	0.30	0.32	0.33	0.33
A_I	0.41	0.41	0.40	0.39	0.36	0.35	0.33	0.30	0.26	0.21	0.15
A_S	0.063	0.068	0.096	0.12	0.14	0.16	0.17	0.20	0.26	0.28	0.32
A_{MS}	0.097	0.083	0.073	0.077	0.091	0.070	0.069	0.074	0.078	0.097	0.12
A_D	0.27	0.26	0.24	0.21	0.18	0.17	0.16	0.12	0.083	0.081	0.072

TABLE IV

COMPONENT RATIOS OF DIFFERENT COMPOSITION OF NATURAL, STYRENE–BUTADIENE AND 1,4-*cis*-POLYBUTADIENE RUBBER

(a) *Rubber*	% *Composition*				
RSS-2	—	12.5	25	37.5	50
SBR-1502	—	12.5	25	37.5	50
BR-11	100	75	50	25	—
Fraction	*Ratio*				
A_B	0.62	0.44	0.42	0.32	0.25
A_I	0.25	0.33	0.30	0.32	0.35
A_S	0.085	0.097	0.11	0.12	0.16
A_{MS}	0.029	0.054	0.047	0.052	0.070
A_D	0.014	0.080	0.12	0.17	0.17
(b) *Rubber*	% *Composition*				
RSS-2	—	12.5	25	37.5	50
BR-11	—	12.5	25	37.5	50
SBR-1502	100	75	50	25	—
Fraction	*Ratio*				
A_B	0.33	0.37	0.35	0.35	0.30
A_I	0.15	0.26	0.31	0.28	0.37
A_S	0.32	0.22	0.15	0.11	0.049
A_{MS}	0.12	0.062	0.052	0.056	0.047
A_D	0.072	0.088	0.15	0.21	0.23
(c) *Rubber*	% *Composition*				
BR-11	—	12.5	25	37.5	50
SBR-1502	—	12.5	25	37.5	50
RSS-2	100	75	50	25	—
Fraction	*Ratio*				
A_B	0.15	0.26	0.28	0.40	0.45
A_I	0.41	0.37	0.36	0.26	0.16
A_S	0.063	0.086	0.098	0.16	0.27
A_{MS}	0.097	0.071	0.048	0.079	0.075
A_D	0.27	0.26	0.21	0.10	0.035

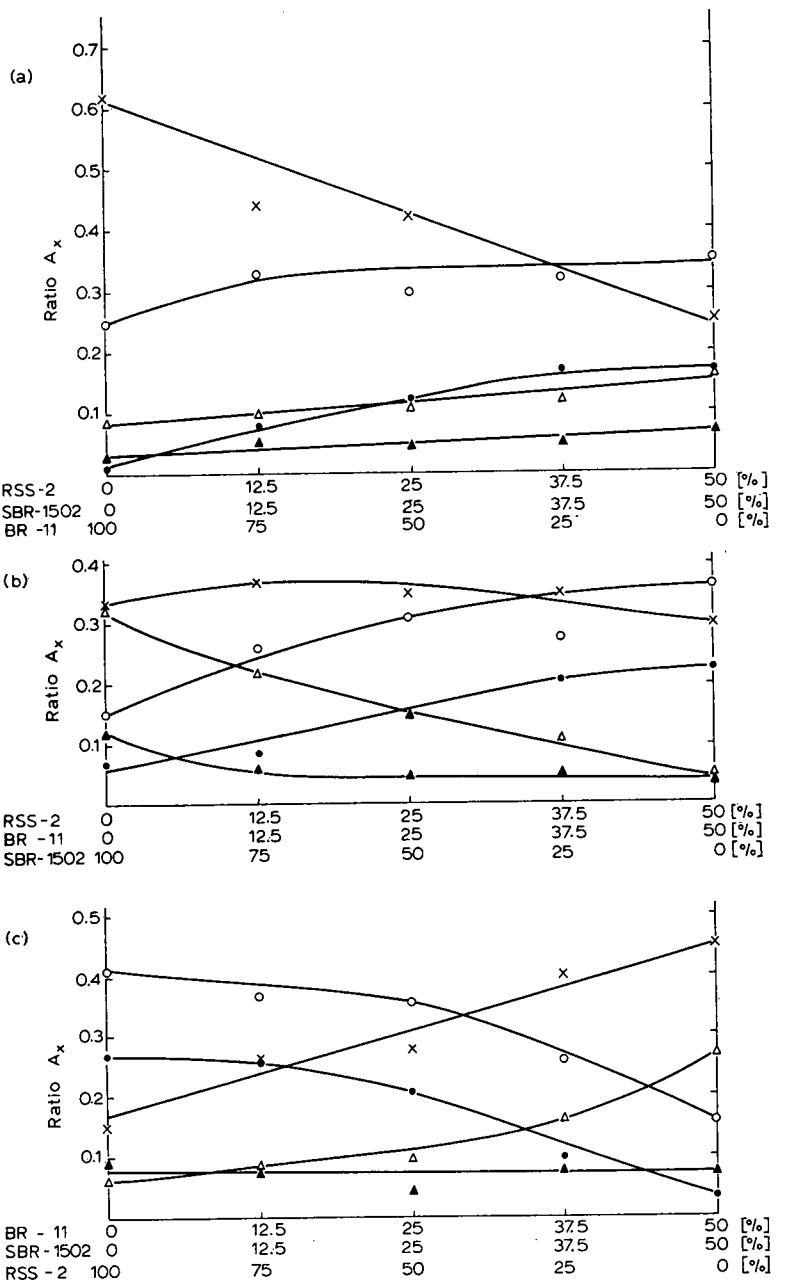


Fig. 4a–c. Standard curves of different compositions of RSS-2, SBR-1502 and BR-11. A_B (×); A_I (○); A_S (△); A_{MS} (▲); A_D (●).

TABLE V

COMPONENT RATIOS OF DIFFERENT COMPOSITIONS OF 1,4-*cis*-POLYBUTADIENE AND STYRENE–BUTADIENE RUBBER

Rubber	*% Composition*				
BR-11	—	25	50	75	100
SBR-1502	100	75	50	25	—
Fraction	*Ratio*				
A_B	0.33	0.41	0.45	0.52	0.62
A_I	0.15	0.13	0.16	0.18	0.25
A_S	0.32	0.33	0.27	0.17	0.085
A_{MS}	0.12	0.086	0.075	0.083	0.029
A_D	0.072	0.047	0.035	0.043	0.014

TABLE VI

COMPONENT RATIOS OF DIFFERENT COMPOSITIONS OF NATURAL, STYRENE–BUTADIENE AND HIGH STYRENE RUBBER

Rubber	*% Composition*				
RSS-2	—	12.5	25	37.5	50
SBR-1502	—	12.5	25	37.5	50
KBS-4581	100	75	50	25	—
Fraction	*Ratio*				
A_B	0.19	0.16	0.21	0.23	0.25
A_I	0.058	0.13	0.19	0.26	0.35
A_S	0.63	0.55	0.41	0.22	0.16
A_{MS}	0.10	0.093	0.082	0.16	0.070
A_D	0.026	0.063	0.11	0.13	0.17

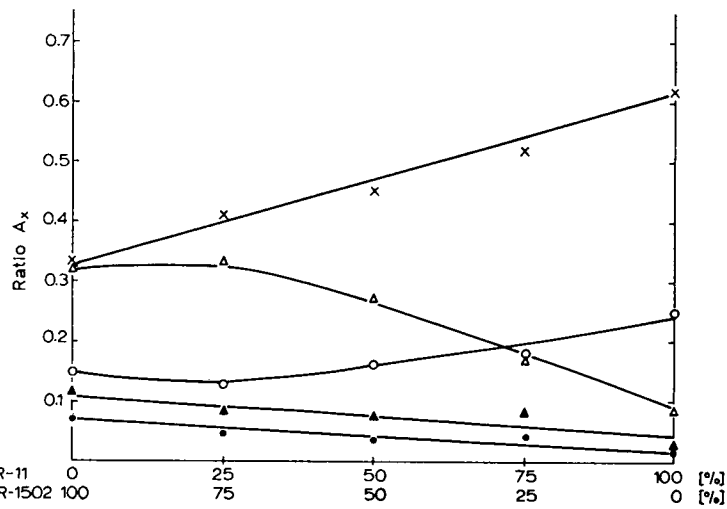


Fig. 5. Standard curves of different compositions of BR-11 and SBR-1502. A_B (×); A_I (○); A_S (△); A_{MS} (▲); A_D (●).

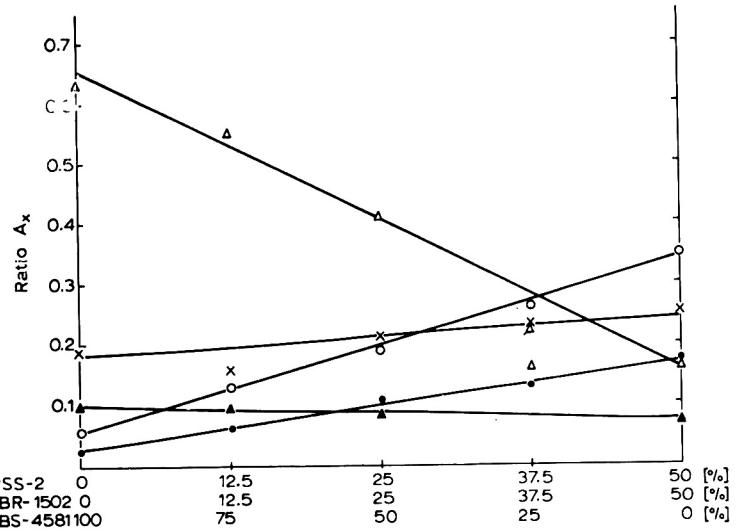


Fig. 6. Standard curves of different compositions of RSS-2, SBR-1502 and KBS-4581. A_R (×); A_I (○); A_S (△); A_{MS} (▲); A_D (●).

composition of the industrial products. The reproducibility was generally good, with σ_{rel} = about 10 %.

Acknowledgements

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CHROM. 3331

Gas chromatographic determination of isotopic molecules by means of open tubular thick layer graphitized carbon black columns*

High efficiency adsorption glass capillary columns may be obtained by replacing the smooth inner wall of a tubular column with a porous layer. This has been done by MOHNKE AND SAFFERT[1] and BRUNER AND CARTONI[2] by etching glass columns by various procedures. HALASZ AND HORVATH[3] obtained adsorption packed capillary columns by filling the columns with a suspension of a very fine support material or by drawing out glass tubes, which were loosely filled with a selected solid of narrow mesh range.

This paper reports on a new type of capillary column, the "open tubular thick layer glass column", which combines the properties of the open tubular and of the packed columns, by using as adsorption material graphitized carbon black. These columns are very effective for separating several isotopic systems.

Experimental

Preparation of the open tubular thick layer graphitized carbon black glass capillary columns. A thin steel wire (0.2 mm diameter) was inserted into a glass tube (2.2 mm I.D. and 6.6 mm I.D.) and one end of it was pulled by hand so as to leave about 30 cm of the steel wire outside the glass tubing. The glass tubing was loosely packed with Sterling MT, a graphitized carbon black (kindly supplied by Dr. SMITH of the Cabot Company) and transferred to a glass-tube drawing apparatus, made according to

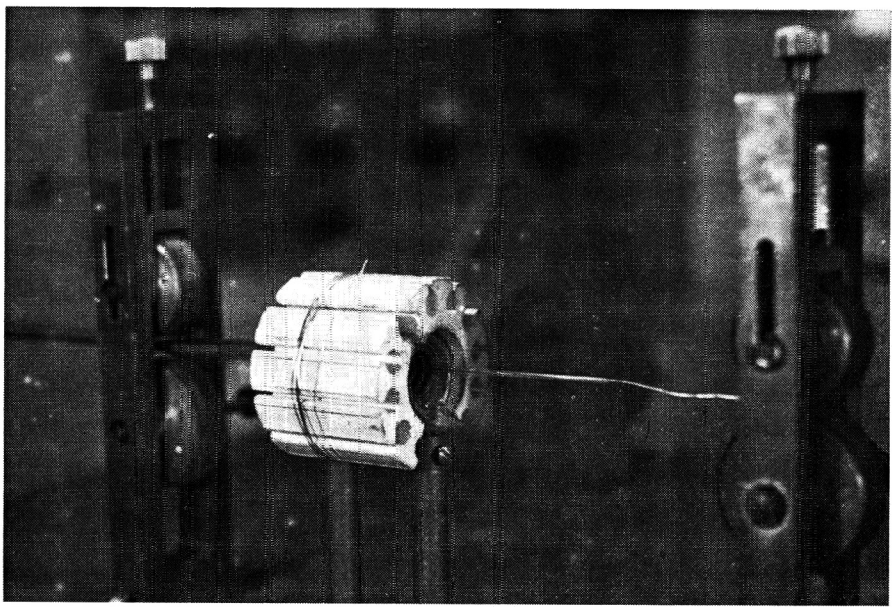


Fig. 1. Set-up for drawing-out the open tubular thick layer graphitized carbon black columns.

* Part of a paper presented at the 4th International Symposium on Advances in Gas Chromatography, New York, April 1967.

DESTY[3], as shown in Fig. 1. The other end of the steel wire was fastened to prevent the steel wire from being pulled into the glass capillary. The tube was drawn out at a ratio of 1:80. By means of this procedure a uniform thick layer of graphitized carbon black with an inner diameter of about 0.15 mm and outer diameter of about 0.7 mm is obtained.

This procedure seems to be of general application for the preparation of thick layers of any solid material in a glass capillary, providing the material does not undergo decomposition at the softening temperature of glass; the size of the packing material, however, seems to be a limiting factor.

Columns of various lengths are easily obtained and are employed as usual in a commercial apparatus (Fractovap C. Erba, Milan) equipped with a flame ionization detector. The behaviour of these columns has been checked by analyzing mixtures of various hydrocarbons. They have characteristics intermediate between the packed capillary and the open tubular columns.

Tailed peaks are usually obtained; to obtain symmetric peaks it has been found necessary to make the elution gas, nitrogen, bubble through a water trap: water vapour acts as an effective reducer of tailed peaks. As the support is a non-porous material it seems that the function of the water vapour is to eliminate the active sites due to the glass capillary.

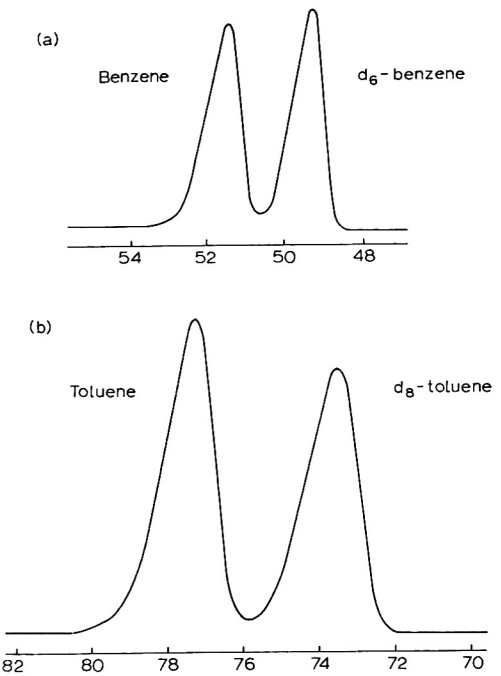


Fig. 2. Chromatographic separation on a 9.6 meter, 0.15 mm I.D. open tubular thick layer graphitized carbon black glass column of (a) C_6D_6–C_6H_6 T col. 69.5°C; P_{N_2} 1.75 atm., (b) C_7D_8–C_7H_8 T col. 98.4° C; P_{N_2} 1.9 atm.

An open tubular thick layer graphitized carbon black column was effective for the separation of the following isotopic species:

Benzene–deuterobenzene (d_6-benzene)
Toluene–deuterotoluene (d_8-toluene)
Acetone–deuteroacetone (d_6-acetone)
Pyridine–deuteropyridine (d_5-pyridine).

Some chromatograms are shown in Figs. 2 and 3. The column, which has a permeability value of 11×10^{-7} cm^2, has a length of 9.6 m. It permits the realisation of a complete separation for the hydrocarbons and a partial separation for pyridine and acetone. Table I shows (at the indicated temperature) the measured separation factors, α; the capacity ratio, K'; and the number of theoretical plates, n. The corresponding column length, L, and the analysis time to obtain a resolution, $R = 1.5$, have been calculated from these values.

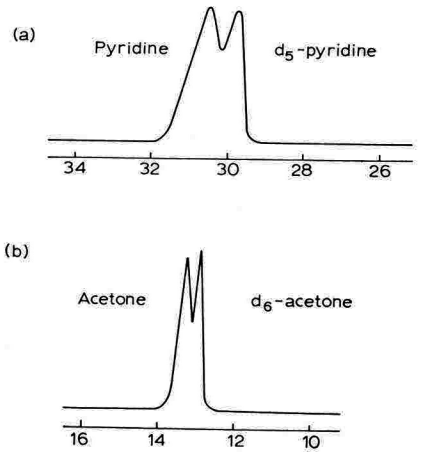


Fig. 3. Chromatographic separation as in Fig. 2 of: (a) C_5D_5N–C_5H_5N, T col. 87.5° C; P_{N_2} 1.25 atm; (b) C_3D_6O–C_3H_6O; T col. 52.8° C; P_{N_2} 1.20 atm.

These figures indicate that the efficiency of these columns for the separation of isotopic systems is much greater than with other partition and adsorption capillary columns[5].

Chromatograms have been run at various temperatures and in all cases an inverse isotopic effect has been observed, the heavier species being always eluted first. The logarithm of the relative retention volumes has been plotted *versus* $1/T$ (Fig. 4)

TABLE I

EXPERIMENTAL SEPARATION FACTORS, CAPACITY RATIOS, NUMBER OF THEORETICAL PLATES, ON A 9.6 m COLUMN, COLUMN LENGTH AND ANALYSIS TIME TO OBTAIN A COMPLETE SEPARATION

System	*T* (°*K*)	α	K'	*n theor.*	*L* (*m*)	*t* (*min*)
Acetone–deuteroacetone	325.0	1.042	2.0	12,000	38.3	52.6
Pyridine–deuteropyridine	360.0	1.030	10.7	12,000	40.7	94.6
Benzene–deuterobenzene	330.4	1.050	32.0	17,000	7.7	65.8
Toluene–deuterotoluene	360.2	1.056	37.7	16,000	8.1	111.4

to obtain the differences of enthalpy and entropy changes, which have been collected in Table II.

Comparison of these data with the chromatographic values obtained on partition and other adsorption columns seems to indicate that the high value of the separation factor obtained on these columns is mainly attributable to an entropy effect.

TABLE II

DIFFERENCES OF ENTHALPY AND ENTROPY CHANGES OF ISOTOPIC SYSTEMS

System	ΔH ($cal \cdot mol^{-1}$)	ΔS ($e.u. \times 10^2$)
d_6 Benzene–benzene	—34.78	— 1.62
d_8 Toluene–toluene	—30.30	+ 1.24
d_6 Acetone–acetone	—74.80	—14.72
d_5 Pyridine–pyridine	—58.31	—10.88

Discussion

The open tubular thick layer graphitized carbon black columns, developed in this investigation, have been found quite effective for the separation of isotopic systems as they combine the properties of packed capillary columns and open tubular columns.

These columns have a considerable amount of adsorbing material and the thickness of the layer may also be changed by using wires of different diameter in the column manufacture. They can be made to any length just as ordinary glass capillary

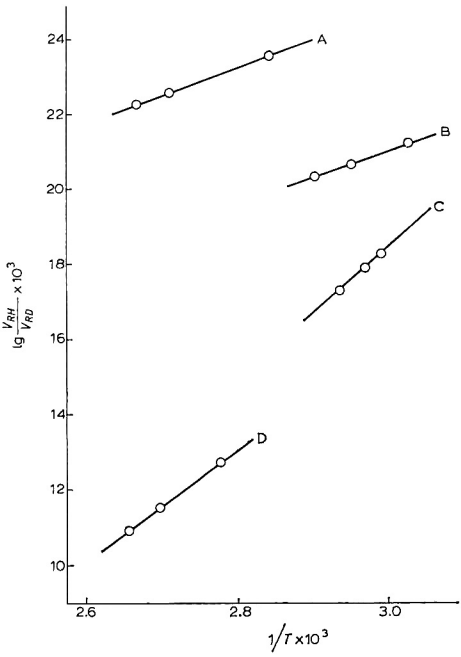


Fig. 4. Plots of logarithm $\times 10^3$ of ratio of retention volumes *vs.* $1/T \times 10^3$ for: (A) toluene–deuterotoluene; (B) benzene–deuterobenzene; (C) acetone–deuteroacetone; (D) pyridine–deuteropyridine.

glass columns and can operate with a low pressure drop. These features therefore permit separations which are not feasible with other columns.

Graphitized carbon black is a thermally stable, inert, non-specific and non-porous adsorbent. Its use in gas–solid chromatography for the separation of some hydrocarbons from their deuterated homologues was pointed out by YASHIN[6].

The interaction energy on this material depends upon the geometrical structure of the molecules and of the adsorbent surface and on non-specific dispersion forces acting between them. It seems that the different sizes of hydrogen and deuterium and the greater mobility of the deuterium compounds should be the determining factors which affect the different adsorption of the isotopic molecules.

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J. Chromatog., 34 (1968) 96–100

CHROM. 3352

Rapid method for calculating percentage recoveries of compounds from gas chromatograms

In studies on food flavours it is often desirable to know the efficiency of isolation of the volatile organic compounds responsible for a particular flavour. This is usually done by experiments on model systems where known amounts of compounds resembling the unknown in boiling point and other characteristics are added to the food, and are isolated by the appropriate technique. The percentage recoveries are often determined by comparing the peak heights of gas chromatograms of a standard solution of the compounds in an organic solvent with a chromatogram prepared with the isolated compounds.

Using this approach FORSS AND HOLLOWAY[1] have investigated the recoveries of added C_{2-10}*n*-alkan-1-ols and C_{3-12} alkan-2-ones from butter oil by molecular distillation and reduced pressure steam distillation. The yields were calculated by measuring the relative peak heights of the compounds in the gas chromatograms.

A more rapid and simpler method for the calculation involves the use of the graph illustrated in Fig. 1. The peak height on the standard chromatogram is measured with a pair of dividers. The point on the graph (B) where this distance (AB) corre-

sponds with the 100 % line, in Fig. 1 is noted. In this example B is 4.45. The equivalent peak height on the chromatogram of the recovered mixture is then measured with the dividers and the percentage recovery can be observed directly from the position of the dividers at 4.45 (B) along the line AB, in this case 85 % (BC).

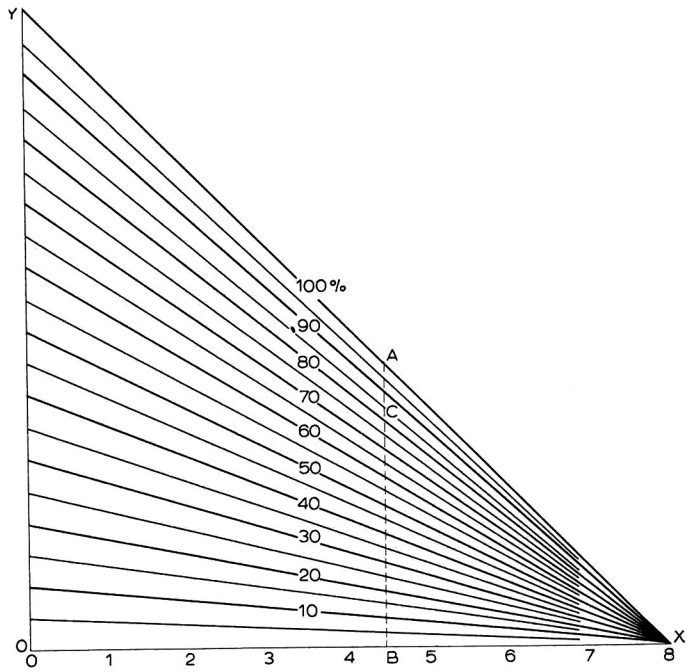


Fig. 1. Graph for determination of percentage recoveries.

A similar device has been described by BARRETT[2] for the measurement of R_F values in paper and thin-layer chromatography. In our case, it was necessary to include a linear scale along the x-axis. Additional lines at 5, 15, 25 % etc. increased the precision and the use of a triangle rather than a trapezium made it possible to work with very small peaks. The use of graph paper made it easy to keep BA vertical. The maximum height of a peak that can be calculated is determined by the distance at OY. In our case 8 in. was sufficient.

Alternatively, the scale may be traced onto celluloid and the device moved from peak to peak on gas chromatographic records. We preferred the former method using graph paper and dividers.

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CHROM. 3322

The progressive loss of aspartic and glutamic acids using a multiple column analytical system

Our laboratory is engaged in the amino acid analysis of proteins isolated from arctic mammals as part of a larger program involving genetic differences and speciation at the molecular level. We are using an ion exchange chromatography method based on the PIEZ gradient system[1] in which a battery of five 6 mm columns is programmed by a sequential switching system.

In the normal daily routine, samples are applied to three or four columns simultaneously. Except for the sample on the initial column which is immediately analyzed, the samples remain adsorbed at the tops of their columns for multiples of

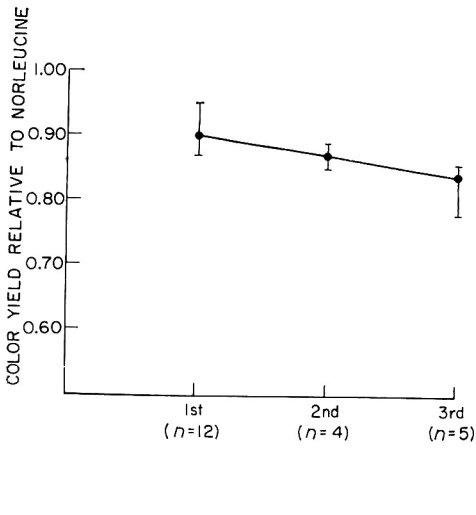


Fig. 1. Loss of aspartic acid on the basis of order of analysis. Differences are significant at the 95% level ("t" test).

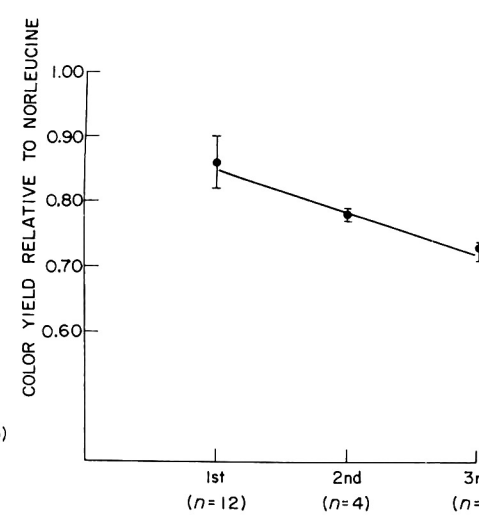


Fig. 2. Loss of glutamic acid on the basis of order of analysis. Differences are significant at the 95% level ("t" test).

six hours before elution begins and are thus exposed to a pH of 2.75, a temperature of 60° and a methanol containing (10%) solvent system[2] for six to eighteen hours.

The values obtained, when compared or grouped on the basis of operational sequence, indicated no significant differences for the majority of the amino acids, but did indicate a progressive decrease in recovery of both glutamic and aspartic acids. Fig. 1 shows the results obtained for the latter.

Fig. 2 shows a similar plot obtained for glutamic acid. Consequently we have incorporated a correction routine into our data handling procedures. The mean values (Table I) are used to correct each analysis on the basis of operational sequence. These factors are included in a computer program which applies the correction on the basis of a sequence number given the analysis.

TABLE I

MEAN RECOVERY OF ASPARTIC AND GLUTAMIC ACID ON THE BASIS OF ORDER OF ANALYSIS RELATIVE TO NORLEUCINE

Acid	*1st* ($n = 12$)	*2nd* ($n = 4$)	*3rd* ($n = 5$)
Aspartic	0.90	0.87	0.84
Glutamic	0.86	0.78	0.73

Differences observed are significant at the 95% confidence level.

To date, we have mostly negative information on the products formed. We have not found any positive correlation with any other peak elsewhere in the chromatogram.

It has been suggested that the color loss we have observed may be due to esterification[3]. In the case of aspartic acid the observed loss may very well be due to color yield differences of the methyl esters. If simple esterification is not the answer, another possibility is the conversion of the aspartic acid to fumaric or maleic acids. The ammonia released by this reaction might not be detected by our system. The large loss of glutamic acid is best explained by its conversion to ninhydrin negative pyrrolidone carboxylic acid[4]. Work to establish the degradation products is now in progress.

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CHROM. 3335

Column separation of phosphatides, acyl carnitines and carnitine

The separation and isolation of carnitine derivatives has been reported[1–6]. Although individual carnitine derivatives may be isolated by these procedures, it is often desirable to separate and isolate the phosphatides, acyl carnitines and carnitine by a single procedure. This is particularly true if the starting material is insect tissue. In these organisms when radioactive carnitine is used as a substrate, the products

in vivo are phosphatidyl β-methylcholine and acyl carnitines[1,7] and *in vitro* acyl carnitines, free carnitine and an unknown[1]. Two column procedures are described which permit the separation of phosphatides, acyl carnitines and carnitine.

Experimental

Extraction of lipid-bound carnitine. The procedure used for the extraction and analysis of the compounds isolated from insects has been described previously[1,7].

Column chromatography. In previous experiments[8] column chromatography on alumina proved to be satisfactory for the quantitative fractionation and isolation of radioactive phosphatidyl choline synthesized from ^{14}C-phosphoryl choline or ^{14}C-choline. If a mixture of phosphatidyl choline, palmityl carnitine and carnitine is placed on a basic alumina (cationatropic, activity grade I, M. Woelm, Eschwege) column, the separation shown in Fig. 1 is obtained. The identity of the peaks was confirmed by thin-layer chromatography[1].

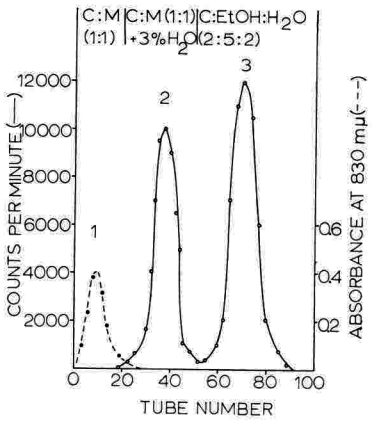
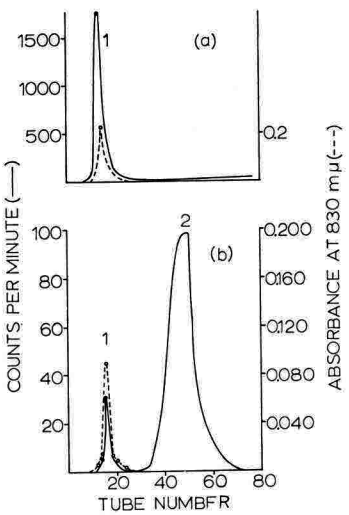


Fig. 1. Alumina separation of mixture of phosphatidyl choline, palmityl-^{14}C-carnitine and ^{14}C-carnitine. The palmityl-^{14}C-carnitine was synthesized by the method of BREMER[9]. 400 μg lipid P (phosphatidyl choline), palmityl-^{14}C-carnitine (150,000 c.p.m.) and ^{14}C-carnitine (80,000 c.p.m.) was applied on a 5 g, 1 × 10 cm alumina column. The column was then washed with 20 ml $CHCl_3$. The eluting solvent is shown on the figure. C = Chloroform; M = methanol. Five ml fractions were collected. Peaks: 1 = phosphatidyl choline; 2 = palmityl carnitine; 3 = carnitine.

When lipid-bound carnitine derivatives formed *in vitro* from either (methyl-^{14}C) or (carboxyl-^{14}C)-carnitine were fractionated on an alumina column, two peaks were found (Fig. 2b). The minor peak (peak 1) behaved similar to lecithin, but its identity is not yet determined[1]. The major peak (peak 2a) behaved similar to palmityl carnitine and was shown to be an acyl carnitine[1]. When a lipid extract from larvae reared on methyl-^{14}C-carnitine was fractionated on an alumina column (Fig. 2b), a major peak (Peak 1) was found as well as a minor peak (Peak 2). Peak 1 was identified as phosphatidyl-β-methylcholine and Peak 2 as an acyl carnitine[1]. The recovery of radioactivity was greater than 90 % on these columns.

In addition to separation on alumina, the lipid extracts were also separated on DEAE-cellulose (Matheson, Coleman and Bell) columns. Typical elution patterns are illustrated in Fig. 3. Peak 1 was shown to be phosphatidyl-β-methyl choline and



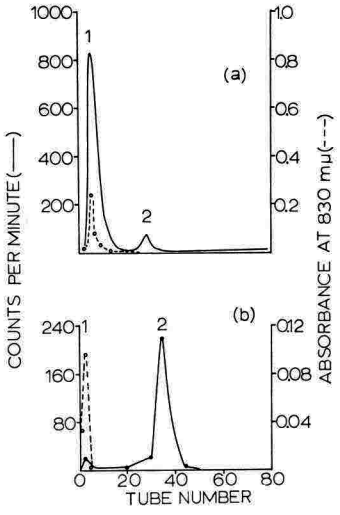


Fig. 2. Alumina separation of lipid extracts. (a) From larvae grown on a defined diet with (methyl-^{14}C)-carnitine as a choline substitute. (b) From fat body preparations of larvae incubated in the presence of (methyl-^{14}C)-carnitine. Conditions same as Fig. 1.

Fig. 3. DEAE cellulose chromatography of lipid extracts. (a) From sterile larvae grown on a defined diet with (methyl-^{14}C)-carnitine as a choline substitute. (b) From fat body preparation of larvae incubated in presence of (methyl-^{14}C)-carnitine. The extracts were applied to a 15 g, 2.2 × 25 cm DEAE-cellulose column prepared according to ROUSER *et al.*[10] The eluting solvent was $CHCl_3$–MeOH (9:1) and 5 ml fractions were collected.

Peak 2 an acyl carnitine[1]. Chromatography on DEAE-cellulose columns was qualitatively reproducible but the recovery of radioactivity was approximately 80 %. For the best recovery it is advisable to prepare new DEAE-columns for each fractionation.

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CHROM. 3351

Zur säulenchromatographischen Trennung von Katecholaminmetaboliten an Amberlite CG 50

Die als ein Hauptabbauprodukt von Adrenalin und Noradrenalin geltende 3-Methoxy-4-hydroxy-mandelsäure[1] wird in der Leber des Menschen und der Ratte über die 3-Methoxy-4-hydroxy-phenylglyoxylsäure[2] und den 3-Methoxy-4-hydroxy-benzaldehyd (Vanillin)[3] zur Vanillinsäure[4–6] abgebaut, die zum Teil ein Konjugat mit Glykokoll (Vanilloylglycin)[7] bildet. Der biologische Abbau der ebenfalls als Metabolit von Adrenalin und Noradrenalin beschriebenen 3,4-Dihydroxy-mandelsäure[8] verläuft teilweise in analoger Weise über die 3,4-Dihydroxy-phenylglyoxylsäure und den 3,4-Dihydroxy-benzaldehyd (Protocatechualdehyd) zur Protocatechusäure[9]. SEKI, INAMORI UND SANO[10] beschrieben 1959 Versuche zur chromatographischen Trennung von Phenolcarbonsäuren an Amberlite mit dem Lösungsmittelgemisch Methyläthylketon–Aceton–0.2 *N* HCl als mobiler Phase. Für die säulenchromatographische Trennung der als Katecholaminmetabolite beschriebenen Phenole hat sich diese Methode sehr gut bewährt.

Experimentelles

Substanzen. 3-Methoxy-4-hydroxy-mandelsäure, 3,4-Dihydroxy-mandelsäure (Calbiochem, Los Angeles), Vanillin (E. Merck A.G., Darmstadt), Protocatechualdehyd (C. Roth, Karlsruhe), Vanillinsäure (T. Schuchardt, München) und Protocatechusäure (Fluka, Buchs) wurden in Form von Handelsprodukten verwendet. 3-Methoxy-4-hydroxy-phenylglyoxylsäure wurde aus Acetovanillon (3-Methoxy-4-hydroxy-acetophenon)[11], 3,4-Dihydroxy-phenylglyoxylsäure aus Piperonal (3,4-Methylendioxy-benzaldehyd)[12] und Vanilloylglycin aus Vanillinsäure[13] dargestellt.

Präparation des Harzes. Die Präparation des im allgemeinen als Ionenaustauscher-Harz verwendeten Amberlite CG-50 II (Gegenion: H, 200–400 mesh, p.A., Serva, Heidelberg) erfolgte nach HIRS, MOORE UND STEIN[14]. Die Äquilibration des Harzes mit dem Laufmittel Methylathylketon–Aceton–0.2 *N* HCl (2:1:9, Vol. Teile) wurde auf einer Nutsche so lange durchgeführt, bis die Titration des Filtrats (mit 0.1 *N* NaOH) mit der des reinen Lösungsmittelgemisches übereinstimmte.

Präparation der Säule. Das in der mobilen Phase suspendierte Amberlite wurde in ein Glasrohr von den Ausmassen 1.0 × 110 cm bis zu einer Höhe von 90 cm eingeschwemmt. Durch einen die Säule umhüllenden Glasrohrmantel wurde aus einem Thermostaten Wasser von 30° gepumpt. Das Aufbringen der Substanzen (5 bzw. 2 μMol von jeder Substanz) erfolgte als Lösung jeweils in insgesamt 1.5 ml mobiler Phase, die Fraktionierung mit Hilfe eines Fraktionssammlers ("Radi Rac", Typ LKB 3400) in Fraktionen von je 32 Tropten. Die Durchflussgeschwindigkeit betrug 32 Tropfen/6 Min.

Quantitative Analyse der Fraktionen. Die quantitative Analyse der einzelnen Fraktionen erfolgte in Anlehnung an die Vorschrift von SEKI *et al.*[10]: sie wurden mit je 1 ml 0.1 *N* Natriumacetat-Lösung versetzt, nach Abdampfen des organischen Lösungsmittelanteils (Eindampfgerät nach HECKER UND KARLSON[15]) mit 0.5 *N* Natriumacetat auf 3.5 ml aufgefüllt, die Extinktionen bei 260 bzw. 280 mμ spektrophotometrisch gemessen.

Identifizierung der getrennten Substanzen. Die Identifizierung der säulenchromatographisch getrennten Substanzen erfolgte im Anschluss an die quantitative Analyse durch Aufnahme von U.V.-Spektren einzelner Fraktionen (Beckman Spektrophotometer DK-2A) sowie durch papierchromatographische Analyse (absteigende Chromatographie, Whatman-Papier Nr. 1) in den Lösungsmittelsystemen Benzol–Propionsäure–Wasser (2:2:1, Vol. Teile)[16] und Butanol–Eisessig–Wasser (4:1:5, Vol. Teile)[17].

Ergebnisse und Diskussion

Wie aus Fig. 1 ersichtlich, lässt sich ein Gemisch von je 5 μMol der 3-Methoxy-4-hydroxy-Verbindungen 3-Methoxy-4-hydroxy-mandelsäure (I), Vanilloylglycin (II), 3-Methoxy-4-hydroxy-phenylglyoxylsäure (III), Vanillinsäure (IV) und Vanillin (V) säulenchromatographisch gut auftrennen.

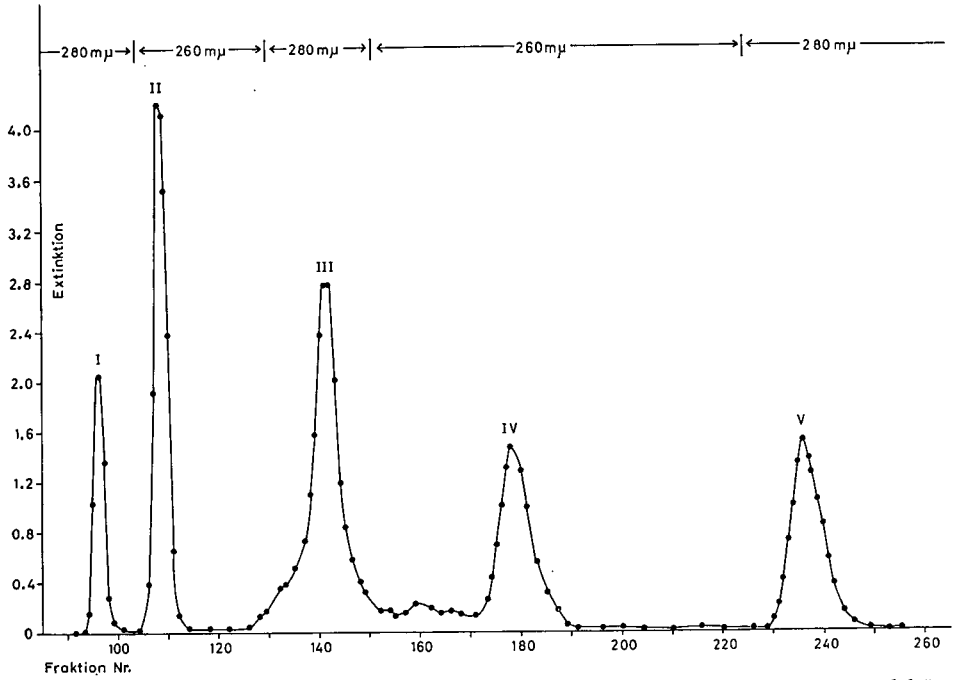


Fig. 1. Säulenchromatogramm eines Gemisches von je 5 μMol 3-Methoxy-4-hydroxy mandelsäure (I), Vanilloylglycin (II), 3-Methoxy-4-hydroxy-phenylglyoxylsäure (III), Vanillinsäure (IV) und Vanillin (V). Amberlite CG-50 II, p.A., 200–400 mesh. Mobile Phase: Methyläthylketon–Aceton–0.2 *N* HCl (2:1:9, Vol. Teile).

Fig. 2 zeigt die chromatographische Trennung eines Gemisches von je 5 μMol der genannten 3,4-Dihydroxy-Verbindungen in folgender Reihenfolge: 3,4-Dihydroxy-mandelsäure (I), 3,4-Dihydroxy-phenylglyoxylsäure (II), Protocatechusäure (III) und Protocatechualdehyd (IV).

Aus Tabelle I sind die für die quantitativen Analysen benutzten Wellenlängen und die prozentuale Wiederfindung der getrennten Substanzen ersichtlich.

Das Säulenchromatogramm eines Gemisches von 3,4-Dihydroxy- und 3-Meth-

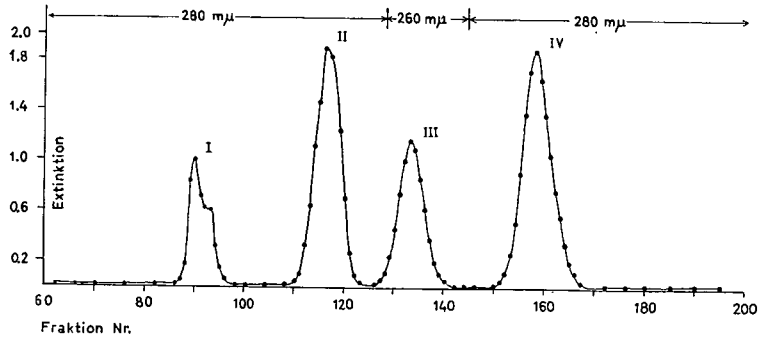


Fig. 2. Säulenchromatogramm eines Gemisches von je 5 μMol 3,4-Dihydroxy-mandelsäure (I), 3,4-Dihydroxy-phenylglyoxylsäure (II), Protocatechusäure (III) und Protocatechualdehyd (IV). Amberlite CG-50 II, p.A., 200–400 mesh. Mobile Phase: Methyläthylketon–Aceton–0.2 *N* HCl (2:1:9, Vol. Teile).

TABELLE I

PROZENTUALE WIEDERFINDUNG DER SÄULENCHROMATOGRAPHISCH GETRENNTEN SUBSTANZEN

Substanz	*Wellenlänge (mμ)*	*Wiederfindung (%)*
3-Methoxy-4-hydroxy-mandelsäure	280	82
Vanilloylglycin	260	103
3-Methoxy-4-hydroxy-phenylglyoxylsäure	280	101
Vanillinsäure	260	102
Vanillin	280	103
3,4-Dihydroxy-mandelsäure	280	96
3,4-Dihydroxy-phenylglyoxylsäure	280	80
Protocatechusäure	260	81
Protocatechualdehyd	280	104

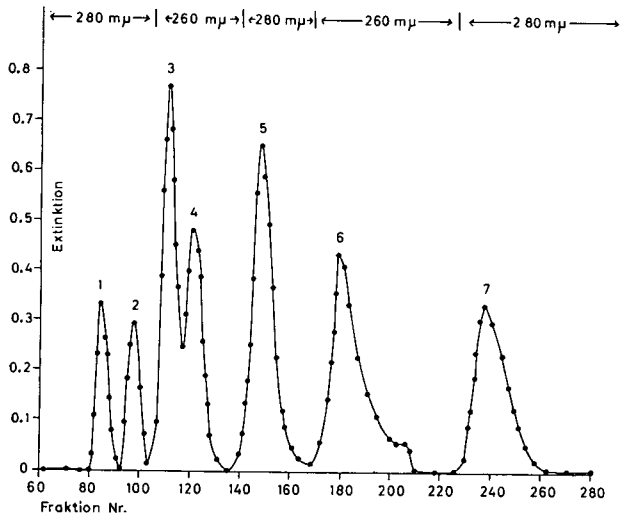


Fig. 3. Säulenchromatogramm eines Gemisches von je 2 μMol 3,4-Dihydroxy-mandelsäure (1), 3-Methoxy-4-hydroxy-mandelsäure (2), Vanilloylglycin (3), Protocatechusäure (4), Protocatechualdehyd (5), Vanillinsäure (6) und Vanillin (7). Amberlite CG-50 II, p.A., 200–400 mesh. Mobile Phase: Methyläthylketon–Aceton–0.2 *N* HCl (2:1:9, Vol. Teile).

oxy-4-hydroxy-Verbindungen zeigt Fig. 3. Es wurden je 2 μMol der folgenden Substanzen aufgetrennt: 3,4-Dihydroxy-mandelsäure (1), 3-Methoxy-4-hydroxy-mandelsäure (2), Vanilloylglycin (3), Protocatechusäure (4), Protocatechualdehyd (5), Vanillinsäure (6) und Vanillin (7).

Die erzielten Trennungen sind nicht aufgrund eines Ionenaustauschvorgangs zu erklären. SEKI *et al.*[10] weisen darauf hin, dass durch den Salzsäureanteil des als mobile Phase verwendeten Lösungsmittelgemisches die Ionisation der Carboxylgruppen des Amberlite-Harzes zurückgedrängt wird, so dass es für die zu trennenden Substanzen nur als nicht polares Adsorbens dienen kann. Die Verteilung der Substanzen erfolgt offenbar nach Massgabe ihrer Polarität, derart, dass die polaren Substanzen schneller wandern als die weniger polaren.

Dank

Die Arbeit wurde durch eine Sachbeihilfe der Deutschen Forschungsgemeinschaft unterstützt. Fräulein E. BÜSCHEMANN und Herrn H. W. NOLDEN ist der Autor für die fleissige Mitarbeit sehr zu Dank verpflichtet.

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CHROM. 3299

Gel filtration of iodoamino acids

Iodoamino acids present in enzymatic digests of thyroglobulin and in blood plasma are commonly separated and identified by paper[1] or thin layer[2] chromatography. To prevent overloading of the chromatograms, a preliminary purification is usually carried out, either by ion exchange chromatography[3] or by gel filtration[4]. Such a purification is essential when the iodoamino acids are grossly contaminated as, for example, in digests of the unencapsulated thyroid gland of teleost fish. Moreover, the sparingly volatile nature of the eluants used in previously described preliminary purification procedures necessitates the extraction or sublimation of each column fraction in order to remove buffer solids prior to chromatography.

The modified gel filtration procedure, now described, uses readily volatile eluants and gives markedly improved separations with the same simplicity of operation.

Method

A 1.5 × 15 cm column was packed with Sephadex G-25, Fine (Pharmacia Corp.), previously equilibrated for a minimum of 2–3 h with a buffer of composition pyridine–acetic acid–water (45:11.5:1943.5, v/v), pH 5.6. The effluent was passed through the well of a crystal scintillation counter connected via a ratemeter to a chart recorder (3 cm/h), prior to collection in round bottom flasks. The sample under investigation was centrifuged, any precipitate washed with buffer and the combined supernatant and washings transferred on to the column. After the elution of the iodotyrosines (MIT and DIT) was complete (100 ml), the buffer was replaced by *tert.*-amylol saturated with 2 *N* ammonia which eluted the iodothyronines (T_3 and T_4). With a flow rate of 0.5 ml/min the separation was completed within 4 h.

Prior to purification by gel filtration, blood plasma samples were digested with papain[4] to release protein bound hormones. Samples of plaice thyroid gland were homogenised in saline–Tris buffer, pH 8.3 and hydrolysed with pancreatin according to the procedure of TONG AND CHAIKOFF[5].

Results

Fig. 1 shows the separation of a model mixture of millimicrogram quantities of ^{125}I labelled amino acids and thyroglobulin. This indicates the complete separation of MIT, DIT and I^- and of T_3 and T_4 from these three compounds. Although separa-

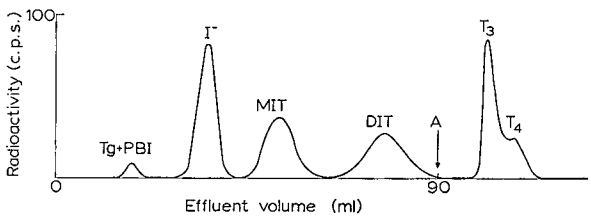


Fig. 1. Separation of iodoamino acids on Sephadex G-25. Tg = Thyroglobulin; PBI = protein-bound iodine; I = iodide; MIT = monoiodotyrosine; DIT = diiodotyrosine; T_3 = triiodothyronine; T_4 = thyroxine. A marks introduction of tertiary amylol saturated with 2 *N* ammonia.

tion of T_3 from T_4 was not sought in this study, it is expected that this may be achieved by elution with a slightly less polar solvent.

The purity of each of the fractions was established by thin layer chromatography; the eluates were prepared for chromatography merely by evaporation, *in vacuo* at 40°. Overall recoveries exceeded 95 % for each of the compounds examined.

Examinations of blood plasma iodoamino acids, performed on samples which had not been subjected to papain digestion, showed that the serum protein bound hormone (PBI) was eluted at the same time, relative to MIT and DIT, as thyroglobulin and that there were marked variations in the ratio PBI/(T_3 & T_4) in replicate analyses. This is in accord with a previous observation[6] that dissociation of PBI occurs during gel filtration, making the ratio dependent on such factors as column size and packing, rate of flow and pH.

The work described here forms a part of the programme of the Department of Agriculture and Fisheries for Scotland and was carried out during the tenure by one of us (R.H.O.) of a Natural Environment Research Council Studentship.

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CHROM. 3333

Separation of scopoletin from fluorescent compounds in roots of *Pisum Sativum* var. meteor by Sephadex gel filtration

Previous methods of separation of fluorescent compounds in plants have relied mainly on the use of paper chromatography and estimation directly on the paper by spot size measurement[1] or alternatively by elution from paper and estimation by fluorimetry[2]. Both methods were found by this author to be unsatisfactory. Methods based on spot area measurements proved unreliable due to fluctuations in the relationship between spot area and the intensity of fluorescence and were further complicated by the rather diffuse periphery of the spots.

Elution from paper was found to be quantitatively unsatisfactory with 5–30 % loss of fluorescence during 30 such separations. The loss increased with increasing concentration of scopoletin applied to the chromatograms.

The technique described here is based on separation using sephadex gel filtration.

For the initial extraction, root systems from twenty plants of *Pisum Sativum* var. meteor, grown in culture solution for 8 days at 22° were removed, frozen, to prevent enzymatic conversion of scopoletin to the glycoside scopolin, for twelve hours and then macerated. Plants grown in vermiculite yield similar results. The ground material was oven dried at 80° and extracted with 2 % acetic acid at 80° for 4 h. The supernatant liquid was drawn off and the residue further extracted with 2 % acetic acid until no fluorescence remained. The supernatant was evaporated at 80° to dryness and taken up in 1 ml of distilled water (phthalate buffer pH 6.8 may equally well be used).

A twelve inch, half inch diameter quickfit column of Sephadex G-15 was prepared and allowed to settle for twelve hours prior to use. Columns allowed to stand longer (up to seven days) behaved not differently from those standing twelve hours. The extract was quantitatively transferred to the column. An extension column was added and distilled water used to wash the extract through the column. Seven ml fractions were collected and examined for fluorescence using an E.I.L. direct reading fluorimeter. The results are shown in Fig. 1. Band I began eluting after 15 cc had run through the column and continued to elute until 90 cc had run off. Band II began to emerge immediately and was collected in 21 cc between 91 and 112 cc. The length of time taken depends on how tightly the column is packed. In the procedure described, band II emerges after 2–3 h. Left on the column at the end of the run are two further bands which move but little. Band III, a blue fluorescing zone penetrates between 50 and 80 mm into the column after 125 cc have been eluted. Band IV, a yellow fluorescing zone penetrates up to 20 mm into the column.

Band III may be separated from the other fluorescing bands using Sephadex G-25. On this grade of Sephadex an incomplete separation of bands I and II was obtained, as shown in Fig. 2. Band IV again penetrates but little, reaching 50 mm into the column after 200 ml have been eluted.

Band I, being a rather diffuse band on G-15 was concentrated and run on Sephadex G-10, no further separation was effected. That this band was due to only one compound was confirmed using paper chromatography (Tab. I). Each fluorescent

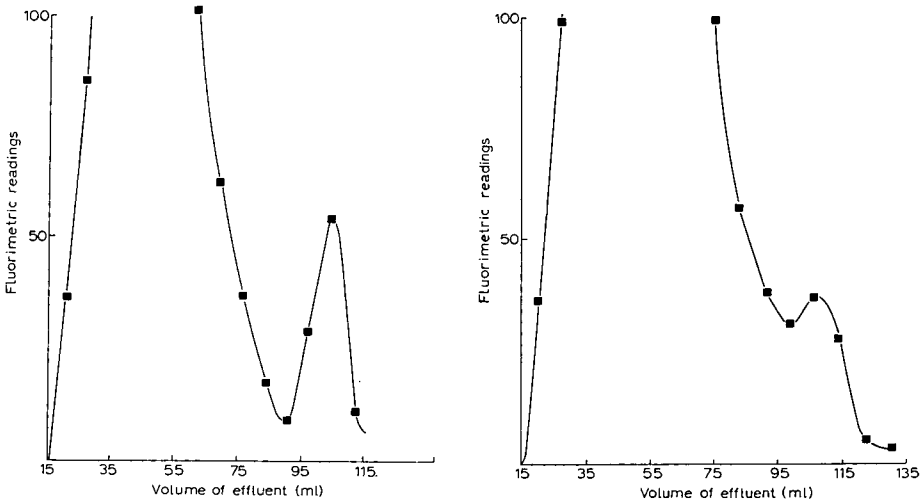


Fig. 1. Elution diagram for the separation of bands I and II on Sephadex G-15 columns.

Fig. 2. Elution diagram for bands I and II on Sephadex G-25 columns.

TABLE I

R_F VALUES OF SCOPOLETIN, SCOPOLIN, CHLOROGENIC ACID, CAFFEIC ACID AND ISOLATED COMPOUNDS

The solvent systems used are: (1) Isopropanol–acetic acid–water (5:0.1:95, v/v/v); (2) butanol–acetic acid–water (4:1:2, v/v/v); (3) 10% acetic acid; (4) benzene–acetic acid–water (6:7:3, v/v/v); (5) ethyl acetate–water (1:1, v/v).

Compound	*R_F values in solvent systems*				
	1	*2*	*3*	*4*	*5*
Band 1	0.72	0.50	0.76	—	0.08
Band II	0.48	0.86	0.47	0.85	0.85
Band III	0.32	0.80	0.38	0.7	0.34
Band IV	0.80	0.69	—	—	—
Scopolin	0.74	0.47–0.63	0.75	—	0.09
Scopoletin	0.48	0.85	0.48	0.85	0.85
Caffeic acid	0.35	0.78–0.83	0.38	0.67	0.35
Chlorogenic acid	0.80	0.68	—	—	—

band was examined and an attempt made to identify each compound by paper chromatographic methods. Synthetic scopoletin[3] was used for reference. This was run alongside each band as well as in combination with the extracted bands. Other reference compounds used include scopolin, caffeic acid and chlorogenic acid.

Band II thus appears to be scopoletin. This was confirmed by means of excitation and emission spectra measurements.:

Emission spectra:	Band I	440–445 mμ.
	Band II	465 mμ.
	Scopoletin	465 mμ.
Excitation spectra:	Band II	max 263, 306, 350 mμ; min 274, 315 mμ.
	Scopoletin	max 262, 305, 355 mμ; min 274, 318 mμ.

It is further suggested on the basis of the chromatography evidence that band I corresponds to scopolin, band III to caffeic acid and band IV to chlorogenic acid.

The value of this technique in separating the scopoletin (band II) from the other fluorescent compounds was illustrated by evaporating 0.4 μg of synthetic scopoletin and 0.4 μg of caffeic acid in 2 % acetic acid to dryness, taking up in distilled water and running on Sephadex G-15, followed by fluorimetric estimation. The reading obtained for the scopoletin containing fraction was compared with a direct reading of an equivalent weight of scopoletin in the same volume of distilled water. Loss from 10 replicate experiments was at most 2 %.

Thanks are due to Dr. J. S. DAVIDSON for the preparation of scopoletin.

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CHROM. 3304

A device for streaking thin-layer chromatograms

Large-scale thin-layer chromatography often requires application of a sample in a band, or streak, along the starting line of the chromatogram. A device which applies a known quantity of sample solution in a uniform, homogeneous streak is described in this note. It is very easy and inexpensive to build, and has the great advantage of requiring no supervision while it is operating.

Fig. 1 shows the principle parts of the streaker. Basically, the device consists of a peristaltic pump with a moving delivery nozzle. The prototype was constructed with a children's "Erector Set" (a toy mechanical construction kit), with a few simple specially-machined parts. It is driven by a small electric motor equipped with a gear-reducer, to give a drive speed of 0.25 r.p.m. The motor drives a brass roller (1.25 cm in diameter). The tube containing the sample is squeezed between this drive roller and a freely rotating roller mounted on a movable shaft; pressure is supplied by an elastic band. As the pump tube is pulled and squeezed by the rollers, the sample solution is forced out through a polyethylene nozzle (a short length of 0.28 mm I.D. polyethylene tubing) attached to the 1.6 mm I.D. rubber pump tube by means of a tapered glass connector. The pump tube is clamped gently to a four-wheeled carriage which rolls on a metal track alongside the thin-layer plate. Obviously, the sample is

forced from the nozzle at a rate proportional to that of the motion of the carriage, for the carriage is drawn along by the pump tube. A freely pivoting arm 5 cm long with a brass roller 1 cm in diameter and 0.5 cm wide mounted at one end is suspended from the side of the carriage, so that the roller rolls along the surface of the thin layer. The nozzle is clamped onto the arm in such a way that its tip just barely touches the layer within a few millimeters of the roller. Also suspended from the side of the carriage is a tube (0.5 cm diameter) through which air or inert gas may be blown to dry the sample.

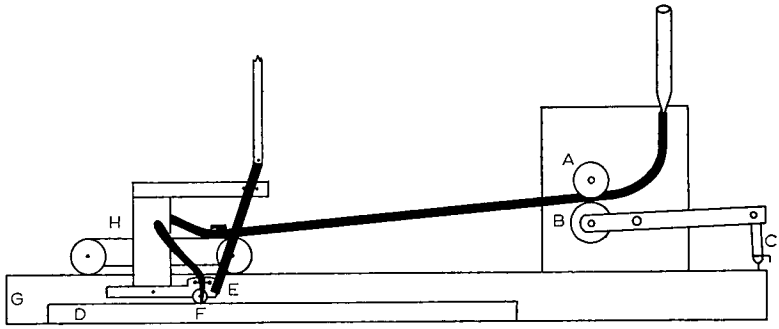


Fig. 1. (A) Drive roller; (B) free roller mounted on movable shaft; (C) elastic band; (D) thin-layer plate; (E) tube for air or inert gas; (F) polyethylene nozzle and brass guide roller; (G) track; (H) carriage.

The sample is introduced into the pump tube and forced through by gentle air pressure (a dropper bulb) until it begins to emerge through the nozzle. Then the pump rollers are clamped together and the motor engaged. A large sample may be applied by multiple passes: to begin a second pass, the pump tube is pinched off, the roller pressure released, and the carriage pulled back to the starting point.

The relevant quantities such as volume of sample and rate of application may easily be varied by varying pump tube diameter, motor speed, and drive roller diameter. With the dimensions given, 0.25 ml is streaked in one 16 cm pass, taking about 16 min. The streaks are of uniform width (variable with solvent, speed, and air flow) and density, and the layer is not damaged. Except for the few minutes required for flushing and refilling, the streaker runs unattended. A buzzer and microswitch may be added to indicate the end of a pass.

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CHROM. 3350

Separation of sugars by thin-layer chromatography

The utilization of thin-layer chromatography (TLC) for the separation of carbohydrates has been much less widespread than its use for the separation of other biological materials. This has been due principally to the poor separation of some sugars by TLC and to the low capacity of the chromatoplates[1-3]. Consequently the inherent advantages of TLC over paper chromatography such as speed and sensitivity have not been entirely realized. In an attempt to improve carbohydrate separations by TLC, a variety of adsorbents, and mixtures of adsorbents, as well as various solvent combinations have been studied (*e.g.* refs. 1–14).

In our experience, good separations of substantial quantities of sugars have been achieved with silica gel and a simple solvent system that, to our knowledge, has not previously been employed. The purpose of this paper is to report some examples of separations that have been obtained.

Experimental

Preparation of chromatoplates. 40 g of Kieselgel D-O (Camag, Muttenz, Switzerland), 5 g of calcium sulfate, and 95 ml of 0.02 *M* sodium acetate buffer were manually shaken in a glass stoppered flask for 1 min. The slurry was transferred to a Desaga adjustable applicator, and layers 0.5 mm thick were deposited on clean glass plates. The use of buffer is not essential, although it seems to yield slightly cleaner separations of certain sugars, for example, arabinose and fructose. The plates were allowed to dry overnight at room temperature, then were used without further preparation, except for scoring into lanes 1 to 2 cm in width.

Procedure. Aliquots containing appropriate quantities of sugars, or sugar mixtures, dissolved in water, were applied to the plates with a Hamilton microsyringe (Hamilton Company, Inc., Whittier, Calif.). Applications were made in small increments (less than 1 μl) under a stream of warm air from a heat gun to facilitate rapid drying, and thus to minimize spot diffusion.

Ascending chromatography was conducted in closed glass tanks lined with Whatman No. 4 paper saturated with the developing solvent, to a distance of 10 cm from the origin. First developments, made at ambient temperature, took about 75 min, subsequent runs took about 60 min. After a run, the plates were dried with the aid of a heat gun then were either returned to the glass tank for another ascension, or were sprayed with the detecting reagent.

Solvent system. A wide variety of solvent systems were surveyed including a number that have previously been utilized for both paper chromatography and TLC; most of these generally gave unsatisfactory results under our conditions. The system that produced distinctly superior separations was chloroform–acetic acid–water (3.0:3.5:0.5, v/v); this system was employed for the studies described in this paper, with one exception to be noted.

Detection of spots. Of a number of spraying agents compared, one containing diphenylamine[15] appeared to produce the best results. The reagent was made up by dissolving 1 g diphenylamine and 1 ml aniline in 100 ml of acetone; prior to spraying, 10 ml of the acetone solution was mixed with 1 ml of 85 % phosphoric acid. The

sprayed plate was heated for 10 min at 130°. Most of the sugars appeared as dark grey spots; pentoses, however, were light bluish-grey, and fructose and fructose-containing compounds were rosy brown (when freshly heated).

Densitometry. Tracings of chromatoplates were made by transmission densitometry, using a Photovolt Model 530 TLC densitometer equipped with a 520 A photometer and a 52 C light source. Filter 610 was utilized in the scanning slit. The TLC stage was driven by a 5138 synchronous motor drive.

Preparation of starch hydrolyzate. To a 20 % commercial wheat starch slurry was added 0.1 % (based on starch) HT-1000 bacterial alpha amylase (Miles Chemical Co., Elkhart, Ind.). The slurry was heated at the rate of 2°–4° per min from 25° to 100°, and held at the latter temperature for 15 min. One μl of the crude hydrolyzate was used for TLC.

Results and discussion

An example of the separations achieved with the procedure outlined above is provided in Fig. 1.

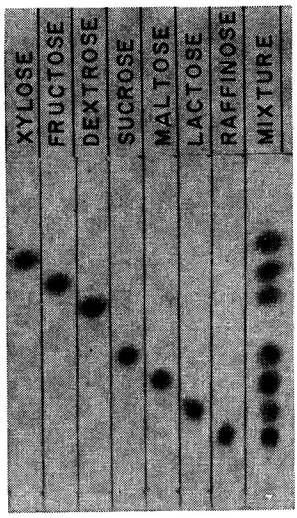


Fig. 1. Separation of seven indicated sugars (100 μg each) on buffered silica gel with chloroform–acetic acid–water (3.0:3.5:0.5, v/v); a threefold development was employed. Spots were visualized with aniline–diphenylamine.

TABLE I

R_G × 100 VALUES OF SUGARS

Xylose	126
Fructose	113
Glucose	100
Sucrose	74
Maltose	61
Lactose	47
Raffinose	32

Good separation of seven sugars of general biological interest, representing a pentose, two hexoses, three disaccharides, and a trisaccharide, may be noted. The system employed produced spots of desirable geometry: compact, circular spots were obtained for all the sugars, with no tailing or bearding evident. Combinations of other solvent systems and/or adsorbents studied in this laboratory yielded sugar spots of less satisfactory configuration, as manifested by tailing and diffusion, and distorted shapes.

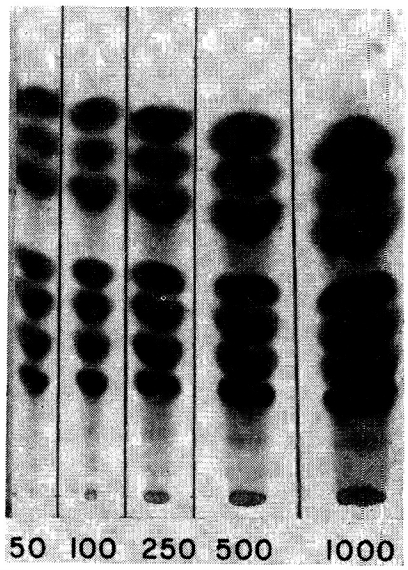


Fig. 2. Separation of indicated quantities (μg) of each sugar. Sugars and conditions same as in Fig. 1, except that a fourfold development was conducted.

The number of developments required to effect adequate sugar separations is largely dependent on the quantities of sugars involved. For the amount of material studied in Fig. 1 (100 μg of each sugar), two ascensions appear to be sufficient, but a threefold development enhances the separation.

The $R_G \times 100$ values of the sugars are summarized in Table I.

Aside from the ability to produce well-separated spots exhibiting good geometry, a desirable TLC procedure should have the property of handling relatively large amounts of material without overloading the chromatoplates. This feature is of particular importance when quantitation by elution of separated sugars followed by colorimetry is contemplated. That the combination of silica gel adsorbent and a chloroform–acetic acid–water solvent system may allow substantial quantities of sugars to be satisfactorily separated is indicated in Fig. 2.

Discrete separations were obtained when as much as 250 and 500 μg of each sugar was deposited on the plate. When larger quantities of sugars are being handled, additional development may be advisable; four developments were employed for this separation.

Such separations suggest that quantitation through elution and colorimetry should be quite feasible; work on this is currently proceeding in our laboratory.

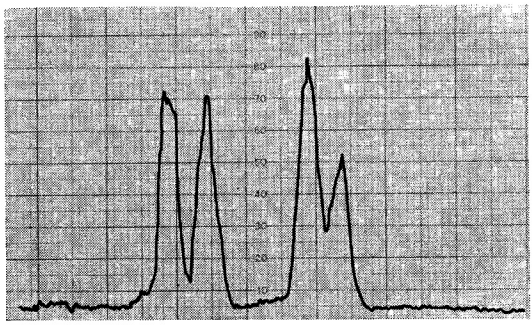


Fig. 3. Densitometer tracing of TLC plate. From right to left peaks represent fructose, glucose, maltose, and lactose; 100 μg of each sugar was deposited. Solvent and detecting system same as in Fig. 1.

Quantitation by densitometry is an alternative route, provided that adequate separations of sugars in a mixture are achieved. A densitometric tracing of a chromatogram of fructose, glucose, maltose, and lactose, separated under the conditions described above, is shown in Fig. 3.

Good peak definition and a steady base line are apparent. These characteristics indicate that a relatively rapid procedure for the quantitative evaluation of sugars in a mixture by TLC and densitometry should be possible.

Among the many applications for TLC in the field of carbohydrates is that of the analysis of commercial corn syrups[16], and of starch hydrolyzates in general. An example of a TLC separation of a crude wheat starch hydrolyzate is furnished in Fig. 4.

Separation of glucose units having D.P. values of from 1 to 9, and possibly to 10,

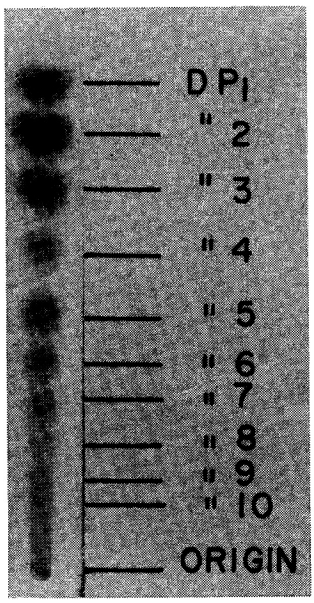


Fig. 4. Separation of oligosaccharides in crude starch hydrolyzate, with chloroform–acetic acid–water (10:79:11, v/v) and four developments. Spots were visualized with aniline–diphenylamine.

are indicated. To obtain this separation, the solvent system proportions were altered in the direction of greater polarity (chloroform–acetic acid–water (10:79:11, v/v)); four developments were conducted.

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CHROM. 3305

A rapid method for the determination of *p,p'*-DDT to *p,p'*-DDE ratios in fish

We wish to describe a more rapid method of analyzing for the presence of DDT in fish than the commonly used method of MILLS, ONLEY AND GAITHER[1]. The following procedure was developed as the result of a need to routinely and rapidly determine the ratio of *p,p'*-DDT to its metabolites and although the recoveries of the pesticides from the tissues were not as high as those obtained using the above method, it was found to be satisfactory for obtaining the ratios of *p,p'*-DDT to *p,p'*-DDE.

Tissue samples ranging from 0.005 to 0.1 g were digested in 2–4 ml of formic acid and maintained at 60° for 1.5–2 h followed by extraction four times with hexane. The combined extracts were evaporated to dryness and the residue was washed with three 2 ml portions of acetonitrile. A small amount of alumina (20 mg, 80–200 mesh) was added and the sample was centrifuged. After evaporating the supernatant to dryness, the residue was dissolved in a small amount of ethyl acetate and spotted on a plate coated with aluminum oxide using the procedure described by KOVACS[2].

Since our samples contained ^{14}C-labeled DDT and its corresponding metabolites, DDE and DDD, a radio chromatogram scanner was used to detect the amounts

of the radioactive compounds. Although the use of heptane as an elution solvent[2] for the separation of *p,p'*-DDT from *p,p'*-DDE was good enough for most tissues, their separation in some tissues was not sufficient to allow the recorder pen of the scanner to return to the base line between the two compounds. However, substitution of low-boiling (30–60°) petroleum ether for heptane provided better separation as evidenced by Table I.

TABLE I

R_{DDD} VALUES OF *p,p'*-DDT AND *p,p'*-DDE IN DIFFERENT ELUENTS[a]

Tissue	*Pesticide*	*Heptane*	*Petroleum ether*
Skin	DDT	1.9	1.9
	DDE	2.6	2.9
Muscle	DDT	2.0	2.0
	DDE	3.1	3.3
Testes	DDT	1.7	1.7
	DDE	2.2	2.4
Kidney	DDT	1.2	1.3
	DDE	1.4	1.8
Ovary	DDT	1.7	1.7
	DDE	2.2	2.2

[a] Eluted for 16.5 cm, other conditions remaining constant for both eluents.

Although the difference in the R_{DDD} values is small, it was sufficient to allow the pen to return to the baseline, thereby giving two distinct peaks with more accurately measurable areas than those in which an overlap occurs.

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CHROM. 3298

The detection of pyruvate and phosphoenolpyruvate on chromatograms

A comprehensive technique has been described by the author[1] for the separation and identification on paper chromatograms of the phosphorylated compounds of the glycolytic pathway and related reactions. Unfortunately, in two of the solvents most useful for the separation of phosphates, namely the third solvent (GW_3) described by GERLACH, WEBER AND DÖRING[2] and the isobutyric acid solvent of KREBS AND HEMS[3], phosphoenolpyruvate and inorganic phosphate have nearly the same R_F value. Since many of the mixtures which may be studied by paper or thin layer chromatography contain considerable amounts of inorganic phosphate the phosphoenolpyruvate spot is obscured by the larger overlying spot of orthophosphate when chromatograms are treated with an acid molybdate reagent and subjected to ultraviolet irradiation. For easy identification of phosphoenolpyruvate a reagent is required that does not depend on reaction with the phosphate group.

Such a reagent has now been developed based upon the rapid catalytic breakdown of phosphoenolpyruvate to pyruvate and inorganic phosphate in the presence of mercuric ion[4] and the detection of pyruvic acid as the highly fluorescent 2-hydroxy-3-methylquinoxaline formed by condensation with *o*-phenylenediamine in acid[5]. The latter reaction also serves as an easy and sensitive means of detecting pyruvic acid itself.

Paper chromatograms are dipped through a solution of 0.1 *M* mercuric nitrate in *N* nitric acid diluted with 2 volumes of ethanol. When the papers are dry they are dipped through a mixture of 5 ml 2 % *o*-phenylenediamine hydrochloride, 5 ml 2 *N* sulphuric acid, and 20 ml of ethanol. Pyruvate and phosphoenolpyruvate appear as pale yellow spots with a green fluorescence under both short and long wave ultraviolet light. The spots appear at room temperature but may be intensified a little by heating for 2 min at 90°; longer heating increases the colour of the background and reduces the fluorescence. After treatment with mercury, 2 $\mu g/cm^2$ of phosphoenolpyruvate and 1 $\mu g/cm^2$ of pyruvate may be detected. If the mercury treatment is omitted phosphoenolpyruvate does not show up but as little as 0.5 $\mu g/cm^2$ of pyruvate may be detected. The previous treatment with mercuric nitrate reduces the fluorescence given by pyruvate by about half. The comparison of fluorescent spots on duplicate chromatograms, one of which has been treated with mercuric ions, and the other that has had this treatment omitted appears to provide a very specific way of detecting phosphoenolpyruvate.

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CHROM. 3325

A rapid method for the estimation of seromucoid synthesis

It is well known that in disease states the serum glycoprotein concentration shows a marked rise[1], probably due to the stimulation of hepatic synthesis by an unknown mechanism[2]. The estimation of the rate of synthesis is more meaningful in the experimental investigation of this problem than are changes in the serum level, but the usual isotopic methods are rather laborious for use in the large numbers of animals required. A rapid method for screening tissue extracts and other substances for their ability to affect the synthesis of seromucoids in mice has therefore been devised, based on the observation that the α-globulin fraction incorporates sulphate[3].

The mice are injected with 50 μC of ^{35}S as sulphate and bled 24 h later. This time interval allows maximum incorporation with little recirculation. A 50 μl sample of serum is placed on Whatman No. 3 M paper strips 5 cm wide, and electrophoresis is carried out in the usual way in barbitone buffer pH 8.6, for 18 h at 2 mA per strip. The papers are dried and scanned in a Packard gas flow counter with 4π configuration, using an 0.5 cm slit. A typical scan is shown in Fig. 1. As stated below, some activity seems to be associated with the transferrins, and therefore only the area of the peak from the edge of the β zone up to the end of the trace is measured and the activity calculated from this. Two standards of 0.1 μC are applied to paper and their mean activity similarly calculated. The results are expressed as the percentage of the original dose per 10 ml of serum.



Fig. 1. Scan of ^{35}S activity in mouse serum proteins after paper electrophoresis.

That inorganic ^{35}S is cleared from the strip during electrophoresis was shown by adding the labelled sulphate to a normal serum. After electrophoresis no detectable activity was present on the strip, whereas with a labelled serum, approximately 50 % of the total serum activity remained on the strip. The reproducibility of the method was determined by repeated electrophoresis of a labelled sample; the mean activity of 8 separations was 246 $\pm$ 1 c.p.m. (S.D. $\pm$ 1.0 % of mean). The electrophoresis method was compared with the more lengthy extraction procedure, in which the serum is

dialysed for 72 h, the seromucoid precipitated and digested, and the activity subsequently counted by a scintillation method using a dioxan phosphor[4]. The comparison is shown in Fig. 2, together with the regression line and 95 % confidence limits; the correlation is statistically highly significant ($P < 0.001$).

In an attempt to define more precisely which fractions were incorporating the isotope, labelled samples were run in a standard vertical starch-gel system[5]. Gasflow scanning of a gel strip showed the major part of the activity to be present in the fast α_2 and post-albumin zones. Two-dimensional paper and starch-gel electrophoresis was also carried out, with subsequent elution and scintillation counting of individual areas. Approximately 25 % of the total serum activity was present in the post albumin zone,

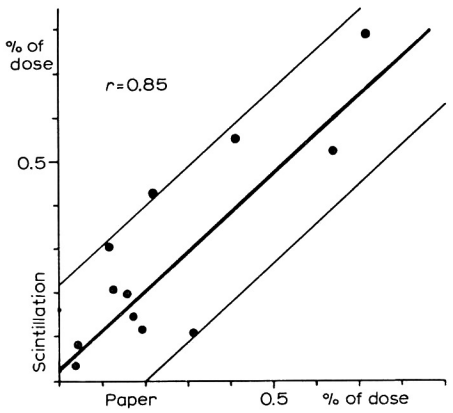


Fig. 2. Comparison of paper electrophoresis and liquid scintillation procedures for the estimation of incorporated ^{35}S in mouse serum. (Fitted regression line with 95% confidence limits).

and 12 % in the fast α_2 zone. Some 10% was present in the β zone and from its position could have been due to trailing of the faster α globulins on the paper, but a further 15 % was definitely associated with the transferrin bands. Other fractions showed negligible activity.

By this method the mean normal value for 17 mice was 0.595 ± 0.05 % of the original dose per 10 ml of serum, and in 8 mice given an injection of turpentine the value was 1.029 ± 0.14 %, indicating the expected marked rise in synthesis rate, ($P < 0.005$).

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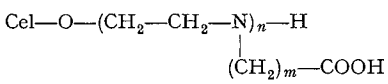
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CHROM. 3342

Trennung von Aminosäuren und Dipeptiden durch Ionenaustauschchromatographie an einem amphoteren Celluloseionenaustauscher

In einer früheren Publikation[1] haben wir über die Synthese amphoterer Celluloseionenaustauscher durch Pfropfpolymerisation von Äthyleniminoessigsäureäthylester sowie β-Äthyleniminopropionsäuremethylester[2] auf vernetzter Cellulose[3] und nachfolgender Verseifung der Estergruppen berichtet. Das komplexchemische Verhalten dieser amphoteren Cellulosederivate gegenüber Schwermetallionen (Cu^{2+}, Ni^{2+} Zn^{2+}) gleicht dem entsprechender Ionenaustauscherharze, die von uns durch Polymerisation von Äthyleniminoalkylcarbonsäureestern erhalten wurden[4–6]. Die Cellulose-Pfropfpolymerisate stellen mit grosser Wahrscheinlichkeit Verbindungen folgenden Strukturtyps dar:



wobei:

m = 1 bzw. 2.

Amphotere Celluloseionenaustauscher wurden bisher noch nicht für säulenchromatographische Trennungen verwendet. Ziel der vorliegenden Arbeit war es, die Anwendbarkeit amphoterer Cellulosederivate für die Trennung organischer Ampholyte, speziell von Aminosäuren und Dipeptiden zu prüfen, um aus den Ergebnissen möglicherweise Rückschlüsse auf die Verwendbarkeit der Austauscher für andere Trennprobleme ziehen zu können.

TABELLE I

POLYÄTHYLENIMINOESSIGSÄURECELLULOSE (CELLULOSE MIT 1,3-DICHLORPROPANOL-(2) VORVERNETZT[3])

Kapazität*	2.3 mÄquiv./g (Zwitterionenform)
Wassergehalt der lufttrockenen Form	8.9%
Schüttvolumen in Wasser	12.4 ml/g (Zwitterionenform)
Atmungsdifferenz zwischen pH 4 und pH 8	*ca.* 10% (McIlvaine-Puffer, Ionenstärke 0.5).

* Berechnet aus dem analytisch bestimmten Stickstoffgehalt.

Experimentelles

Die Untersuchungen wurden mit einem Polyäthyleniminoessigsäurecellulosederivat durchgeführt, dessen Eigenschaften aus Tabelle I hervorgehen. Als Testsubstanzen dienten Gemische ausgewählter Aminosäuren (Fluka, puriss.) und Dipeptide (Mann Research Lab., New York, N.Y., chromatograph. rein).

Der amphotere Celluloseionenaustauscher wurde bei allen in dieser Arbeit beschriebenen Versuchen in Form seines Alkalisalzes eingesetzt. Zur Elution wurden Phosphat- oder Citratpuffer benutzt. Die quantitative Bestimmung der Aminosäuren und Dipeptide im Eluat erfolgte mit Ninhydrin nach der Methode von MOORE

TABELLE II

SÄULENCHROMATOGRAPHISCHE TRENNUNG VON AMINOSÄURE- UND DIPEPTIDGEMISCHEN

Versuch I, II, IV durch pH-Gradientenelution; Versuch III sowie V bis IX durch Konzentrationsgradientenelution.

Versuch-Nr.	*Verbindung*	*ml Eluat bis zum Peak**	*Elutionsbedingungen*
I	Glu, Asp	39	*M*/15 Phosphatpuffer, pH 4.9 bis 8.3; 40°
	Gly, Ala, Phe	50	
	Try	59	
	Lys	66	
	Arg	69	
II	Asp	36	*M*/30 Phosphatpuffer pH 4.9 bis 8.0; 40°
	Gly' His	51	
	Try	60	
	Lys	94	
	Arg	102	
III	Gly	53	Phosphatpuffer pH 6 *M*/30 bis *M*/15; 40°
	Try	66	
	His	74	
	Lys	94	
	Tyramin	121	
IV	Asp, Glu,	44	*M*/10 Citratpuffer pH 3.95 bis 6.49; 40° (Trennungen Try, Lys, Arg nur unvollständig, vgl. Text)
	Gly, Phe, Alu	51	
	Try	59	
	Lys	62	
	Arg	64	
V	Val–val	39	Phosphatpuffer pH 8 *M*/30 bis *M*/15; 40°
	Val	49	
VI	Gly–val	42	
	Gly, Val	50	
VII	Leu–try	46	(Die Trennversuche V bis IX wurden unter identischen Bedingungen durchgeführt)
	Gly–try	50	
	Try	63	
VIII	Gly–Phe	43	
	Phe–gly	46	
	Phe	53	
IX	Leu–tyr	43	
	Gly–tyr	50	
	Tyr	59	

* Angegeben sind ml Eluat bis zur Peakspitze.

UND STEIN[7,8], die zur Anpassung an die experimentellen Gegebenheiten etwas modifiziert wurde.

Apparatur. Die Chromatographie-Säulen hatten eine Länge von 750 mm und einen Innendurchmesser von 10 mm. Die Elutionsgeschwindigkeit wurde mit einer peristaltischen Pumpe auf 3.5 bis 3.7 ml/h eingestellt. Das Eluat wurde mittels eines Fraktionssammlers unter Benutzung eines Tropfenzählers (LKB-Produkter, Stockholm) aufgefangen.

Ergebnisse und Diskussion

In Tabelle II sind die Trennergebnisse angegeben, die unter verschiedenen pH- und Konzentrationsbedingungen erzielt wurden.

Die Auswertung der in Tab. II zusammengestellten Trennergebnisse macht folgendes deutlich: Eine Gruppentrennung in saure, neutrale und basische Aminosäuren gelang in jedem Fall. Saure und neutrale Aminosäuren mit sehr ähnlichen isoelektrischen Punkten konnten untereinander nicht aufgetrennt werden (Versuch I, II und IV). Die Aminosäuren wurden in der Reihenfolge eluiert, die durch die Lage der isoelektrischen Punkte gegeben war. Die unter den Versuchsbedingungen überwiegend als Anion vorliegenden Asparagin- sowie Glutaminsäure wurden von dem überwiegend als Anion vorliegenden Austauscher praktisch nicht festgehalten. Die neutralen Aminosäuren lagen im untersuchten pH-Bereich wohl überwiegend als Zwitterion vor, bei pH 8 teilweise als Anion, unterhalb pH 6 teilweise als Kation. Sie wurden demzufolge stärker festgehalten als die sauren Aminosäuren. Eine Sonderstellung nahm das nach der Lage seines isoelektrischen Punktes (5.89) den neutralen Aminosäuren zuzurechnende Tryptophan ein (zum Vergleich: Glycin 5.97, Alanin 6.00). Es konnte stets von allen anderen Aminosäuren abgetrennt werden. Hierfür könnte eine nichtionische Adsorption an dem amphoteren Celluloseaustauscher verantwortlich gemacht werden. Nichtionische Adsorptionseffekte sind für die Trennungen von Aminosäuren mit fast identischen isoelektrischen Punkten bereits diskutiert worden[9–11]. Die als bivalentes Kation vorliegenden basischen Aminosäuren Lysin und Arginin wurden im pH-Bereich 5 bis 8 vom Austauscher relativ stark festgehalten und auch voneinander getrennt. Auch liessen sie sich von der Base Tyramin abtrennen.

Wenn die Elution im schwach sauren Bereich vorgenommen wurde (Versuch IV), wurde die Auftrennung der Aminosäuren sehr viel schlechter. Es kann angenommen werden, dass der amphotere Austauscher zwischen pH 4 bis 6 wenigstens teilweise als Zwitterion vorliegt. Die als bivalentes Kation vorliegenden basischen Aminosäuren werden nicht mehr so stark fixiert und die sauren Aminosäuren müssen relativ stärker gebunden werden. Durch diesen Effekt rücken alle Banden zusammen und auch eine vollständige Gruppentrennung ist nicht mehr möglich.

Die Trennversuche von Dipeptiden sowie Dipeptiden und Aminosäuren (Versuch V bis IX) an dem amphoteren Celluloseaustauscher ergaben ein von den oben beschriebenen Verhältnissen abweichendes Bild. So konnten alle untersuchten Dipeptide von ihren einzelnen Aminosäurekomponenten vollständig abgetrennt werden, obwohl die Unterschiede in den isoelektrischen Punkten nur äusserst geringfügig waren. (Beispiel: Glycyltyrosin 5.69; Tyrosin 5.66 oder Glycylvalin 5.71; Valin 5.96.) Auch bei den untersuchten Dipeptidgemischen (Versuche VII bis IX) waren gute Trennerfolge zu beobachten. So konnte auch Glycylphenylalanin von Phenylalanylglycin getrennt werden. Er scheint, dass sich bei der Trennung von Dipeptiden an dem

amphoteren Celluloseaustauscher sowohl ein Einfluss der pK-Werte der ionischen Gruppen als auch ein Einfluss der Molekülgrösse und des nichtionischen Teils der Moleküle bemerkbar machten.

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CHROM. 3334

Kontinuierliches Elutionssystem fur die Ionenaustausch-Chromatographie unter Verwendung linearer Gradienten

Die von COHN[1,2] für die Auftrennung der Nucleotide eingeführte Ionenaustausch-Chromatographie wurde von HURLBERT *et al.*[3] zu einem System weiterentwickelt, in dem für diese Auftrennung die Konzentration des Elutionsmittels kontinuierlich ansteigt. Nachteilig sind dabei aber der oftmalige Wechsel der Elutionsmittelvorratsflaschen, die schlechte Konstanz des Flüssigkeitsvolumens in der Mischflasche und die nach kurzer Zeit eintretende starke Verflachung des Gradienten. Dem letzten Nachteil begegneten GILBERT UND YEMM[4] später durch schrittweises Reduzieren des Mischungsvolumens.

Der einfachste Gradient wird durch Verbindung zweier gleicher zylindrischer Behälter an der Basis erreicht. Durch das Hintereinanderschalten solcher Systeme kann der Gradient steiler gelegt bzw. ein zweites Elutionsmittel eingeführt werden.

Für die Auftrennung der freien Nucleotide und Zuckerphosphate an Dowex 1 × 10 (Formiatform) wurde das in Fig. 1 schematisch dargestellte Mischflaschensystem verwendet, wobei die Verbindungen aus Silikonschläuchen und die Unterbrechungen als Schlauchquetschhähne ausgeführt waren. Für die Inbetriebnahme wurden bei den geschlossenen Schlauchquetschhähnen a, b, c und den offenen d, f, g die einzelnen Flaschen mit folgenden Elutionsmitteln gefüllt.

A_I = H_2O (400 ml)
B_I = 0.3 *N* HCOOH (400 ml)
A_{II} = 0.3 *N* HCOOH (500 ml)
B_{II} = 4.0 *N* HCOOH (500 ml)
A_{III} = 4.0 *N* HCOOH (500 ml)
B_{III} = 1.0 *M* $HCOONH_4$ in 4.0 *N* HCOOH (500 ml).

In den Glaskapillaren S_I, S_{II} und S_{III} steigt dabei das Flüssigkeitsniveau

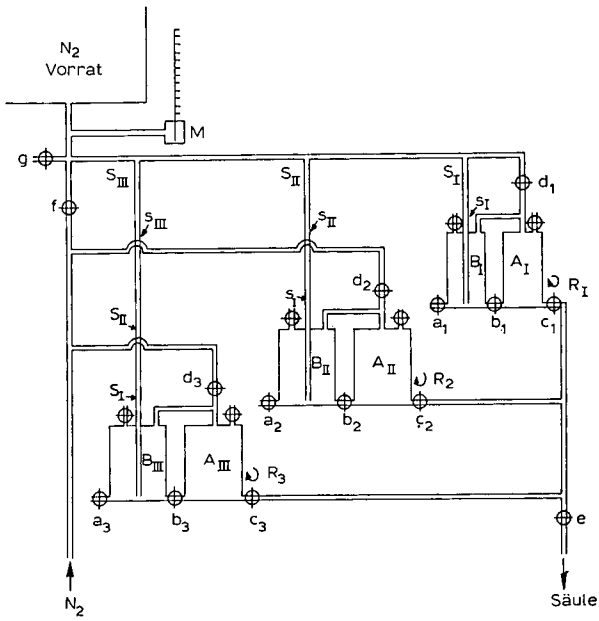


Fig. 1. Schematische Darstellung des Mischflaschensystems zur kontinuierlichen Elution unter Verwendung von drei linearen Gradienten. (Erklärung und Funktion siehe Text).

jeweils bis zum Punkt s_I. Nach Schliessen von d_1 wird mit Stickstoff ein zusätzlicher Druck an das Flaschensystem gelegt, wodurch in S_{II} und S_{III} jeweils der Punkt s_{II} erreicht wird. Nach Schliessen von d_2 wird dann in S_{III} der Flüssigkeitsspiegel bis s_{III} gedrückt. Sobald e geschlossen und die Magnetrührwerke R in Betrieb genommen sind, können in allen Mischsystemen die Verbindungen bei b und c wiederhergestellt werden. Es hat sich als vorteilhaft erwiesen, die Flaschen A_I, A_{II} und A_{III} nicht durch horizontales Rühren von unten, sondern durch vertikales von der Seite zu durchmischen. Nach Öffnen des Hahnes e, der gleichzeitig auch als "safety catcher" für Stromausfall oder Kollektorüberfüllung dient, steigt bei Ablauf des ersten Mischsystems A_I B_I die Konzentration an Ameisensäure im Elutionsmittel linear von 0 bis 0.3 *N*. Dabei sinkt der Flüssigkeitsspiegel bis auf das Bodenniveau des obersten Flaschenpaares, wobei im Kapillarrohr S_{II} der Punkt s_I bzw. in S_{III}, der Punkt s_{II} erreicht wird. Bei weiterem Sinken des Flüssigkeitsspiegels beginnt das zweite Flaschenpaar A_{II} B_{II} sich zu entleeren, wodurch die Ameisensäurekonzentration im Elutionsmittel linear auf 4.0 *N* weitersteigt. Das dritte Mischflaschensystem A_{III} B_{III} bewirkt dann in der 4.0 *N* Ameisensäure einen linear verlaufenden Ammoniumformiat-Gradienten von 0 bis zu einer Endmolarität von 1.0.

Wird das in Fig. 1 wiedergegebene Mischflaschensystem zur Erreichung grösserer Auftrennungsgeschwindigkeiten unter erhöhtem Druck gefahren, so soll der Gaspolster über der Flüssigkeit in den einzelnen Flaschen möglichst klein und das Volumen des Druckbehälters im Vergleich zum Volumen der übrigen Anlage möglichst gross gehalten werden. Nach Schliessen von g wird die gesamte Anlage inklusive des Druckbehälters unter einen am Manometer M ablesbaren Überdruck gesetzt und nach Schliessen von d_1 bzw. d_2 das Flüssigkeitsniveau in den drei Kapillarrohren S_I S_{II} und S_{III} auf gleiche Ausgangshöhe eingestellt. Dann wird, wie bereits vorher beschrieben, vorgegangen.

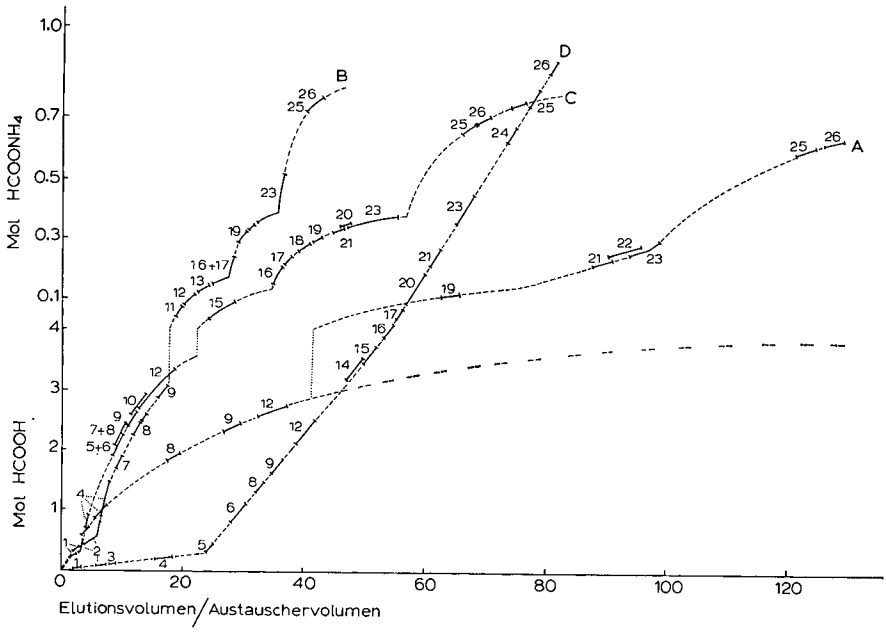


Fig. 2. Elutionsdiagramm der freien Nucleotide und Zuckerphosphate (Dowex 1 × 10; Formiatform). A = Auftrennung der Nucleotide nach HURLBERT *et al.*[3]. B = Auftrennung der Nucleotide nach der Modifikation von GILBERT AND YEMM[4]. C = Auftrennung der Nucleotide und Zuckerphosphate nach der Modifikation von STEHLIK UND ALTMANN[5]. D = Auftrennung der Nucleotide und Zuckerphosphate mit dem in Fig. 1 angegebenen Mischflaschensystem. 1 = Cytidin-5′-monophosphat; 2 = Diphosphopyridinnucleotid; 3 = Cytidin-5′-diphosphat-deriv.; 4 = Adenosin-5′-monophosphat; 5 = Hexose-monophosphat; 6 = Triose-monophosphat; 7 = Triphosphopyridinnucleotid; 8 = Guanosin-5′-monophosphat; 9 = Uridin-5′-monophosphat; 10 = Anorganisches Phosphat; 11 = Inosin-5′-monophosphat; 12 = Adenosin-5′-diphosphat; 13 = Riboflavindiphosphat; 14 = Phosphorglycerat; 15 = Guanosin-5′-diphosphat-mannose; 16 = Uridin-5′-diphosphat-N-acetylglucosamin; 17 = Uridin-5′-diphosphat-glucose; 18 = Adenosin-2′-monophosphat-5′-diphosphat-ribose; 19 = Guanosin-5′-diphosphat; 20 = Fructose-1,6-diphosphat; 21 = Cytidin-5′-triphosphat; 22 = Uridin-5′-diphosphat; 23 = Adenosin-5′-triphosphat; 24 = 2,3-Diphosphoglycerat; 25 = Guanosin-5′-triphosphat; 26 = Uridin-5′-triphosphat.

Die durch dieses System erfolgende Auftrennung der freien Nucleotide und Zuckerphosphate an Dowex 1 × 10 ist in der Fig. 2 wiedergegeben (Gradientenverlauf D). Zum Vergleich sind die ebenfalls an Dowex 1 × 10 erzielten Auftrennungen, sowie die dafür verwendeten Gradienten an HCOOH und $HCOONH_4$ von HURLBERT *et al.*[3] (Gradientenverlauf A) und deren Modifikationen von GILBERT UND YEMM[4] (Gradientenverlauf B) sowie von STEHLIK UND ALTMANN[5] (Gradientenverlauf C) eingezeichnet.

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* Leiter: Prof. Dr. K. KAINDL.

News

New apparatus

A *detector for liquid chromatography* with the same order of sensitivity and quantitative accuracy of detectors for gas–liquid chromatography has been announced by Packard Instrument Co., Downers Grove, Ill. The Model 7101 Liquid Chromatography Detector System enables direct analysis of steroids, polymers, lipids, and other organic compounds without the need for time-consuming preparation of derivatives. The instrument may be utilized for immediate direct analysis of the effluent from small liquid chromatographic columns (flow rates up to 0.15 ml/min), and for rapidly locating components of interest from larger columns by adding a stream splitter. With most organic compounds, minimum sensitivity is 5 μg/ml.

The operation of the Model 7101 is unique. The effluent from the column is deposited on a moving conveyor. The solvent is evaporated, leaving the solute which is then transported to a high-temperature area where vaporization or pyrolysis occurs depending on the sample structure. The volatilized or pyrolyzed solute is aspirated through the hydrogen flame detector, and the response recorded on a strip chart. Because the conveyor does not pass through the hydrogen flame detector, noise levels are extremely low.

The system incorporates a stable electrometer with 14 operating ranges from 3×10^{-6} to 1×10^{-12} AFS; high voltage power supply; single pen, 6-inch recorder; temperature controller; and patented detector.

Packard Instrument Co. also manufactures gas chromatographs, gas fraction collectors, radiochromatogram scanners, and other instruments for detecting radioactivity.

For further information apply to the publisher under reference No. Chrom. N-93.

A *lambda dispenser* which accurately ($\pm$ 1 %) pipets microliter quantities of liquid reagents by one finger press on the plunger is announced by Misco Scientific, Berkeley, Calif., U.S.A. The volume to be delivered is set by a micrometer type dial. The storage reservoir is of inert plastic. Several sizes are available, dispensing 1–250 μl.

For further information apply to the publisher under reference No. Chrom. N-96.

Gel filtration is a chromatographic technique which may be scaled up if suitable column equipment is used. Sephadex gels form stable beds which, with proper use, give extremely reproducible results and require no regeneration. Gel filtration with Sephadex is, therefore, highly suited to large scale work.

The *Sephadex column K 100/100* is a new member of the series of precision laboratory columns manufactured by Pharmacia Fine Chemicals, Uppsala, Sweden. It is intended for preparative gel filtration on a relatively large scale; bed volumes can be varied from 4.8 to 7.3 l, allowing samples from 0.2 to 2 l to be treated in one experiment, depending on the type and degree of separation required.

It is possible to carry out gel filtration on a very large scale. Pharmacia Fine Chemicals has developed columns for gel filtration on beds with volumes in the 1000 l range. Those interested in working with columns larger than the K 100/100 column are invited to inform us about their particular requirements.

For further information apply to the publisher under reference No. Chrom. N-95.

Manufacturers' literature

Barber-Colman Company, Rockford, Ill. announces the following new literature:

(1) New literature on the *Series Po2T Pressure Transmitter*. The general purpose transmitter measures process pressures and generates an Autronic® signal in a variety of ranges of 0–40 to 0–5000 p.s.i.g. with an accuracy within ± 0.5 % of the calibrated span. Pressure changes are sensed by a durable 316 stainless steel diaphragm assembly. The Series Po2T is applied in continuous industrial processes such as those in the petrochemical, waste treatment, distillation, and pipeline control applications. All standard units are supplied suitable for weather-proof and explosion-proof service in Class I, Division 1, Group D Hazardous Location Classification areas.

(2) New literature on the *Model A09C-2 proportional controller*. The Model A09C-2 is a field mounted proportional controller with manual reset which has been designed to compare a process variable input signal with an internally adjustable setpoint voltage. The adjustable proportional band with manual reset is very useful on liquid level applications. All units are supplied suitable for weather-proof and explosion-proof service in Class I, Division 2, Group D service.

(3) New literature on the *Model A20D Indicating Digitizer*. The Model A20D, a null balance instrument, indicates a process variable and converts the transmitted signal of the variable into a digital code suitable for telemetering, data logging, data processing, card punching, tape punching or any compatible form of digital recording. The instrument operates either alone or in parallel with recording and controlling instruments directly from the field signal of any Autronic® transmitter or converter. A silicon transistorized plug-in amplifier in the instrument provides high encoding accuracy.

For further information apply to the publisher under reference No. Chrom. N-97.

The latest edition of *Instrument News*, Vol. 18, No. 1, is available from the Perkin-Elmer Corporation, Norwalk, Conn.

It contains five instrument application articles, *viz.* Application of Atomic Absorption Spectroscopy to Water Pollution Control; The Identification of D-Lysergic Acid Diethylamide (LSD) by IR Spectrophotometry; High Resolution Gas Chromatographic Analysis of Auto Exhaust Gas; Polymer Solid Crystallization Measurements with the Differential Scanning Calorimeter; and, Matrix Isolation with the Perkin-Elmer Model 225 IR Spectrophotometer.

Also included are articles describing the use of Perkin-Elmer instruments for luminescence spectroscopy, spin decoupling with the NMR spectrometer, and CHN analysis of refractory compounds.

New products described in detail include the Model 457 Infrared Spectrophotometer, the Model 270 Mass Spectrometer, the KLA 3-B Amino Acid Analyzer, and the new Coleman Junior II Spectrophotometer.

Free copies are available.

For further information apply to the publisher under reference No. Chrom. N-81.

The *JEOL News*, Vol. 5, No. 8, 1967 contains details of: (i) Applications of the JLC-3BC amino acid and nucleic acid analyzer. The analysis of free amino acids and peptides in jellied tuna. (ii) Application of the JRA electronic computer to a gas chromatograph. Some of the applications are: (a) the determination of the retention time of each peak, the integrated value of each peak, the integrated value of each peak as a percentage, the percentage of each peak multiplied by its sensitivity, and (b) the separation of overlapping wave forms.

For further information apply to the publisher under reference No. Chrom. N-84.

New material

Pierce Chemical Company announces the *TRI-SIL Poly Solvent Kit* for the rapid, one-step production of trimethylsilyl derivatives of polar compounds such as alcohols, organic acids, sugars and related carbohydrates, phenols, steroids, sterols and other materials containing an active hydrogen. Included in the kit are TRI-SIL Concentrate reagent and four silylation grade solvents: pyridine, dimethylformamide, dimethylsulfoxide and tetrahydrofuran. The TRI-SIL Concentrate is based upon the work of C. C. SWEELEY, R. BENTLEY, M. MAKITA AND W. W. WELLS (*J. Am. Chem. Soc.*, 85 (1963) 2497–2507) and enables the user to adapt SWEELEY's method to a choice of solvents. An instructional pamphlet is enclosed.

The kit components are individually packaged in convenient Hypo-Vials, which allow immediate access yet maintain complete protection of the sensitive contents. The kit is housed in an attractive and unbreakable molded polyolefin case that will serve as a personal kit for years after the contents have been used.

For further information apply to the publisher under reference No. Chrom. N-82.

Appointments

LKB Instruments Ltd. are pleased to announce that due to the continued expansion of their Company, occasioned by the increased demand within the United Kingdom for the specialised scientific instruments manufactured by LKB Produkter AB, they have strengthened their management team with the appointment of Mr. DEREK B. EASTELL, M.I.E.E. as their Managing Director.

Mr. DEREK B. EASTELL, prior to assuming this post, was Managing Director of Easco Electrical Ltd., where he was responsible for the development and marketing of his company's range of lighting equipment, and later Managing Director of Specto Ltd., manufacturers of photographic and electro-mechanical equipment.

Mr. HORACE G. MCDONELL has been named head of the Instrument Group of the Perkin-Elmer Corporation, which has been announced by CHESTER W. NIMITZ, Jr., President of the scientific instrument manufacturer.

Mr. MCDONELL, who is a Vice President of Perkin-Elmer, will be responsible for the company's analytical instrument operations. He succeeds Mr. JOHN H. ZAUNER, who is leaving the position for reasons of health. Mr. ZAUNER is continuing with the Instrument Group in a staff capacity.

Prior to his new position, Mr. MCDONELL was General Manager of the Electronic Products Division. Mr. PAUL C. HUTCHINSON will succeed Mr. MCDONELL as General Manager of the Electronic Products Division.

Mr. MCDONELL joined Perkin-Elmer in 1952. Prior to managing the Electronic Products Division, he served as Director of Corporate Planning, as Director of the Process Control Instrument Department and in several engineering and marketing positions in the Instrument Division. Mr. MCDONELL holds a B.S. degree in physics from Adelphi College. He resides in Ridgefield, Conn.

Perkin-Elmer is a major producer of chemical analytical instrumentation for industrial, scientific and medical research and testing. In addition to operations in Connecticut, Illinois and California, it has integrated companies in West Germany, England and Australia, a joint company in Japan, and assembly operations in Mexico and a world-wide instrument marketing organization.

Exhibition

From December 6th to 14th, 1967 the United States Department of Commerce, Bureau of International Commerce held an *Exhibition of Chromatography, Fluorescence and Electrophoresis Equipment* at the United States Trade Centre, London, W.1.

This exhibition of new American equipment was designed especially to meet the British needs for analytical instruments, both for research and development work. Twenty three companies displayed a wide range of instruments some of which were completely new to the British market. The exhibits were staffed throughout the duration of the show by technical representatives.

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19. PEPTIDES; CHEMICAL STRUCTURE OF PROTEINS

19a. Peptides

See PC section.

20. PROTEINS

20a. Proteins

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CHROM. 3327

ÜBER DIE PROGRAMMIERTE GASCHROMATOGRAPHIE

IV. THEORETISCHE GRUNDLAGEN DER STRÖMUNGSPROGRAMMIERTEN GASCHROMATOGRAPHIE. TEIL 1

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SUMMARY

Programmed gas chromatography. IV. The theoretical basis of flow programming in gas chromatography. Part 1

The theoretical basis of programming the flow rate in gas chromatography was examined from the point of view of experimental and literature data. The principles and mathematical arguments with respect to the programming are defined in a general way.

Die Ergebnisse und weiteren Möglichkeiten der strömungsprogrammierten Gaschromatographie, welche die temperaturprogrammierte Gaschromatographie sehr gut ergänzt, rechtfertigen das zunehmende Interesse für diese Methode. Neuere Publikationen berichteten über Versuche zur Vereinigung dieser beiden programmierten Techniken. Man versucht, durch Klärung einzelner theoretischer und praktischer Probleme die Grundlagen für eine doppeltprogrammierte Gaschromatographie zu schaffen[1–4]. Aus diesem Grunde wächst immer mehr das Interesse, die grundlegenden theoretischen Fragen der strömungsprogrammierten Methode zu untersuchen, was auf Grund der Fortschritte der letzten Jahre auch möglich geworden ist[5–27].

Die Theorie dieser Methode lässt sich auf die Theorie der "klassischen" Gaschromatographie zurückführen, die wir wegen ihrer konstanten Versuchsverhältnisse als den Normalfall definieren[28]. Auf Grund unserer Erfahrungen kann man unsere Ergebnisse als "Grundsätze" und "Hilfssätze" formulieren. Die Grundsätze sind unter bestimmten gaschromatographischen Bedingungen (z.B. bei konstanter Temperatur) gültige, allgemeine Zusammenhänge. Hilfssätze sind hingegen Behauptungen, die mit einem der Grundsätze in Zusammenhang stehen und aus diesem mathematisch abgeleitet werden können.

Der Zusammenhang zwischen den beiden gaschromatographischen Methoden gibt der *erste Grundsatz* der strömungsprogrammierten Gaschromatographie wieder:

Bei konstanter Temperatur, unter unveränderten gaschromatographischen Verhältnissen, besitzt das netto Retentionsvolumen einer beliebiger Komponente einen konstanten Wert; diese Beständigkeit von V_N schliesst aber die gut definierten und mit-

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einander zusammenhängenden Änderungen von p_i, j, F, t_R und t_M nicht aus. Man kann nämlich die folgende Gleichung aufstellen:

$$V_N = j_1F_1(t_{R(1)} - t_{M(1)}) = j_2F_2(t_{R(2)} - t_{M(2)}) = \ldots = j_kF_k(t_{R(k)} - t_{M(k)}) = \\ = \int_0^{t_{Rp}} jF[p_i(t)]\,dt - \int_0^{t_{Mp}} jF[p_i(t)]\,dt \qquad (1)$$

Diese Parameter stellen die wichtigsten Operationselemente der strömungsprogrammierten Gaschromatographie dar und verbinden auf engster Weise die beiden gaschromatographischen Methoden.

COSTA NETO, KÖFFER UND DE ALENCAR haben wichtige theoretische Grundlagen der strömungsprogrammierten Methode erkannt[29]. Sie haben u.a. experimentell bewiesen, dass unter unveränderten gaschromatographischen Bedingungen bei konstanter Temperatur die Retentionszeit der untersuchten Kohlenwasserstoffe eine lineare Funktion der reziproken Volumströmungsgeschwindigkeit des Trägergases ist.

Unsere Untersuchungen an verschiedenartigen Verbindungen bewiesen die allgemeine Gültigkeit dieses Satzes, den man folgendermassen ausdrücken könnte:

Bei konstanter Temperatur ist unter unveränderten gaschromatographischen Verhältnissen die Retentionszeit einer jeden Komponente eine lineare Funktion der reziproken Volumströmungsgeschwindigkeit des Trägergases. Dies ist der *zweite Grundsatz* der strömungsprogrammierten Gaschromatographie:

$$t_R = m_1(1/F) + b_1 \qquad (1.1)$$

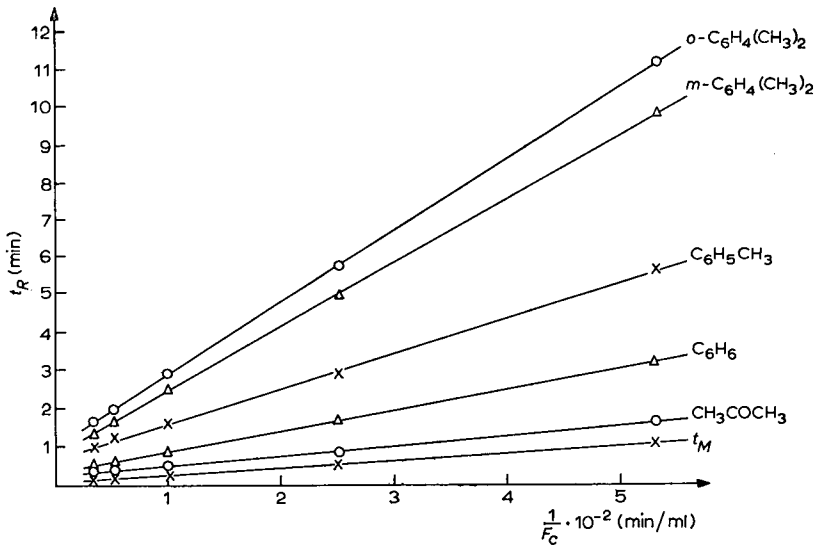


Fig. 1. Erhaltene Linearität bei einem Modellgemisch. Nachweis des zweiten Grundsatzes. Detektor: Thermistor; Brückenstrom: 20 mA; Empfindlichkeit: 50%. Kolonne: 2.0–2.5 m Länge, innere Durchmesser 5 mm; Füllung: 20% Silikonöl-550 auf Celite 545 Träger von 0.2 mm Korndurchmesser. Temperatur des Verdampfers: 270°, bzw. 300°. Thermostattemperatur: 160°. Einwaage: 1–4 μl mit Hamilton-Syringe. Trägergas: Wasserstoff; Strömungsgeschwindigkeit: bei jenem Versuch verändert. Schreibgerät: Speedomax G; 2.5 mV; 1 sec; Geschwindigkeit des Papiers: 1.27 cm/min.

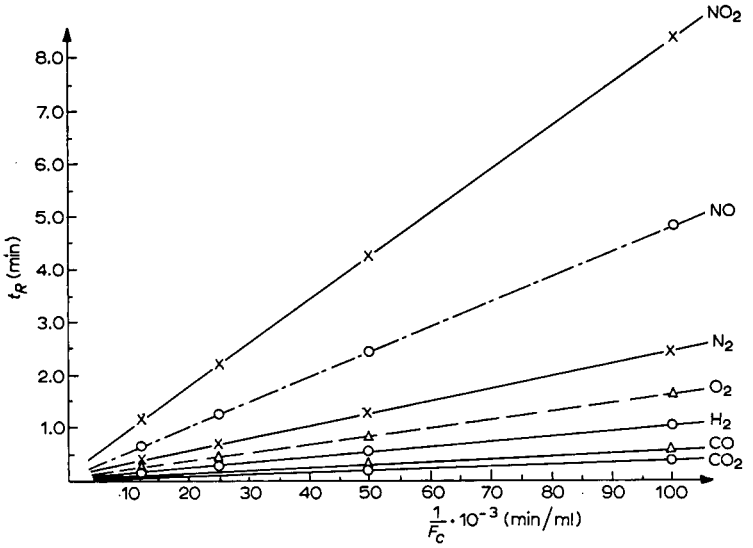


Fig. 2. Bestätigung Beweisführung des zweiten Grundsatzes durch permanente Gase. Detektor: Thermistor; Brückenstrom: 20 mA; Empfindlichkeit: 1/512–1/2. Kolonne: Länge 0.8 m; innerer Durchmesser 5 mm; Füllung: aktiv Trocken-Perlen Kieselgel von 0.2 mm Korndurchmesser. Thermostattemperatur: 25.0°. Einwaage: 0.5–12.0 ml. Trägergas: Argon; Strömungsgeschwindigkeit: bei jenem Versuch verändert. Schreibgerät: Speedomax G; 2.5 mV; 1 sec. Geschwindigkeit des Papiers: 1.27 cm/min.

Da unsere Untersuchungen zeigten, dass auch die zum Messen der t_M-Werte üblichen Edelgase (Argon, Helium) keine Ausnahmen bilden, kann der zweite Grundsatz als allgemein gültig betrachtet werden. Aus der Reihe unserer diesbezüglichen Versuche sind einige Ergebnisse in graphischer Form in Fig. 1 und 2. gegeben. Die Linearität der Geraden wurde mathematisch kontrolliert. Als Beispiel soll die Berechnung für den Fall des Sauerstoffs dargestellt werden. Die aus den Messungen erhaltenen und zu den Berechnungen nötigen Daten sind der Tabelle I zu entnehmen.

Aus den Daten ergibt sich:

$$\Sigma 1/F = 194.99 \cdot 10^{-3} \quad \Sigma t_R = 3.225$$

$$\Sigma 1/F \cdot t_R = 214.958 \cdot 10^{-3}$$

$$\Sigma 1/F^2 \cdot 10^{-6} = 13347.165 \cdot 10^{-6}$$

$$(\Sigma 1/F)^2 = 38021.1 \cdot 10^{-6}$$

TABELLE I

DATEN DES SAUERSTOFFS

$1/F \cdot 10^{-3}$	t_R	$1/F \cdot 10^{-3} \cdot t_R$	$1/F^2 \cdot 10^{-6}$
100.00	1.600	160.000	10000.000
50.00	0.800	40.000	2500.000
25.00	0.425	10.625	625.000
13.33	0.250	3.333	177.729
6.66	0.150	1.000	44.436

TABELLE II

GEMESSENE UND BERECHNETE WERTE DER RETENTIONSZEIT DES SAUERSTOFFS

$1/F \cdot 10^{-3}$ (*min/ml*)	$t_{R(gemessen)}$ (*min*)	$t_{R(berechnet)}$ (*min*)	t_R (*min*)	*Abweichung* (%)
100.00	1.600	1.593	+0.007	+0.46
50.00	0.800	0.816	—0.016	—1.97
25.00	0.425	0.427	—0.002	—0.56
13.33	0.250	0.246	+0.004	+1.76
6.66	0.150	0.143	+0.007	+5.00

Substituiert man die entsprechenden Daten in Gleichungen 2, 3 und 4, so können die Werte von $T_{R(\text{gerechnet})}$ ermittelt werden:

$$m = \frac{\Sigma 1/F \cdot \Sigma t_R - n \Sigma 1/F \cdot t_R}{(\Sigma 1/F)^2 - n \Sigma (1/F)^2} \tag{2}$$

$$b = \frac{\Sigma 1/F \cdot \Sigma 1/F \cdot t_R - \Sigma 1/F^2 \cdot \Sigma t_R}{(\Sigma 1/F)^2 - n \Sigma 1/F^2} \tag{3}$$

$$t_{R(\text{gerechnet})} = 15.5364 \cdot 1/F + 0.0390 \tag{4}$$

In Tabelle II sind die gemessenen und berechneten Werte zusammengestellt.

Die Formulierung des dritten Grundsatzes wurde auch durch praktischen Ergebnisse ermöglicht. Zuvor ist es aber notwendig, die netto Retentionszeit eindeutig zu definieren, da diesbezüglich heute keine Eindeutigkeit besteht. Der von uns benutzte Ausdruck ist:

$$t_N = (t_R - t_M) \tag{4.1}$$

und ist nicht gleich mit dem in Lit. 21 gegebenen.

Der dritte Grundsatz lautet dann:

Bei konstanter Temperatur, unter unveränderten gaschromatographischen Verhältnissen, ist die netto Retentionszeit einer beliebiger Komponente eine lineare Funktion der reziproken Volumströmungsgeschwindigkeit des Trägergases:

$$t_N = m_2(1/F) + b_2 \tag{4.2}$$

Auf Grund mathematischer Überlegungen konnte der erste Hilfssatz der strömungsprogrammierten Gaschromatographie aufgestellt werden: Man wähle zwei beliebige Geraden:

$$t_R^* = m^*(1/F) + b^* \tag{5}$$

$$t_R^{**} = m^{**}(1/F) + b^{**} \tag{6}$$

Man subtrahiere Gleichung (5) von Gleichung (6):

$$t_R^{**} - t_R^* = (m^{**} - m^*)(1/F) + (b^{**} - b^*) \tag{7}$$

Gleichung (7) ist ebenfalls die Gleichung einer Geraden, was durch Einführung folgender Bezeichnungen veranschaulicht wird:

$$t_R{}^{**} - t_R{}^{*} = \Delta t_R \quad (m^{**} - m^{*}) = m_3, \text{ und } (b^{**} - b^{*}) = b_3$$

So erhält man für Gleichung(7):

$$\Delta t_R = m_3(1/F) + b_3 \tag{8}$$

Die Linearität von Gleichung (8) lässt sich auch auf andere Weise zeigen: Der Ausgangswert der unabhängigen Veränderlichen soll $1/F_1$ betragen. Das Argument soll um $\Delta 1/F$ erhöht werden. So beträgt der Funktionszuwachs Δy

$$\Delta y = m\{(1/F_1) + \Delta(1/F)\} + b - \{m(1/F_1) + b\} = m\Delta(1/F) \tag{9}$$

Bei unverändertem $1/F$ soll nun $1/F_2$ als Ausgangswert genommen werden. Dann gilt:

$$\Delta y = m\{(1/F_2) + \Delta(1/F)\} + b - \{m(1/F_2) + b\} = m\Delta(1/F) \tag{10}$$

Gleichungen (9) und (10) zeigen, dass bei verschiedenen Ausgangswerten gleiche Argumentzunahmen gleiche Funktionszunahmen liefern, d.h. die Funktion ist zwar proportional zum Argumentzuwachs, aber doch unabhängig von deren Ausgangswert. Da ausser einer Geraden keine andere Funktion über diese Eigenschaft verfügt, ist die Linearität von Gleichung (8) bewiesen[30,31].

Der Satz lässt sich auch graphisch beweisen, da die Änderung von m und b bzw. von beiden die Linearität der Gerade nicht beeinträchtigt, während es sich beim

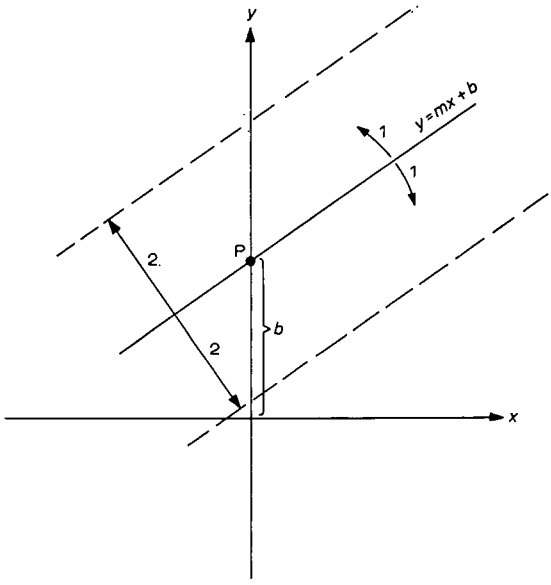


Fig. 3. Graphischer Nachweis der Linearität des ersten Hilfssatzes. (1) b = Konstant; (2) m = konstant.

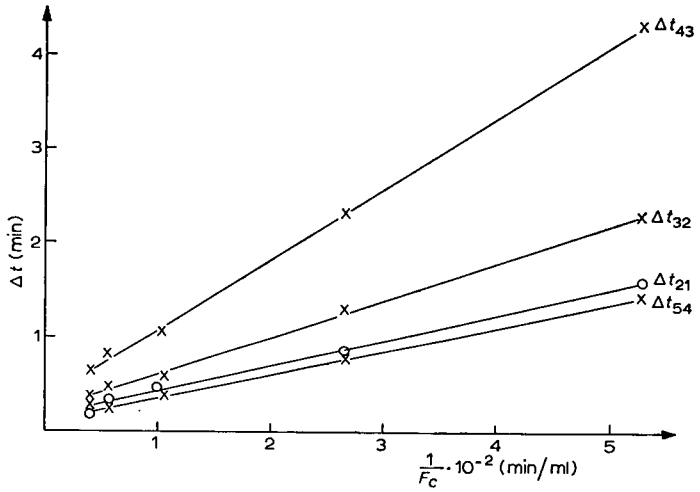


Fig. 4. Nachweis des ersten Hilfssatzes mit Modellpaaren *o*- und *m*-Xylol (Δt_{54}), Aceton–Benzol (Δt_{21}), Toluol–Benzol (Δt_{32}) und *m*-Xylol–Toluol (Δt_{43}). Für Messbedingungen s. Unterschrift Fig. 1.

Subtrahieren der beiden Gleichungen nur um die Änderung dieser beiden Werte handelt (Fig. 3).

Den ersten Hilfssatz kann man daher wie folgt definieren:

Bei konstanter Temperatur ist unter unveränderten gaschromatographischen Verhältnissen die Differenz der Retentionszeiten von zwei beliebigen Komponenten eine lineare Funktion der reziproken Volumströmungsgeschwindigkeit des Trägergases:

$$\Delta t_R = m_3(1/F) + b_3 \tag{11}$$

Dieser Hilfssatz wurde auch experimentell nachgewiesen. Als Beispiel sind in Fig. 4 die Resultate einer derartigen Messung gegeben.

Die weiteren Hilfssätze der strömungsprogrammierten Gaschromatographie lauten wie folgt, wenn:

$$t_R' = t_R - t_M, \text{ und } \Delta t_N = t_{N(k)} - t_{N(k-1)}$$

Bei konstanter Temperatur ist unter unveränderten gaschromatographischen Verhältnissen die reduzierte Retentionszeit einer beliebiger Komponente eine lineare Funktion der reziproken Volumströmungsgeschwindigkeit des Trägergases:

$$t_R' = m_4(1/F) + b_4 \tag{12}$$

Bei konstanter Temperatur ist unter unveränderten gaschromatographischen Verhältnissen die Differenz der netto Retentionszeiten von beliebigen Komponenten eine lineare Funktion der reziproken Volumströmungsgeschwindigkeit des Trägergases:

$$\Delta t_N = m_5(1/F) + b_5 \tag{13}$$

Auch diese Hilfssätze wurden durch die Versuchsresultate weitgehend bestätigt.

Alle Messungen wurden an Carlo Erba Geräten, Typ Fractovap C bzw. D durchgeführt.

BEZEICHNUNGEN

F_c = Strömungsgeschwindigkeit des Trägergases (ml/min), bei Kolonnen-Temperatur
F = Strömungsgeschwindigkeit des Trägergases (ml/min)
j = Korrektionsfaktor des Druckgefälles nach JAMES UND MARTIN
p_i = Eintrittsdruck des Trägergases (kp/cm^2)
t = Zeit (min)
t_R = Retentionszeit der Komponente (min)
t_R' = Reduzierte Retentionszeit der Komponente (adjusted retention time) (min)
t_N = Netto Retentionszeit der Komponente (min)
t_M = Durchlaufgeschwindigkeit von Luft (Argon, Helium) (gas hold up time) (min)
t_{Mp} = Programmierte Durchströmzeit von Luft (Argon, Helium) (min)
t_{Rp} = Programmierte Retentionszeit der Komponente (min)
Δt_R = Differenz der Retentionszeiten von zwei Komponenten (min)
Δt_N = Differenz der netto Retentionszeiten von zwei Komponenten (min)
k = laufende Nummer
V_N = netto Retentionsvolumen (ml Trägergases).

MATHEMATISCHE BEZEICHNUNGEN

n = Zahl der Messungen
m = Richtungstangente der Gerade
b = die durch die Gerade aus der y-Achse herausgeschnittene Strecke
Δy = Funktionszuwachs
$\Delta(1/F)$ = Argumentzuwachs.

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ZUSAMMENFASSUNG

Es wurden auf Grund von Versuchen und Literaturdaten die theoretischen Grundlagen der strömungsprogrammierten Gaschromatographie untersucht und die Grund- und Hilfssätze dieser Methode in allgemeiner Form definiert.

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PREDICTION OF RETENTION TIMES IN THE GLC OF DIASTEREOISOMERS OF METHYL-BRANCHED FATTY ACIDS

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SUMMARY

Examination of the order of elution of certain diastereoisomers of a number of multiple methyl-branched fatty acid esters from polar GLC columns suggests a general guide based on molecular rotations. Thus diastereoisomers having molecular rotations of the highest magnitude should elute first followed by others in order by decreasing order of magnitude of molecular rotation. In lieu of experimental molecular rotations which are lacking for most diastereoisomers an attempt has been made to correlate additive molecular rotations $\Sigma[M]_D$ with GLC data. It is believed that some discrepancies from predicted order of elution are due to complex interactions among asymmetric centers which modify one or more of the $[M]_D$ values assigned on the basis of data for fatty acids with a single methyl branch.

It has recently been noted in the gas–liquid chromatography (GLC) of the methyl esters of 2L,4L- and 2D,4L-dimethylhexanoic acids, and of methyl esters of four racemic 2,4,6-trimethyloctanoic acids, that the optical isomers with the same configurations at all of the asymmetric carbons had shorter GLC retention times than isomers where different configurations were present in any one molecule[1].

A different series of naturally occurring methyl-branched fatty acids, based on isoprenoid units, also exists. Recently resolution of methyl esters of certain diastereoisomers of some of these acids has been achieved with efficient polar (~ 45,000 theoretical plates) open tubular (capillary) GLC columns[2,3]. In the GLC of methyl esters of the saturated isoprenoid acids there is apparently a definite reversal of the above observation, with optical isomers having the same configurations at all of the asymmetric carbon atoms having lengthened retention times relative to some other diastereoisomers.

It is possible to calculate the retention time (as equivalent chain length) of esters of a number of methyl-branched fatty acids by the addition of increments of fractional chain length corresponding to the positions of individual methyl group substituents on the aliphatic chain[4,5]. When optically active centers occur in aliphatic chains it is often possible to add molecular rotations for individual asymmetric centers to obtain a reasonable approximation of the experimental molecular rotation for the whole molecule[1,6]. The analogy suggested that molecular rotations ($[M]_D$) might be usefully compared with GLC retention times.

Since experimental $[M]_D$ values were available for only a limited number of materials of interest this comparison was extended to include calculated molecular rotations ($\Sigma[M]_D$) derived from generally accepted $[M]_D$ values for individual optically active centers[6]. The latter, as employed below (Table I), are all to some

TABLE I

SIGNS AND MAGNITUDES OF INDIVIDUAL MOLECULAR ROTATIONS ASSIGNED TO MONOMETHYL SUBSTITUTED FATTY ACIDS (*cf.* ref. 6)

Methyl position	*Assigned $[M]_D$ for D-methyl substituent*
C_2	−28
C_3	+14
C_4	+ 0.4
C_5	0
ω-8	− 0.4
ω-7	− 0.8
ω-6	− 1.4
ω-5	− 2.0
ω-4	− 2.6
ω-3	− 3
ω-2	−14
ω-1	−12

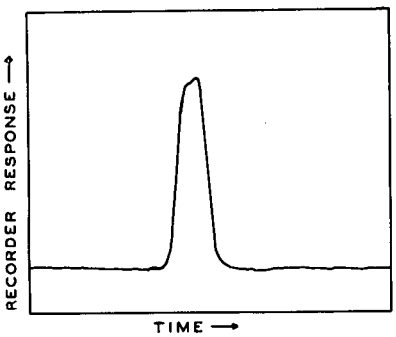
degree "typical" rather than exact values. The precise point in a long chain at which the sign of rotation for a "D" methyl substituent alters from (+) to (—) is arbitrarily taken as near the C_5 carbon. The designations for carbon atoms relative to the terminal methyl group should be noted. In GLC the *iso* methyl substituent has a significant effect on retention time and was designated ω_1. In optical rotation studies the *iso* methyl substituent is inactive and following literature practices the *anteiso* substituent is designated ω-1. In agreement with GLC practice greater weight is given to the relation between centrally located substituents and the terminal methyl group than to the carboxyl group, excepting in the case of the C_2 and C_3 substituents of shorter chain acids. Since optical isomers totally opposite in configuration cannot be separated by GLC on optically inactive materials the magnitude of $[M]_D$ or $\Sigma[M]_D$ for only one of each diastereoisomeric pair of acids need be considered and sign of rotation is of no significance.

NON-ISOPRENOID FATTY ACIDS

A number of these acids, of the type studied by ODHAM, occur in the preen glands of water birds and are all-"D" in configuration[1,7–9]. As an adjunct to work on these materials the simpler diastereoisomers 2L,4L- and 2D,4L-dimethylhexanoate were synthesized and separated by preparative GLC, the 2L,4L diastereoisomer appearing first when either polypropylene glycol or Versamid was the liquid phase[1]. Four diastereoisomeric pairs of 2,4,6-trimethyloctanoates have also been synthesized and separated by analytical GLC on a polar open-tubular column[7,9]. The order of elution is indicated as: 2D,4D,6D slightly before 2D,4L,6D, followed by a well-separated peak for 2D,4D,6L and finally by a well-separated peak for 2L,4D,6D.

In both instances the order of GLC elution is by decreasing order of magnitude

for $\Sigma[M]_D$ values. In the 2,4-dimethylhexanoates the difference in $\Sigma[M]_D$ values (Table II) is large, and the observed $[M]_D$ values show an even greater difference. This suggests a relation to the facile GLC separation. In the 2,4,6-trimethyloctanoates there is a rough correlation between $\Sigma[M]_D$ magnitudes ($43 > 37 \gg 19 \gg 13$) and observed component retention times in GLC. However, the data of Table II indicate that in this series of acids it is unusual for $[M]_D$ and $\Sigma[M]_D$ values to be in good agreement.



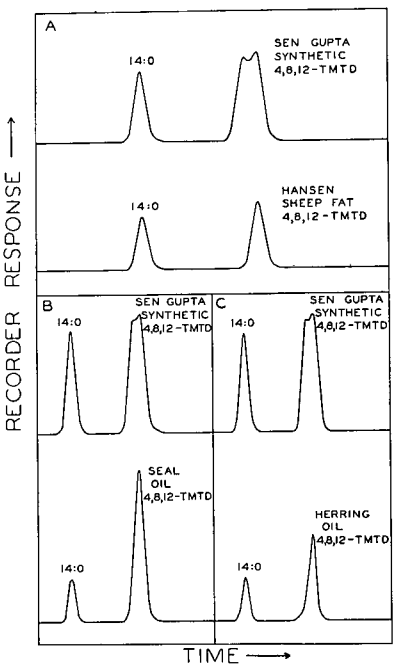


Fig. 1. Resolution of diastereoisomers of methyl esters of 3,7,11-trimethyldodecanoic acid (prepared from farnesol). Coupled columns (300 ft.) operated at 140° and 80 p.s.i.g. helium. Retention time 28 min.

Fig. 2. Resolution of diastereoisomers of methyl esters of 4,8,12-trimethyltridecanoic acid (prepared from farnesol) compared with materials isolated from natural sources. Coupled columns (300 ft.). Operating conditions: (A) 140°, 80 p.s.i.g. helium; (B and C) 150°, 80 p.s.i.g. helium. Retention times: (A) 14:0, 47 min; 4,8,12-TMTD, 50 min. (B and C) 14:0, 32 min; 4,8,12-TMTD, 34 min.

ISOPRENOID FATTY ACIDS

There is only partial resolution of the diastereoisomeric pairs of synthetic (from farnesol) 3,7,11-trimethyldodecanoic acid esters (Fig. 1). The $\Sigma[M]_D$ values of (C_3) + (ω-4) are respectively (—) 16.6 for the 3L,7D isomer and (+) 11.4 for the 3D,7D isomer. It is accordingly presumed that the decrease in $\Sigma[M]_D$ magnitude corresponds to the actual order of elution in GLC since the all-"D" component elutes last in several members of this series.

Somewhat more information is available for evaluation of data in the diastereoisomeric pairs of synthetic (from farnesol) 4,8,12-trimethyltridecanoic acid (Fig. 2). The $\Sigma[M]_D$ values of (C_4) + (ω-4) are respectively (—) 3.0 for the 4L,8D isomer

TABLE II

OBSERVED[1,7–9] AND CALCULATED MOLECULAR ROTATIONS FOR METHYL ESTERS OF DIASTEREOISOMERS OF NON-ISOPRENOID METHYL-BRANCHED FATTY ACIDS

Fatty acid methyl ester	*Molecular rotation* (°)		
	Experimental ($[M]_D$)	*Calculated* ($\Sigma[M]_D$)	
2L,4L-dimethylhexanoate	+50.9	(C_2) + (ω-1)	+40
2D,4L-dimethylhexanoate	−12.8		−16
2D,4D,6D-trimethyloctanoate	−60.4	(C_2) + (ω-3) + (ω-1)	−43
2L,4D,6D-trimethyloctanoate			+13
2D,4D,6L-trimethyloctanoate			−19
2D,4L,6D-trimethyloctanoate			−37
2D,4D,6D-trimethylnonanoate	−48.8	(C_2) + (ω-4) + (ω-2)	−44.6
2D,4D,6D,8D-tetramethyldecanoate	−80.6	(C_2) + (ω-5) + (ω-3) + (ω-1)	−44.4
2D,4D,6D,8D-tetramethylundecanoate	−59	(C_2) + (ω-6) + (ω-4) + (ω-2)	−46

TABLE III

$\Sigma[M]_D$ VALUES FOR DIASTEREOISOMERS OF PRISTANIC (2,6,10,14-TETRAMETHYLPENTADECANOIC) ACID

Substituent	*Diastereoisomer*			
	2D,6D,10D	2L,6D,10D	2D,6D,10L	2D,6L,10D
C_2	−28	+28	−28	−28
C_6 (ω-8)	− 0.4	− 0.4	− 0.4	+ 0.4
ω-4	− 2.6	− 2.6	+ 2.6	− 2.6
$\Sigma[M]_D$	−31	+25	−25.8	−30.2

TABLE IV

$\Sigma[M]_D$ VALUES FOR DIASTEREOISOMERS OF PHYTANIC (3,7,11,15-TETRAMETHYLHEXADECANOIC) ACID

Calculation	*Substituent*	*Diastereoisomers*			
		3D,7D,11D	3L,7D,11D	3D,7D,11L	3D,7L,11D
I	C_3	+14	−14	+14	+14
	C_7 (ω-8)	− 0.4	− 0.4	− 0.4	+ 0.4
	ω-4	− 2.6	− 2.6	+ 2.6	− 2.6
	$\Sigma[M]_D$	+11.0	−17.0	+16.2	+11.8
II	C_3	+14	−14	+16	+16
	C_7 (ω-8)	− 0.4	− 0.4	− 0.4	+ 0.4
	ω-4	− 2.6	− 2.6	+ 2.6	− 2.6
	$\Sigma[M]_D$	+11.0	−17.0	+18.2	+13.8

TABLE V

RELATIVE RETENTION DATA FOR METHYL ESTERS OF VARIOUS ISOPRENOID FATTY ACIDS COINJECTED WITH METHYL ESTERS OF ALIPHATIC FATTY ACIDS (BDS COLUMN NO. 10, 140°, 40 P.S.I.G. HELIUM)

Compound	*Bacterial "all-D" pristanate and phytanate*	*Phytol-derived phytanate*	*All-synthetic phytanate*	*Mixed all-synthetic + phytol-derived phytanate*	*Phytol-derived 4,8,12,16-TMHD*
14:0[a]	1.000	1.000	1.000	1.000	1.000
DDD-Pristanate	2.414	—	—	—	—
16:0	2.467	2.468	2.466	2.469	2.473
17:0	3.857	3.857	3.855	3.855	3.853
?-Phytanate	—	—	4.099	4.097	—
LDD-Phytanate	—	4.146	4.148	4.148	4.149
?-Phytanate	—	—	4.157	Not obvious	—
DDD-Phytanate	4.212	4.211	4.213	4.210	4.208
18:0	6.018	6.025	6.025	—	6.017
?	—	—	—	—	7.081
4L,8D,12D,16-TMHD (?)	—	—	—	—	7.161
4D,8D,12D,16-TMHD (?)	—	—	—	—	7.273

[a] Adjusted retention time 13 min.

and (—) 2.2 for the 4D,8D isomer. The 4,8,12-trimethyltridecanoic acids isolated from one terrestrial source and two marine sources[2,3] show only one peak on GLC and correspond to the synthetic component of longer retention time. There is strong evidence that these naturally occurring acids are derived from phytol (3,7D,11D,15-tetramethyl-2-hexadecen-1-ol) and are therefore all-"D" in configuration. The decrease in $\Sigma[M]_D$ magnitude is therefore probably in accord with the order of elution on GLC.

The pristanates (2,6,10,14-tetramethylpentadecanoates) isolated from natural sources show two GLC peaks[2,3] of which that with the longer retention time corresponds to the peak for a 2D,6D,10D isomer prepared from all-"D" phytanate. It is inferred that the other peak is the 2L,6D,10D isomer since both of these isomers should be formed when phytol is degraded to pristanic acid (see below). Unfortunately $\Sigma[M]_D$ values calculated for the four pristanic diastereoisomers (Table III) include respectively (—) 31 for the 2D,6D,10D isomer and (+) 25 for the 2L,6D,10D isomer. Decreasing $\Sigma[M]_D$ values are thus not in agreement with GLC retention time for the two isomers in question.

The phytanates (3,7,11,15-tetramethylhexadecanoates) isolated from natural animal sources also normally show two peaks. That of longer retention time coincides with the peak for 3D,7D,11D,15-phytanate prepared from the lipids of a halophilic bacterium[2,10]. Reduction of the double bond in phytol produces 3L and 3D asymmetric centers and hence the phytanate component of shorter GLC retention time is reasonably assumed to be the 3L,7D,11D isomer. Since the $\Sigma[M]_D$ values (Table IV) are respectively (—) 17.0 for the 3L,7D,11D isomer and (+) 11.0 for the 3D,7D,11D isomer the principle relating increasing GLC retention time and decrease in magnitude for $\Sigma[M]^D$ values appears to be operative for these particular isomers (see below).

Esters of 4,8,12,16-tetramethylheptadecanoic acids derived from phytol would have respective $\Sigma[M]_D$ values of (—) 3.4 for the 4L,8D,12D isomer and (—) 2.6 for the 4D,8D,12D isomer. Two GLC peaks are observed (Fig. 3; Table V) but as in the case of 3,7,11-trimethyltridecanoate there is no definite evidence that the component of longer retention time is the all-"D" isomer.

VALIDITY OF $\Sigma[M]_D$ VALUES FOR ISOPRENOID FATTY ACIDS

Experimental $[M]_D$ values for authentic all-"D" materials were respectively (—) 37.8° for methyl pristanate and (+) 10.8° for methyl phytanate (corrected for 3 % pristanate impurity)[10]. These compare with $\Sigma[M]_D$ values of (—) 31° and (+) 11°. It must be recognized that the $[M]_D$ values of Table I are adopted from literature values for acids[6], not esters, and that experimental values are sensitive to solvent and other factors. It is not possible, with present evidence, to rationalize interactions among the asymmetric centers of isoprenoid fatty acids which would modify the $\Sigma[M]_D$ values for the 2D,6D,10D and 2L,6D,10D pristanates. Possibly the discrepancy between decreasing magnitude of $\Sigma[M]_D$ values and increasing GLC retention times arises from the methyl substituent at C_2 in this case.

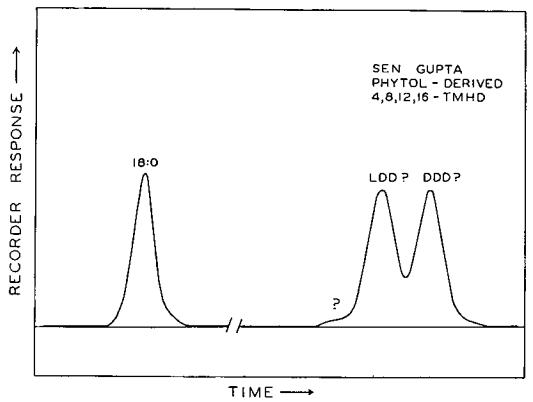


Fig. 3. Resolution of diastereoisomers of methyl esters of phytol-derived 4,8,12,16-tetramethylheptadecanoic acid. Single column (No. 10) operated at 140° and 40 p.s.i.g. helium. Retention data are given in Table V.

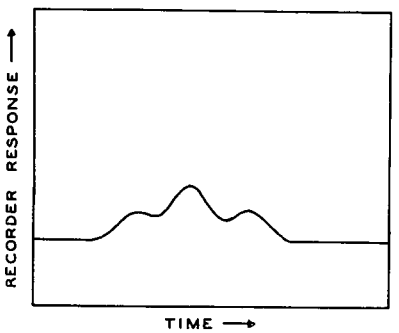


Fig. 4. Resolution of diastereoisomers in ethyl phytanates of completely synthetic origin. Single column (No. 7) operated at 150° and 40 p.s.i.g. helium. Retention time 64 min.

An opportunity to investigate possible asymmetric center interactions in the phytanates arose when a completely synthetic[11] sample of ethyl phytenate was obtained. This was hydrogenated to ethyl phytanate (Fig. 4) and subsequently converted to methyl phytanate (Fig. 5; Table V). The ethyl and methyl phytanates,

which have similar GLC peak patterns, each contain four diastereoisomeric pairs of phytanates. Careful GLC study suggests that these appear, on GLC, with the retention time and degree of separation as follows: unknown $\ll$ 3L,7D,11D $<$ unknown $\ll$ 3D,7D,11D. The original straightforward calculation I (Table IV) is correct only insofar as the $\Sigma[M]_D$ magnitudes indicate that 3L,7D,11D will precede 3D,7D,11D and that 3D,7D,11D will appear last. One example of the many possible interactions which might affect the magnitude, and possibly even the sign of a low $[M]_D$ value asymmetric center, such as the C_7 (ω-8), is given in calculation II, Table IV. Should the presence of opposite rotations at the C_7 (ω-8) and ω-4 asymmetric centers allow a slight increase in the $[M]_D$ value for the C_3 center in the 3D,7D,11L and 3D,7L,11D isomers, then the GLC retention times of the four diastereoisomers would be, in decreasing order of $\Sigma[M]_D$ magnitude, 3D,7D,11L $<$ 3L,7D,11D $<$ 3D,7L,11D$<$ 3D,7D,11D. This rudimentary Procrustean adjustment, although producing a reasonably attractive interpretation of the observed distribution of GLC peaks, is undoubtedly a gross oversimplification.

There are other aspects of these separations, such as the relatively poor separation among 3,7,11-trimethyldodecanoate and 4,8,12-trimethyltridecanoate diastereoisomers on comparison with 3,7.11,15-tetramethylhexadecanoate and 4,8,12,16-tetramethylheptadecanoate diastereoisomers, which are not easily explained.

OBSERVATIONS ON THE GLC HEIGHT RATIOS OF PHYTOL-DERIVED PHYTANATES

An additional minor component immediately preceded the 4,8,12,16-TMHD peaks (Fig. 3). This observation is of interest since it could be an additional diastereoisomer originating in the source phytol or as an artifact from the extensive chemical

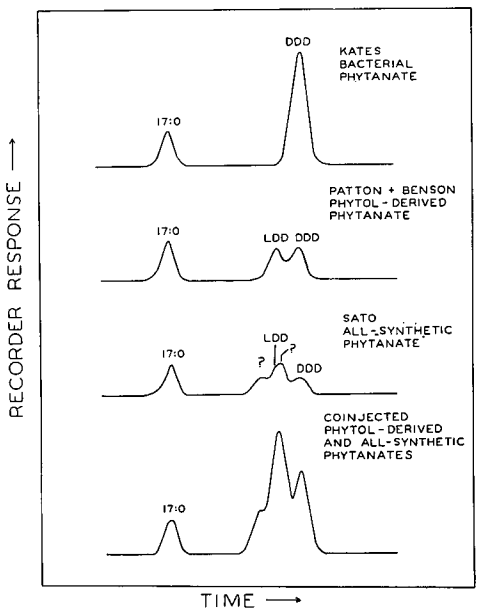


Fig. 5. Resolution of diastereoisomers in methyl phytanates of different degrees of complexity. Single column (No. 10) operated at 140° and 40 p.s.i.g. helium. Retention data are given in Table V.

treatment. If a fourth diastereoisomer were present, and if the order of elution were similar to the phytanates (Fig. 5), this would be superposed on the presumed 4L,8D, 12D,16-TMHD peak which it will be noted is of height equal to the presumed 4D,8D, 12D,16-TMHD peak.

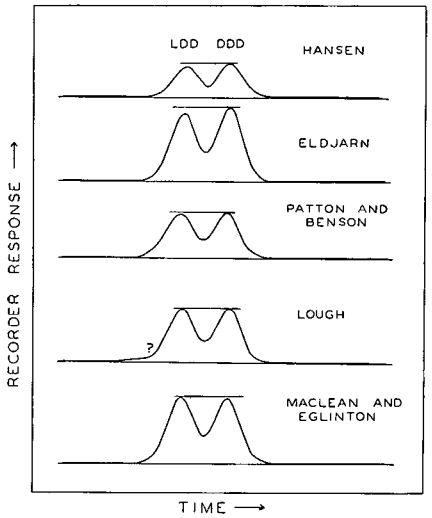


Fig. 6. Examination of peak height ratios for similar sized samples of phytol-derived phytanates prepared in different laboratories. All analyses were carried out with coupled column (300 ft.) operated at 150° and 80 p.s.i.g. helium. Retention time 108 min.

In the original publication on the resolution of diastereoisomers of phytanic acid a definite deviation from a 1:1 peak height relationship for phytanates derived from phytol was noted[2]. Since peak resolution was poor it was possible to explain this as due to tailing of the 3L,7D,11D-phytanate peak raising the peak height of the 3D,7D,11D-phytanate. Alternatively stereospecificity in chemical reactions was suggested (*cf.* refs. 1 and 7). During a study carried out with coupled columns samples of phytanate prepared in five different laboratories were examined under the same conditions (Fig. 6). It is evident that in respective samples prepared by HANSEN, ELDJARN and PATTON AND BENSON, the second or 3D,7D,11D-phytanate peak is higher than the 3L,7D,11D-phytanate peak. The sample prepared by LOUGH also possibly shows the same characteristics, but in the sample prepared by MACLEAN AND EGLINTON the peak heights are unequivocally equal.

The degree of resolution achieved in these analyses is such that it is difficult to consider that tailing of the earlier peak is responsible for the differences in peak height. If the phytol were not configurationally homogeneous then the additional diastereoisomers produced should be detectable as a separate peak well before the 3L,7D,11D-phytanate peak or should increase the height of this particular peak (*cf.* Fig. 5). Neither effect is observed in the majority of cases. The alternative is to assume stereospecificity in one or more of the chemical steps from phytol to methyl phytanate. It is, however, necessary to make the observation that inadvertent slight enrichment in one diastereoisomer could take place during preparative GLC of these materials on efficient packed columns coated with polar liquid phase, since an apparently homoge-

neous peak would actually be enriched in the 3L,7D,11D phytanate in the leading edge and correspondingly deficient in this diastereoisomer in the tailing edge.

EXPERIMENTAL

GLC operations were similar to those described previously[2,3]. Two butanediol–succinate coated open-tubular columns (Nos. 7 and 10) purchased from the Perkin-Elmer Corp., Norwalk, Conn., U.S.A., were used either singly or coupled in series. The latter combination achieved about 80,000 theoretical plates for methyl hexadecanoate at 150°. All retention data given are adjusted (measured from leading edge of solvent peak) and measured to the intercept of the tangent drawn at the leading edge of the peaks with the baseline.

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NOTE ADDED IN PROOF

Results of collaborative studies with I. MACLEAN AND G. EGLINTON (unpublished) have established that 3D,7D,11-trimethyldodecanoate and 4D,8D,12-trimethyltridecanoate coincide with the components of longer retention time in the respective esters of synthetic acids representing mixtures of diastereoisomers.

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CHROM. 3337

CLASS SEPARATION OF FLAVOUR VOLATILES BY LIQUID CHROMATOGRAPHY ON SILICA GEL AT 1°

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SUMMARY

A simple technique is described for the fractionation of complex flavour mixtures by liquid chromatography on silica gel using low boiling solvents at 1°. Its application to the examination by combined gas chromatography–mass spectrometry of the volatiles from peas and bananas is outlined.

INTRODUCTION

The use of the gas chromatograph directly coupled to a fast-scanning mass spectrometer has, in recent years, become the principal technique for the identification of volatile food flavour constituents. However, it is now apparent that the complex nature of volatile food flavours is such that, even with the customary use of high resolution capillary columns, a considerable proportion of the flavour constituents is not fully resolved by the gas chromatograph, and mixed mass spectra result. Only in those cases where the spectra are especially characteristic can mixed spectra be interpreted, and at best the resulting evidence is by itself not completely acceptable for the unequivocal identification of the components. Unless components of a mixture are first fully resolved by the gas chromatograph the full potential of the combined use of the gas chromatograph and the mass spectrometer is not being realised.

Greater separation of components prior to their mass spectral scanning is obviously required and in this laboratory has been effected in two ways: (a) by pre-fractionation of the mixture into classes by liquid chromatography and (b) by the development of a workable system to transfer "single peaks" from one high resolution column to another of different polarity[1]. The first approach has been employed in current investigations of the volatiles from frozen peas and bananas.

Alcohols were found to predominate in the volatiles from peas[2], but their identification by combined gas chromatography–mass spectrometry (GC–MS) was made difficult by the presence of minor unresolved non-alcoholic components. The preliminary separation of the alcohols as a group required a simple technique capable of handling the small amount of volatiles available from peas. Thin layer chromatography (TLC) at low temperature, as suggested by STAHL[3], proved unsatisfactory due to the loss of the more volatile constituents (boiling below approximately 100°).

To prevent this loss of volatiles the adsorbent was enclosed in a narrow bore plastic tube, which could be readily sectioned to recover the fractions. The literature shows that flexible tubing (cellophane[4–6] and teflon[7]) has been used previously to contain the adsorbent and has also been sectioned to recover the separated zones[5,6]. Techniques have been described which use dry columns of adsorbent developed by the solvent front moving horizontally[6], or vertically upwards[8] or downwards[9].

To minimize the loss of the more volatile components, the use of low boiling solvents was considered vital. Monofluorotrichloromethane (b.p. 24.7°) and methyl ethyl ether (b.p. 7.9°) were chosen for column development and the elution of fractions respectively, but the two were also mixed for development. The temperature of operation was reduced to 1°. Since the risk of contamination was very real in view of the small amounts of material involved, teflon tubing was chosen, and a grade of neutral silica gel was selected with a negligible content of extraneous matter[10].

EXPERIMENTAL

Glassware

All glassware was cleaned by chromic acid mixtures, rinsed with distilled water and oven dried.

Solvents

Since methyl ethyl ether was not readily available commercially it was prepared as follows: Ethyl chloride (300 ml) and sodium methoxide in methanol (sodium 115 g in methanol 1500 ml) were charged at 0° into a stirred stainless steel bomb which was slowly heated. When the reaction began at 55° the heating was discontinued and the temperature rose to a maximum of 140°. The reaction mixture, cooled to —20°, was transferred to a 3 l flask, from which the crude ether was distilled through a reflux condenser at 20° to reduce the carry over of methanol, and thence to a further condenser cooled by solid carbon dioxide. The ether was redistilled through a column 20 in. by 0.6 in. packed with 1/8 in. glass helices. The distillate containing about 1 % methanol was stood over calcium chloride overnight and the remaining traces of methanol were removed by percolation through a column of Linde Molecular Sieve 4A.

Monofluorotrichloromethane ("Isceon 11" Monsanto Chemicals Aust. Ltd.) was fractionally distilled through the above column. Carbon tetrachloride, the main impurity in the commercial product, was thereby reduced to less than 10 parts per million.

Adsorbents

Silica gel (HR Merck) was partially deactivated by the addition of 25 %, w/w of water. Silver nitrate impregnated silica gel (Adsorbosil CABN, Applied Science Labs.) was stored over phosphorus pentoxide until required.

Apparatus and method

The teflon tube, 9 in. in length, 1/4 in. O.D. 3/16 in. I.D. was thoroughly washed in hot soapy water followed by distilled water and then oven dried. For support and protection from contamination the teflon tube was slid into a glass tube of similar length. The column was packed as follows: One end of the teflon tube was plugged with 1/8 in. of glass wool, a few crystals (*ca.* 1 mg) of azobenzene added above

the plug, and with gentle suction and vibration the adsorbent was added until 1 in. remained unfilled. Subsequent operations were conducted in a cold room at 1°. A charge (30–100 μl) of the flavour concentrate was added to the top of the packing, quickly followed by the remainder of the packing which was retained by a second glass wool plug.

The loaded column was inverted and stood in a beaker to which the developing solvent was added to a depth of 1/4 in. Development began when the ascending solvent front reached the 1 in. level and was complete, usually in about 4 h, when the orange colour of the azobenzene appeared in the upper glass wool plug. The column (Fig. 1.A) was removed from its glass tube and cut into selected sections of 1/2 or 1 in.

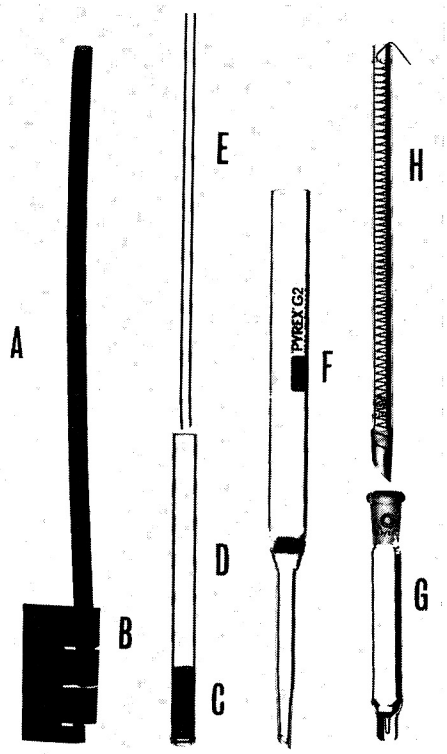


Fig. 1. (A) Teflon tube packed with silica gel; (B) template for sectioning column; (C) column section; (D) glass holder for column section; (E) extruding rod for packing; (F) filter tube for eluting fraction; (G) collection tube; (H) fractionating column.

with a scalpel and the sheet metal template (B). Cut sections (C) were transferred with forceps to glass tubular holders (D) and the packing, still wet with solvent, extruded by means of flat-ended rods (E) into sintered glass filter tubes (F). Each fraction was eluted from the adsorbent with four additions each of 0.5 ml of methyl ethyl ether, the combined washings being collected in tubes (G). The ether was distilled off through simple wetted-wall fractionating columns (H) each of which consisted of a 25 SWG Nichrome wire helix closely fitting a 5 mm I.D. glass tube. For simplicity no reflux head was employed since adequate reflux was provided by partial condensation of the ether on the column wall. The distillation rate was adjusted by the depth

of immersion of the tubes in a water bath a 25°. When distillation had ceased in about 1 h the column hold-up (about 0.1 ml) was allowed to drain back into the tube, which was then disconnected from the column. Most of the remaining solvent was driven off by careful warming with the fingers. When the amount of recovered material was small each tube was stoppered and centrifuged to gather the material in the capillary cavity in the thickened base. Fractions were stored in the same tubes at —20° until examined by GC–MS.

The technique employing silver nitrate impregnated silica gel, applied as described below, was identical to the above except that development of the column was made with a 3:5 mixture of "Isceon 11" and methyl ethyl ether.

APPLICATIONS

As a preliminary trial to examine the order of separation of five classes of compounds, 50 μl of a mixture of hexan-1-ol, pentan-1-al, heptan-2-one, ethyl hexanoate and dodecane was chromatographed on silica gel as described. Fractions recovered from 1 in. sections of the column were examined by gas chromatography and the distribution of the five compounds among the fractions was indicated to be as follows:

0–1 inch: discarded
1–2 inch: hexanol only
2–3 inch: pentanal, heptanone; hexanol
3–4 inch: heptanone, pentanal
4–5 inch: heptanone, pentanal (small)
5–6 inch: ethyl hexanoate, heptanone (small)
6–7 inch: ethyl hexanoate only
7–8 inch: nil
8–9 inch: dodecane.

The result showed a complete separation of three of the components. Hexanol overlapped slightly with pentanal and heptanone which separated only slightly, the ketone running ahead and becoming the major component in the 4–5 in. fraction. The overlap between ketone and ester appeared to be slight. The hydrocarbon obviously ran with the solvent front.

The above result was sufficiently encouraging to immediately apply the technique to actual flavour mixtures. Experience with such mixtures has shown that the resolution between classes is improved if the column is not heavily loaded. A charge of 30 μl is considered the optimum for 3/16 bore columns as used above.

Pea volatiles

As indicated above the technique was developed primarily to aid the identification by GC–MS of the alcoholic constituents of the volatiles from frozen peas. The total pea volatile alcohols were separated as a fraction on silica gel essentially as described. Their spectra then showed removal of the interfering background, which was found to be due largely to a range (C_{10}–C_{15}) of branched and straight chain paraffins (also separated as a fraction).

Although this pre-separation of the alcohols served to strengthen greatly the mass spectra evidence for most of the saturated alcohols and for *cis*-hex-3-en-1-ol, it

also revealed the presence of other unsaturated alcohols, many only in trace amounts and some with peaks incompletely resolved. In view of their possible role in contributing to the overall pea flavour, stronger evidence for their identification was essential. Their further separation from the much more abundant saturated alcohols was therefore attempted using silver nitrate impregnated silica gel as the adsorbent. The total alcohol fraction above was chromatographed on silver nitrate–silica gel as described. This effectively spread the alcohols between eight fractions, the examination of which by GC–MS resulted in the positive identification of nine unsaturated alcohols and the tentative identification of three.

The composition of the eight fractions showed that complete separation between saturated and unsaturated alcohols as classes was not fully realised, as the carbon number, the presence of methyl branches, whether the hydroxyl is primary or secondary, and whether double bonds are *cis* or *trans* influence the rate of travel on the column. However a clean separation was effected between saturated and unsaturated primary straight-chain alcohols of the same carbon number. For instance, hexan-1-ol, a major component, ran ahead of *trans*-hex-3-en-1-ol, which was followed and partly overlapped by *cis*-hex-3-en-1-ol, another major component. The above *trans* isomer was indicated in the gas chromatogram of the total alcohol fraction as a slight shoulder on the tail of the large hexan-1-ol peak. Yet in two fractions from the silver nitrate column it was fully resolved to the base line and in one of these in sufficient concentration to give a strong clear mass spectrum and an informative infra red spectrum. Similar separations were observed for saturated and unsaturated alcohols of other chain lengths.

Banana volatiles

In connection with studies in this laboratory on banana ripening the need arose for a clear knowledge of the identity and relative amounts of the volatile saturated alcohols present in the ripe fruit. Although investigation of the volatiles from banana has been extensive, the complex nature of the mixture, especially the predominance of esters, made it impossible to extract this information from the published data (reviewed by WICK *et al.*[11]). Pre-fractionation on silica gel has made the investigation of this point a relatively simple matter. A concentrate of the volatiles from ripe bananas (30 μl) was chromatographed on silica gel as described and the column divided into seven fractions. The first fraction (1–2 in.) contained exclusively the saturated alcohols, which, being relatively few in number and well resolved, presented no difficulties in identification by their mass spectra. The esters formed a broad band (2 1/2–5 in.) well resolved from the alcohols. Within this band separation of some esters according to type had taken place and in several instances the separation of two esters of identical GC retention times had been achieved. Details of this work will be published elsewhere.

DISCUSSION

In a recently published discussion on the division of flavour volatiles into groups[12], two objections to the use of thin layer or column chromatography for this purpose were: the possibility of chemical changes to sensitive compounds induced by the adsorbent, and the difficulty of removing the solvent without loss of the more

volatile components. In adopting the present technique the authors recognize the possibility of isomerization due to a double bond shift and the hydrolysis of sensitive esters. For these reasons silica gel was chosen in preference to alumina. Its extensive deactivation and use at lower than normal temperatures would both weaken any such activity. In the application of this technique to flavour mixtures no instance of chemical change due to the adsorbent has been observed. Lower esters are apparently unaffected, since both methyl and ethyl acetates were found in fractions of banana volatiles separated in this way.

With the use of low boiling solvents the loss of the lower boiling volatiles becomes a much less serious problem. Methanol is clearly recoverable in high yield. Even acetaldehyde was observed in fractions of pea volatiles.

Besides the prime advantage of making possible a greater overall resolution of flavour constituents, this separation by liquid chromatography often effects a considerable concentration of minor components in certain fractions. This is especially so when one class of compound or even a single compound predominates. In addition the classification of fractions according to their location on the column is valuable supporting evidence for identification, and may well provide the vital clue for the interpretation of mass spectra. This has been the experience in this laboratory especially in the interpretation of the mass spectra of alcohols and esters when the intensity of the molecular ion is often low.

The successful use of silver nitrate impregnated silica gel for the specific separation of unsaturated alcohols has suggested the possible use of other adsorbents with specific activity. For instance sulphur compounds are of considerable interest to the flavour chemist, but their occurrence usually as very minor components poses great difficulties in their separation and identification. Their pre-separation as a class or into several classes would appear to be possible on specific adsorbents such as weak Raney metal catalysts[13] or supports impregnated with mercury salts[14]. Possible extension of the described technique in this direction is being examined.

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CHROM. 3340

SEPARATION OF MOUSE IMMUNOGLOBULINS ON THE BASIS OF WATER-SOLUBILITY ON SEPHADEX G-25

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SUMMARY

Whole mouse serum was placed on columns of Sephadex G-25 "Fine" or "Superfine" equilibrated with distilled water or 0.0001 *M* phosphate, and eluted with 0.01 *M* phosphate, pH 7.4, containing 0.15 *M* NaCl. Proteins were eluted in two peaks. The first emerged in approximately the void volume of the column; the second emerged with and slightly in advance of the salt front. IgM was almost entirely confined to the 2nd peak, which also contained 30–50 % of the IgG, 15–35 % of the IgA, some β-2C and occasionally small amounts of other non-immunoglobulin proteins. Most of the IgA, 50–70 % IgG, and sometimes small traces of IgM appeared in the 1st peak, which also contained the bulk of the non-immunoglobulin proteins. Further purification of the 2nd peak proteins was capable of yielding IgM free of contaminants detectable by immuno-diffusion. The best results were obtained with "Superfine"-grade Sephadex G-25.

INTRODUCTION

The differential solubility of serum proteins in distilled water or solution of low ionic strength has long been known, and permits the selective precipitation of euglobulins by dialysis. The advent of dextran gels (Sephadex) has made it possible to change the salt concentration of protein solutions with greatly increased speed and efficiency, and may facilitate the separation of proteins on the basis of solubility differences. This possibility has been to some extent explored by EPSTEIN AND TAN[1,2] and FJELLSTRÖM[3], who passed human serum through columns of Sephadex G-25 in solutions of low ionicity, and by PORATH[4] who employed a gradient of increasing concentrations of ammonium sulphate.

In the present paper we describe a simple method for fractionating mouse serum proteins by passage through a column of Sephadex G-25 equilibrated with distilled water or 0.0001 *M* phosphate, phosphate-buffered NaCl being used for elution. 'Euglobulins' are selectively retarded on the column. By means of this technique it is possible to obtain immunoglobulins free from the bulk of other serum proteins. In particular IgM may be obtained free of α2M-globulin in such a way that

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it can be completely purified by subsequent passage through a column of Sephadex G-200; moreover, IgA can be obtained completely free of IgM.

METHODS

Sera

Mouse serum was obtained from adult A/H and C57BL/H animals. Usually the mice had previously received two subcutaneous injections of *Bordetella pertussis* vaccine (4×10^8 organisms), which were given to boost the serum immunoglobulin levels. The two injections were separated by a week and the mice were bled 10 days after the second.

Unless otherwise specified sera were separated and used within 5 h of bleeding.

Gel filtration

Sephadex G-25 (Pharmacia) was suspended in twice-distilled water and packed in columns of the following dimensions: 1.5×90 cm, 2.5×85 cm, and 5×100 cm. Initially "Fine" grade Sephadex (particle size 20–80 μ) was used, but for most of the work we employed "Superfine" (particle size 10–40 μ), the grade primarily intended by the makers for thin-layer chromatography. Before use, the bed was washed with at least 2 l of twice-distilled water or phosphate buffer (0.0001 M; pH 7.0).

Freshly prepared serum was applied to the top of the column—about 1.5 ml for a 1.5×90 cm column, 1–3 ml for a 2.5×85 cm column and 8–10 ml for a 5×100 cm column—and eluted with 0.01 M phosphate buffer, pH 7.4, containing 0.15 M NaCl. The flow rate was adjusted to between 10 ml/h and 30 ml/h according to the size of the column, although higher rates were obtainable even with superfine Sephadex and did not seem seriously to affect the separation.

Separation proceeded at room temperature. A column could be used several times provided that it was thoroughly washed with at least 3–4 l of 0.0001 M phosphate or distilled water.

Fractions of 2–6 ml were collected and their protein content was estimated spectrophotometrically at 280 mμ. Until the characteristics of a given column were established the specific resistance of each fraction was determined with a Phillips PR 9500 conductivity measuring bridge (cell constant = 1.3).

Fractions were pooled and concentrated by vacuum dialysis, dialysis against polyethylene glycol (mol. wt. 40,000: SERVA), or Aquacid I (Calbiochem).

Immunochemical analysis

Fractions were analysed by immunoelectrophoresis[5] in agar or agarose, by double diffusion in agar[6], and by single radial diffusion[7]. Antisera for the analysis were prepared in rabbits against whole mouse serum; mouse IgA; mouse IgM; mouse IgG1; mouse IgG2. The specific anti-mouse-Ig sera were prepared as described by BAZIN[8]. The same nomenclature for mouse immunoglobulins is employed as in previous papers[8,9], being based on that introduced by FAHEY *et al.*[10]

Protein concentration

The Biuret method as described by GORNALL and coworkers[11] was used for all protein determinations with a standard preparation of bovine serum albumin titrated by the Kjeldahl method.

RESULTS

The proteins of mouse serum emerged from a column of Superfine-grade Sephadex equilibrated with distilled water or 0.0001 *M* phosphate buffer, pH 7.0 in two well-separated peaks (Fig. 1). The first peak corresponded to the void volume of the column (*i.e.* that part of the total bed volume which lies outside the dextran gel granules), as measured by the passage of coloured Dextran 5000 (Pharmacia). The proteins of this peak had no tendency to precipitate even after prolonged storage. The second main protein peak emerged just before, and with, the salt front. The proteins in the earliest fractions of the second peak, *i.e.* those in which the salt concentration was lowest, tended to precipitate after a few hours in the tube at room temperature, but this could easily be prevented by the addition of a small amount of NaCl, or by using a refrigerated fraction collector.

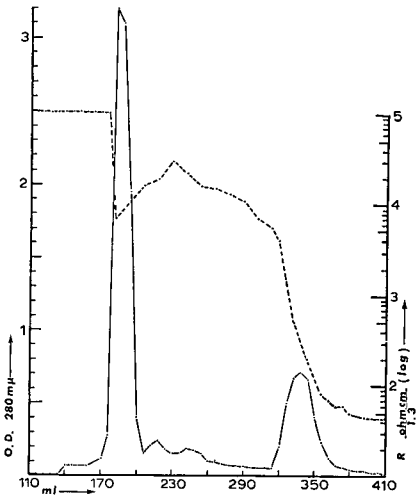


Fig. 1. Protein concentration (absorbance at 280 mμ) and specific resistance (cell-constant 1.3) of fractions eluted from a 2.5 × 85 cm column of Sephadex G-25 "Superfine" equilibrated with distilled water. 2 ml mouse serum eluted with 0.01 *M* phosphate, pH 7.4, 0.15 *M* NaCl.

At its best, the separation effected by fine-grade Sephadex was similar to the above. However, results with this grade were very much less reproducible. Some or even all of the water-insoluble proteins normally present in the second peak would often arrive with the first peak and form a dense cloudy precipitate in the collecting-tubes. The addition of NaCl to such tubes within one or two hours caused the precipitate to dissolve immediately.

Immunoelectrophoretic analyses showed that most of the serum proteins appeared in the first peak. These included pre-albumin, albumin, α_2-macroglobulin, transferrin, some IgG and many less easily recognizable proteins (Fig. 2a). It also contained some IgA. IgM was never detectable in immunoelectrophoretic analyses of the first peak, although traces were occasionally discernible in OUCHTERLONY plates and by the technique of single radial diffusion. The second peak consisted principally of immunoglobulins (IgM, IgG1, IgG2a, IgG2b) together with β-2C (part of the third component of complement), some IgA and sometimes traces of some other

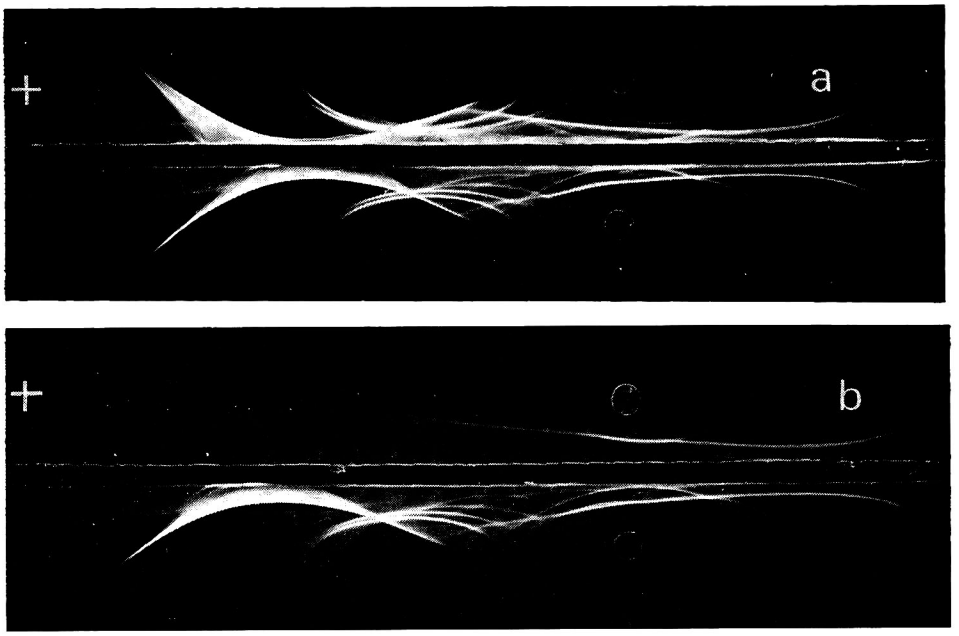


Fig. 2. Immunoelectrophoretic analysis of reconcentrated fractions of mouse serum eluted from a column of Sephadex G-25 "Superfine"; rabbit antiserum to whole mouse serum in troughs. (a) Upper well: 1st peak from column, lower well: unfractionated mouse serum. (b) Upper well: 2nd peak from column, lower well: unfractionated mouse serum.

unidentified proteins (Fig. 2b). The small amount of material eluted between the two main peaks (Fig. 1) consisted of proteins characteristic of the first peak.

Quantitative investigation, by single radial diffusion, of the immunoglobulins in the two peaks of five separated sera gave the approximate values shown in Table I.

TABLE I

DISTRIBUTION OF IMMUNOGLOBULINS BETWEEN THE TWO MAIN PROTEIN FRACTIONS ELUTED FROM SEPHADEX G-25 "SUPERFINE" COLUMNS, ESTIMATED BY SINGLE RADIAL DIFFUSION (5 POOLS OF SERUM)

	1st peak	*2nd peak*
IgA	65–85 %	15–35 %
IgG1	about 65 %	about 35 %
IgG2	50–80 %	30–50 %
IgM	traces or none	100 %

The best separations were obtained with fresh serum. Storage at —20° for three weeks or at 4° for one week resulted in trailing of the first peak.

The results were similar whether the column was equilibrated with distilled water or with 0.0001 *M* phosphate, pH 7.0. It is possible that manipulation of the buffering conditions and/or salt concentrations may produce further refinements in the technique. However, preliminary experiments indicate that columns equilibrated with 0.0001 *M* phosphate at pH 5.2 or 7.4 yield less useful separations.

Further purification of IgM on Sephadex G-200 followed by sucrose gradient ultracentrifugation

The second peak eluted from a G-25 column was concentrated and passed through a column of Sephadex G-200 equilibrated with 0.1 *M* Tris–HCl buffer, pH 8.2, containing 1 *M* NaCl. The same buffer was used for elution. The first half of the first peak so obtained consisted of IgM as judged by immunoelectrophoresis or double diffusion against a rabbit serum against whole mouse serum (Fig. 3). The yield of the recovered IgM was 15–20 % of the quantity in the original serum before all chromatography. It was sometimes contaminated with a small amount of α_2-lipoprotein. However, virtually complete purification, as judged from OUCHTERLONY plates, was obtained by subsequent ultracentrifugation on a sucrose gradient as described by ADLER[12], without further loss of IgM.

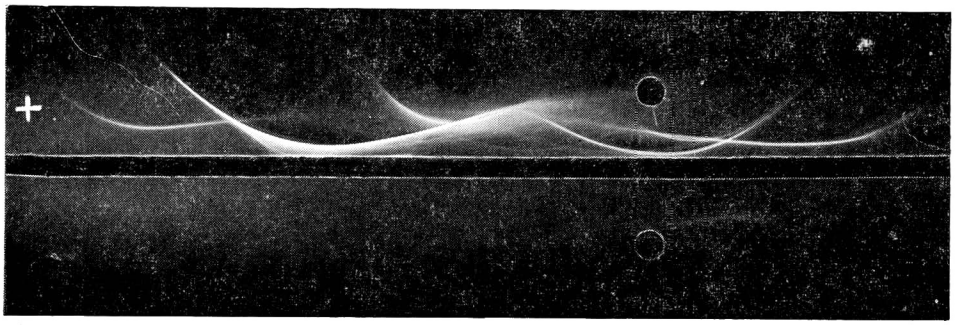


Fig. 3. Purification of mouse IgM. Rabbit antiserum to whole mouse serum in trough. Upper well: unfractionated mouse serum, lower well: IgM prepared from 2nd peak of a G-25 "Superfine" column by filtration through Sephadex G-200 (concentration of protein: 12 mg/ml).

DISCUSSION

In principle the technique described resembles ordinary dialysis against distilled water. Proteins move down the Sephadex G-25 column faster than do salts until they reach a zone of such low ionicity that some begin to precipitate. Once precipitated, the water-insoluble proteins may be arrested until redissolved at the arrival of the saltfront. This process may be envisaged as occurring repeatedly down the length of the column, with the euglobulins being continually precipitated and redissolved within a narrow zone of low ionicity. The critical salt concentration at which the precipitates begin to redissolve corresponds to a measured resistance of about 5,000 ohms/cm (representing an approximately 0.002 *M* solution of NaCl). However, not all the proteins remain stably in solution at this ionicity, since, as noted above, reprecipitation tends to occur unless the ionic strength is raised.

The critical ionicity zone may well not be the same for all the proteins in the second peak. During elution of the second peak the ionicity of the eluate was rising rapidly. Possible differences between proteins emerging at the beginning and end of the second peak were not investigated.

The two main advantages of the present method over standard dialysis are, first, that proteins are not held for protracted periods in the precipitated state, and second, that they have no opportunity to form large aggregates. Both factors should tend to reduce denaturation of the molecules. It is possible that reversible adsorption

of some protein molecules onto the surface of the Sephadex granules[13–15] may have been a minor complicating factor; such a phenomenon could account for the gradual emergence of a small quantity of protein between the first and second main peaks, and for the occasional contamination of the second peak with small amounts of material characteristic of the first peak.

The best and most reproducible results were obtained with Superfine-grade Sephadex. This may be due to a more complete arrest of fine protein precipates by the smaller size dextran particles.

The method described effects, simply and rapidly, separations which can usefully be employed in the purification of immunoglobulins. In particular, since α_2-macroglobulin appears entirely in the first peak, the method can be used in conjunction with Sephadex G-200 to prepare nearly pure IgM.

Most of this work was performed while one of us (H.B.) was a visiting worker at the Medical Research Council's Radiobiological Research Unit, Harwell, and was in receipt of a grant from Euratom.

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CHROM. 3346

CONTRIBUTION DE L'ANALYSE PAR CHROMATOGRAPHIE EN COUCHE MINCE À L'ÉTUDE DE LA SYNTHÈSE DES TANINS—SÉPARATIONS QUALITATIVE ET ANALYSE QUANTITATIVE

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SUMMARY

The contribution of thin-layer chromatographic analysis to the study of the synthesis of tannins—qualitative separation and quantitative analysis

A thin-layer chromatographic method is described for tanning agents resulting from the condensation of naphthalenesulphonic acid and of dihydroxydiphenyl sulphone in the presence of formaldehyde.

The following reactions were studied:

Condensation of naphthalenesulphonic acid in the presence of formaldehyde alone.

Condensation of the dihydroxydiphenyl sulphone in the presence of formaldehyde alone, in which condensed sulphones are formed.

Formaldehyde condensation of the above condensed sulphones with naphthalenesulphonic acid, pre-condensed or non-condensed, the formaldehyde condensation forming the final tanning agent.

INTRODUCTION

Après la présentation de la méthode analytique par chromatographie en couche mince que nous avons utilisée, cette étude expose les résultats obtenus dans l'analyse des mélanges complexes constitités par des tanins issus de la condensation d'acide naphtalène sulfonique et de dihydroxydiphényl sulfone en présence de formaldéhyde.

Nous avons étudié successivement:

La condensation de l'acide naphtalène sulfonique (ANS) sur lui-même en présence de formol.

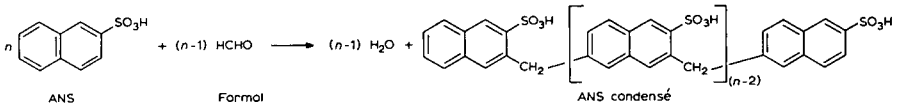
La condensation de la dihydroxydiphényl sulfone (DPS) sur elle-même en présence de formol qui donne le résol de sulfone.

La condensation formaldéhydique du résol sur de l'acide naphtalène sulfonique condensé au préalable ou non condensé en présence de formaldéhyde pour donner le tanin définitif.

PRÉSENTATION DE LA SYNTHÈSE DES TANINS

Schéma des synthèses réalisées

Le schéma ci-dessous représente les formules développées de l'acide napthalène sulfonique et de la sulfone, ainsi que le mode de condensation de ces deux produits avec le formaldéhyde:

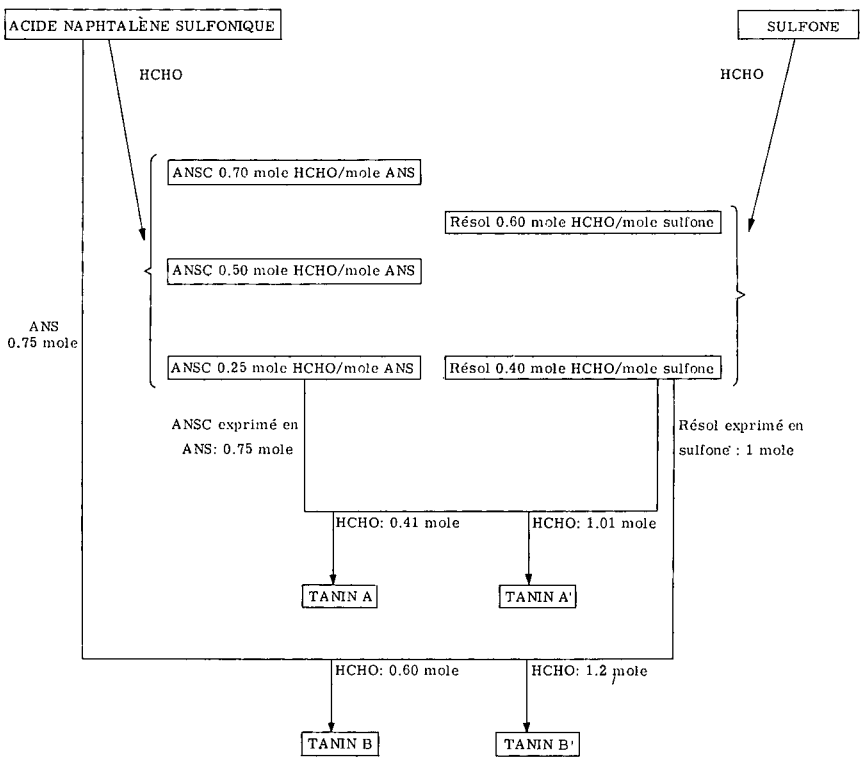


Le Tableau I donne la stoéchiométrie des différentes synthèses réalisées.

Nous sommes en présence d'une part de trois condensations d'acide naphtalène sulfonique (ANS), différentes par la quantité de formol mise en jeu, d'autre part, de deux condensations de dihydroxydiphényl sulfone (DPS) différentes pour la même

TABLEAU I

SCHÉMA DE LA SYNTHÈSE DES TANINS



raison. Les tanins A et A' sont issus de la condensation formaldéhydique d'un résol sur de l'acide naphtalène sulfonique préalablement condensé.

Les tanins B et B' sont issus de la condensation du même résol sur l'ANS non condensé avec des quantités de formaldéhyde identiques aux quantités totales mises en jeu précédemment. Ainsi la comparaison de A et B d'une part et de A' et B' d'autre part, permettra de voir l'influence de la précondensation de l'ANS sur la composition finale des tanins.

Aperçu du mode opératoire

Synthèse de l'acide naphtalène sulfonique condensé. On condense l'acide naphtalène sulfonique en présence de formol vers 100° à reflux.

Synthèse des résols. La dihydroxydiphényl sulfone est condensée avec le formaldéhyde en milieu alcalin à reflux vers 100°. Ensuite on réalise sur la résine obtenue une fixation de radicaux CH_2SO_3Na par du formol et du sulfite de sodium en autoclave sous pression vers 120°. Le mélange réactionnel est acidifié et le résol précipite.

Synthèse des tanins. On met à reflux à 100° l'acide naphtalène sulfonique condensé ou non avec le résol et le formaldéhyde.

PRÉSENTATION DE LA MÉTHODE ANALYTIQUE UTILISÉE

Analyse qualitative

L'essentiel de la séparation qualitative par chromatographie en couche mince proposée a fait l'object d'une récente publication[1]. Nous rappellerons cependant succinctement les principaux points de cette étude:

Les tanins testés sont des condensats d'acide naphtalène sulfonique, de dihydroxydiphényl sulfone et de formaldéhyde.

Les séparations sont réalisées par chromatographie en couche mince d'après les conditions opératoires suivantes:

Support: Silice fluorescente à 2500 Å et activée à 110°.

Chromatographie monodimensionnelle. Éluant acide: phase supérieure du mélange *n* butanol–acide acétique–eau (40:10:50), le temps d'élution est de 2h. Éluant basique: *n* butanol–ethanol–ammoniaque–eau (75:10:15:10), le temps d'élution est de 2 h.

Chromatographie bidimensionnelle. On entreprend d'abord une chromatographie acide puis dans le sens perpendiculaire une chromatographie alcaline.

Révélation. L'absorption à la lumière ultraviolette à 2500 Å sur support fluorescent visualise finement tout l'ensemble des composés.

Depuis le premier travail publié de nouvelles révélations ont amélioré encore la visualisation des composés et ce sur un plan spécifique: le fluoborate de *p*-nitrobenzène diazonium permet de révéler tous les composés phénoliques par copulation.

Les plaques pulvérisées de jaune de pinacryptol et examinées en lumière ultraviolette sur support fluorescent, présentent pour la sulfone condensée et pour les tanins des taches bleu noir et pour l'acide naphtalène sulfonique condensé, des taches jaune brun, ce qui permettra de différencier l'acide naphtalène sulfonique non réagi dans le tanin final.

Analyse quantitative

Nous avons mis au point une méthode d'analyse quantitative sur deux produits

résiduels de réaction: l'acide naphtalène sulfonique (ANS) non réagi et la dihydroxydiphényl sulfone (DPS) non réagie.

Ce dosage est réalisé à partir des chromatoplaques par photodensitométrie en lumière ultraviolette à 2500 Å au moyen du densitomètre Joyce et de son adaptateur pour chromatographie en couche mince.

Le dosage est réalisé en lumière réfléchie et la méthode adoptée est celle des étalons externe: on dépose sur une largeur de 15 mm les mélanges à doser; à côté et de la même façon on dépose des étalons synthétiques de quantités connues de composés à doser; après élution chromatographique on densitomètre l'ensemble et on dose la DPS ou l'ANS dans les mélanges par rapport aux étalons. L'erreur inhérente à la méthode est de: $\pm$ 10 % en valeur relative.

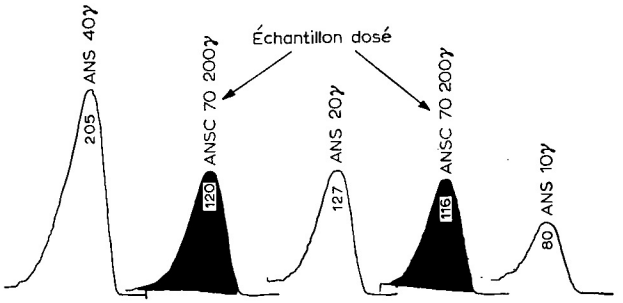


Fig. 1. Enregistrement densitométrique de dosage d'ANS libre dans l'ANS condensé.

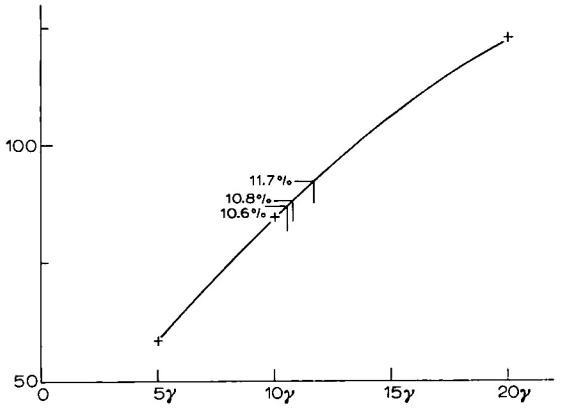


Fig. 2. Dosage de l'ANS dans l'ANSC 70, 1 h, 11 %.

Les Figs. 1 et 2 représentent l'enregistrement densitométrique d'un dosage d'ANS ainsi que la courbe correspondante.

ÉTUDE DE LA CONDENSATION DE L'ACIDE NAPHTALÈNE SULFONIQUE

Séparation qualitative

Sur silice fluorescente à 2500 Å les produits sont bleu foncé sur fond bleu, tandis qu'après réaction au jaune de pinacryptol ces produits forment des taches jaune brun sur fond vert clair.

Élution alcaline. Les composés se séparent en 8 différents niveaux bien nets compris entre $R_F = 0$ et $R_F = 0.5$.

Élution acide. Les composés se séparent en 7 à 8 différents niveaux compris entre $R_F = 0$ et $R_F = 0.8$. Les mêmes remarques signalées pour l'élution alcaline sont à noter.

Élution bidimensionnelle. L'ANS condensé se situe sur le chromatoplaque sur une diagonale caractéristique de $R_F = 0$ à 1 en élution acide et de $R_F = 0$ à 0.5 en élution alcaline.

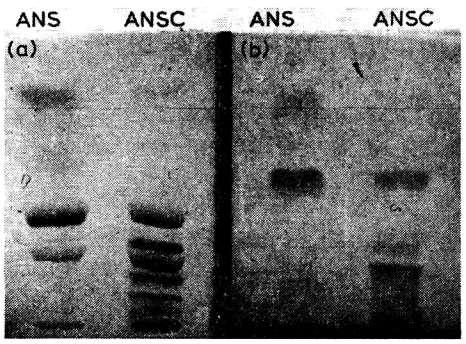


Fig. 3. Condensation de l'acide naphtalène sulfonique. (a) Élution monodimensionnelle alcaline; (b) élution monodimensionnelle acide.

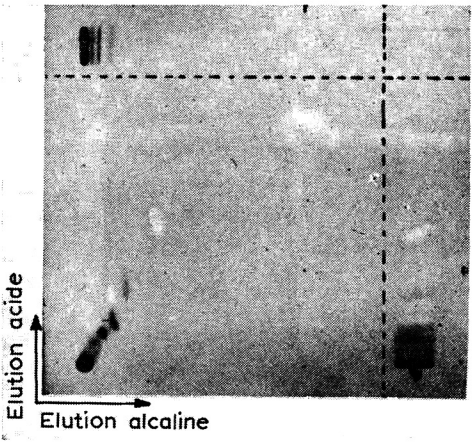


Fig. 4. Élution bidimensionnelle avec témoins élutions monodimensionnelles acide et alcaline.

Étude de la condensation en fonction du temps et de la concentration en formaldéhyde—Dosage de l'acide résiduel

On constate peu de variations pour les échantillons condensés pendant un temps différent à taux de formaldéhyde constant; par contre, les différences sont notoires pour les échantillons condensés à temps constant mais avec un taux de formaldéhyde variable; on constate un résidu d'acide naphtalène sulfonique non réagi qui diminue lorsque la concentration en formaldéhyde augmente, le phénomène inverse se produisant pour les composés de R_F les plus bas.

Résultats du dosage de ANS résiduel:

Le domaine de dosage densitométrique de l'acide résiduel est situé entre 5 et 20 μg d'ANS pur. La limite de détection est de l'ordre de 0.5 μg.

Pourcentage d'ANS résiduel en fonction du temps: la réaction de condensation est très rapide puisque nous avons noté des pourcentages identiques au bout de 1/4 d'heure et au bout de 9 h.

Pourcentage d'ANS résiduel en fonction de la quantité de formol mise en jeu. La courbe (Fig. 5) présente ces résultats.

ÉTUDE DE LA CONDENSATION DE LA DIHYDROXYDIPHENYLSULFONE (DPS) EN RÉSOL

Séparation qualitative

Sur silice fluorescente à 2500 Å, les produits présentent une révélation identique

à celle de l'ANS condensé; par contre, après pulvérisation de jaune de pinacryptol les constituants des résols sont noirs sur un fond vert clair ce qui permet de différencier ces composés de l'ANS condensé.

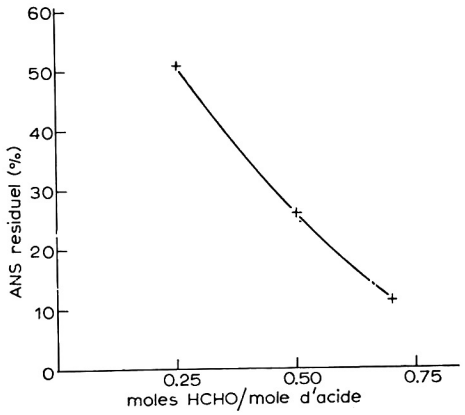
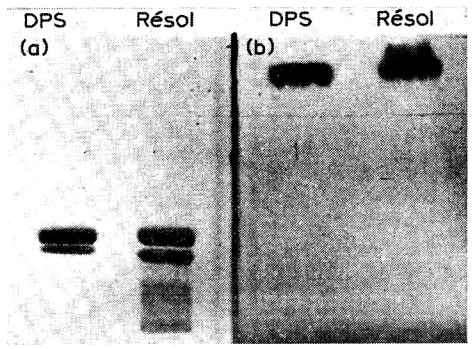


Fig. 5. Pourcentage d'acide naphtalène sulfonique résiduel dans l'acide condensé en fonction du taux de formaldéhyde utilisé.

Élution alcaline. Les composés présentent 8 à 10 niveaux différents de R_F compris entre 0 et 0.5; ils se trouvent donc au même endroit que les condensats d'ANS.

Élution acide. Tous les composés rentrant dans la constitution des résols se retrouvent non séparés au front du solvant ($R_F = 1$) ce qui permet de les séparer de l'ANS condensé.

Élution bidimensionnelle. Étant donné la position des résols en élution acide, la chromatographie bidimensionnelle situe ces produits le long de la ligne de $R_F = 1$ en élution acide et $R_F = 0$ à 0.5 en élution alcaline.



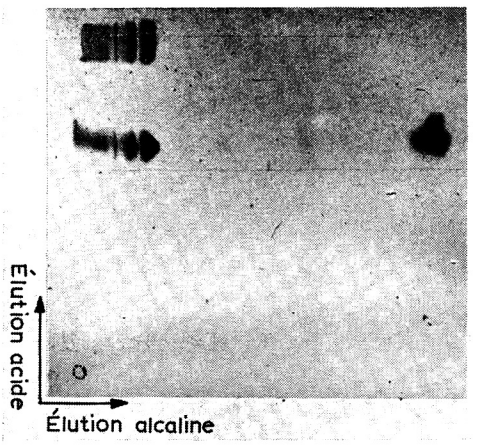


Fig. 6. Condensation de la dihydroxydiphényl sulfone. (a) Élution monodimensionnelle alcaline; (b) élution monodimensionnelle acide.

Fig. 7. Élution bidimensionnelle de résol.

Étude de la condensation en fonction du temps et de la concentration en formaldéhyde—Dosage de la DPS résiduelle

Le temps a peu d'effet sur la réaction. Après 1 heure la réaction est très avancée; entre 1 heure et 6 heures, on constate cependant qualitativement une augmentation des produits de $R_F = 0$ tandis que le taux de la sulfone résiduelle varie assez peu.

Par contre, l'augmentation de la concentration en formaldéhyde a pour effet de diminuer la concentration en DPS résiduelle que nous avons évaluée au paragraphe qui suit:

Résultats du dosage de DPS résiduelle:

Le domaine de dosage est compris entre 1 et 10 μg, la limite de détection descendant bien au-dessous de 0.5 μg.

Pourcentage de DPS résiduelle en fonction du temps: on a pu constater que la réaction était terminée au bout d'une heure, car les pourcentages de DPS sont identiques après 6 h de réaction.

Pourcentage de DPS résiduelle en fonction de la quantité de formol utilisée.

Deux concentrations seulement ont été testées (Tableau II):

TABLEAU II

ÉVALUATION DE LA DPS RÉSIDUELLE DANS LE RÉSOL

Concentration de formaldéhyde	*1 mole HCHO/mole DPS*	*1.5 mole HCHO/mole DPS*
% DPS résiduelle	32 %	21 %

ÉTUDE DE LA CONDENSATION DES TANINS

Séparation qualitative

Sur silice fluorescente à 2500 Å les chromatoplaques présentent comme pour le reste des taches bleu foncé sur fond fluorescent bleu. Après réaction au jaune de pinacryptol, les composés appartenant à l'acide naphtalène sulfoné résiduel sont bruns, les composés appartenant au résol résiduel ainsi qu'au tanin condensé apparaissent en noir sur fond vert clair.

Élutions monodimensionnelles. Les domaines des produits élués se trouvent:

pour l'élution alcaline à des R_F: $0 \leqslant R_F \leqslant 0.5$; pour l'élution acide à des R_F: $0 \leqslant R_F \leqslant 1$.

En élutions alcaline et acide les produits de $R_F \sim 0$ semblent se trouver en quantité importante. La réaction différente des constituants de l'ANSC résiduel permet de repérer facilement ces derniers (Fig. 8).

Élution bidimensionnelle (Fig. 9). L'élution bidimensionnelle permet de visualiser finement: le résol résiduaire au front de l'élution acide y compris la sulfone non réagie; les constituants de l'ANSC résiduel (en brun) y compris l'ANS de départ qui n'a pas réagi (sur la diagonale); des constituants nouveaux qui ne se trouvent ni dans le résol ni dans l'ANSC de départ. Il y a donc bien eu réaction entre ces deux derniers constituants et formation de composés plus condensés.

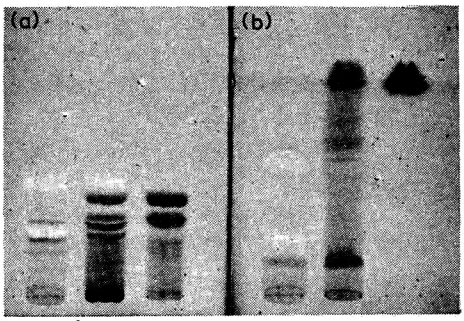


Fig. 8. Élutions monodimensionnelles alcaline (a) et acide (b) de ANSC tanin A résol.

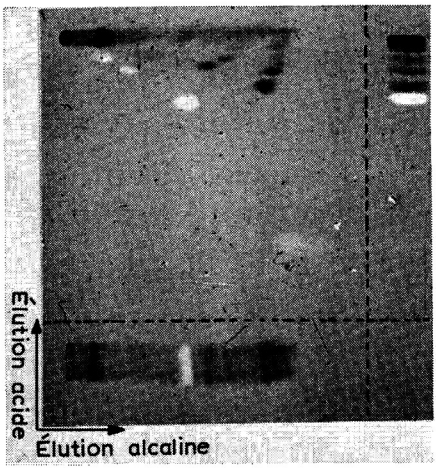


Fig. 9. Élution bidimensionnelle avec élutions monodimensionnelles témoins acide et alcaline.

Étude quantitative

Sur le plan quantitatif nous avons pu évaluer les quantités de sulfone et d'ANS résiduelles. Ces dosages ont été réalisés par densitométrie U.V. après élution alcaline. Les résultants obtenus sont consignés dans le Tableau III.

TABLEAU III

ÉVALUATION DE L'ANS ET DE LA DPS RÉSIDUELLE DANS LES TANINS

	DPS résiduelle (%)	*ANS résiduel* (%)
Tanin A	3.2	8
Tanin A′	0.4	2.2
Tanin B	1.1	8.5
Tanin B′	0.7	6.5

De la comparaison en bidimensionnelle des différents tanins, on peut tirer les conclusions suivantes:

(1) Plus la quantité totale de formol rentrant en réaction est importante, plus faibles sont les résidus d'ANS ou de sulfone libre ou condensée.

(2) La précondensation de l'ANS avant réaction avec le résol diminue l'importance de la condensation avec ce résol dont une partie reste libre dans le mélange tanin formé.

CONCLUSION

Sans prétendre avoir résolu l'ensemble du problème analytique que constituent des mélanges aussi complexes que les tanins synthétiques, cette étude montre l'intérêt que présente la technique de chromatographie en couche mince dans la séparation

des produits constituant ces mélanges ainsi que dans la recherche des conditions de synthèse les plus intéressantes.

RÉSUMÉ

La technique de chromatographie en couche mince associée sur le plan quantitatif à la photodensitométrie a permis d'aborder l'analyse de mélanges tannants complexes. Ces mélanges étaient issus de la condensation de l'acide naphtalène sulfonique et de la dihydroxydiphényl sulfone en présence de formaldéhyde.

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CHROM. 3368

FRACTIONATION OF A NATURAL MIXTURE OF ALKENYL ACYL- AND DIACYL CHOLINE PHOSPHATIDES BY ARGENTATION ADSORPTION THIN-LAYER CHROMATOGRAPHY

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SUMMARY

Argentation adsorption thin-layer chromatography has been used for the fractionation of a natural mixture of alkenyl acyl- and diacyl choline phosphatides. This produced not only concentrates of alkenyl acyl choline phosphatides but also species of alkenyl acyl- and diacyl choline phosphatides that varied in unsaturation in the fatty acid moieties.

INTRODUCTION

The first attempt to separate intact plasmalogens (alkenyl acyl phosphatides) from the corresponding ester (diacyl) phosphatides was made by GRAY AND MACFARLANE[1] on a mixture of alkenyl acyl- and diacyl choline phosphatides obtained from beef heart muscle. They used partition chromatography with secondary cellulose acetate as a support and cyclohexane–methanol as a solvent system. Although they concluded that it was difficult to obtain pure native plasmalogen by this technique, they obtained a fraction in 20% yield from the original mixture, which contained 75–85% plasmalogen, whereas the original material had a plasmalogen content of only 50%. They noted also that the plasmalogens had a very low content of saturated fatty acids, and that polyene fatty acids predominated. They attributed their fractionation to the degree of unsaturation, and, as a further possibility, to the chain length of the fatty acids.

Other attempts to prepare native plasmalogens from natural mixtures of alkenyl acyl and diacyl phosphatides by selective enzymatic[2,5] and alkaline hydrolysis[3,4] were successful, although the corresponding ester phosphatides were destroyed.

Fractionation of egg and soy diacyl choline phosphatides into species varying in their degree of unsaturation by argentation adsorption thin-layer chromatography (TLC) was reported by KAUFMANN, WESSELS AND BANDOPADHYAYA[6]. ARVIDSON[7] used a similar technique to fractionate diacyl choline phosphatides from egg yolk and livers of various animal species, and also reported the quantitative fatty acid composition of the components. Recently, ARVIDSON[8] subfractionated by reversed-phase partition TLC four fractions obtained by argentation TLC of intact rat liver

diacyl choline phosphatides, and obtained eight components, each containing one saturated and one unsaturated fatty acid.

The observations of GRAY AND MACFARLANE[1], GOTTFRIED AND RAPPORT[2], and KLENK AND KRICKAU[9] that alkenyl acyl phosphatides generally contain very small amounts of saturated fatty acids, and that the main fatty acids are polyenes and the fact that an additional unsaturation due to vinyl linkage is present in the alkenyl acyl phosphatides as compared to the diacyl phosphatides, pointed to the possibility exploited in the study of argentation adsorption TLC for the fractionation of a mixture of alkenyl acyl- and diacyl choline phosphatides. This produced not only concentrates of alkenyl acyl choline phosphatides, but also species of alkenyl acyl- and diacyl choline phosphatides that varied in unsaturation in the fatty acid moieties.

EXPERIMENTAL

Preparation of phosphatides

Fresh beef heart was freed of adipose and connective tissues and ground to a paste. The lipids were extracted with chloroform–methanol (2:1) by the method of FOLCH as modified by DAS AND ROUSER[10]. The total lipids (50 g) were dissolved in 200 ml of chloroform, and the phosphatides were precipitated with 2 l of acetone. Phosphatides free of neutral lipids were obtained by redissolving the precipitate in 200 ml of chloroform and reprecipitating the phosphatides with 2 l of acetone. A final repetition of this procedure yielded 25 g of an essentially 100 % phosphatide fraction. Absence of neutral lipids in the phosphatide fraction was confirmed by TLC. In the TLC system Silica Gel G/chloroform–methanol–ammonia (70:30:5), nothing was seen to migrate close to the solvent front where lipids less polar than cerebrosides migrate.

Isolation of a mixture of alkenyl acyl- and diacyl choline phosphatides

Two grams of this phosphatide mixture were fractionated into different classes of phosphatides by preparative TLC on Silica Gel G plates (20 × 20 cm with a silica gel layer thickness of 250 μ activated for 1 h at 110°) with chloroform–methanol–ammonia (70:30:5) as the solvent. Fifty mg of phosphatides were spotted per plate on 40 plates. The bands of different phosphatides were made visible under ultraviolet light after spraying the plates with 0.2 % alcoholic solution of 2′,7′-dichlorofluorescein. The fraction containing alkenyl acyl- and diacyl choline phosphatides was scraped off each plate with a razor blade and eluted three times with 100 ml of chloroform–methanol (2:1). After removing the chloroform–methanol under reduced pressure at 40° on a rotary evaporator, the residue was redissolved in chloroform, filtered and evaporated to dryness. This yielded 500 mg of a mixture of alkenyl acyl- and diacyl choline phosphatides. The purity of this mixture was tested in the above mentioned system where a single spot was obtained having the same R_F value as a sample of diacyl choline phosphatide synthesized in the laboratory and purified by preparative thin-layer chromatography. The synthetic sample was homogeneous as shown by thin-layer chromatography on Silica Gel G with chloroform–methanol–ammonia (70:30:5) as the developing solvent and the infrared spectrum of this sample was identical with those reported in the literature.

Fractionation of a mixture of alkenyl acyl- and diacyl choline phosphatides by argentation TLC

The silver nitrate-impregnated plates were prepared by developing activated Silica Gel G plates (silica gel thickness, 250 μ, plates, 20 × 20 cm) in a saturated solution of silver nitrate in 95 % ethanol contained in a chromatographic chamber. After the solution rose to the top of the plates, the plates were removed, air dried for 10 min, and activated for 2 h at 100°. Subfractionation of a mixture of alkenyl acyl- and diacyl choline phosphatides (10 mg per plate) into several species was carried out on 45 silver nitrate–impregnated Silica Gel G plates with chloroform-methanol–water (70:25:3) as the developing solvent. The different bands, which were visible under ultraviolet light, after spraying the plates with 0.2 % alcoholic solution of 2′-7′-dichlorofluorescein, were scraped off separately into separate 125 ml Erlenmeyer flasks and eluted three times with 50 ml of chloroform–methanol (1:2). The purity of the fractions was verified by argentation adsorption TLC in the same system when they showed single spots and their identities confirmed by plain adsorption TLC (system Silica Gel G/chloroform–methanol–ammonia (70:30:5)) where all the fractions gave single spots with the same R_F values and their R_F values were the same as that of a synthetic sample of diacyl choline phosphatide.

Methanolysis of alkenyl acyl- and diacyl choline phosphatides

Individual fractions (20 mg of each) were dissolved separately in 20 ml of diethyl ether in 125 ml Erlenmeyer flasks and immersed in a dry ice–acetone bath maintained at −30°. While the contents of each flasks were being stirred with a magnet, 2 ml of 100 % sulfuric acid[11] were added and stirring was continued for 10 min, during which time the temperature of the bath rose to 0°. The bath temperature was lowered again to −30°, 15 ml of absolute methanol were added and the stirring was continued for 10 min. The reaction mixture was carefully neutralized with 20 ml of 35 % KOH in methanol, followed by continuous rapid swirling until the jelly-like mixture turned into a white, milky solution. After 10 min of stirring, the contents of the flasks were transferred to a separatory funnel containing 200 ml of cold, distilled water, and the flasks rinsed twice with 20 ml of ether. The products obtained by methanolysis were extracted completely with 150 ml of ether, the ether extract washed free of alkali with distilled water and dried over anhydrous sodium sulfate. The silica gel containing adsorbed alkenyl acyl- and diacyl choline phosphatides may also be methanolyzed directly by the above cited technique after suspending the silica gel in 20 ml of chloroform–methanol (1:2) and extracting the products obtained by methanolysis with ether.

Separation of fatty acid methyl esters (ME) and dimethyl acetals (DMA) by TLC and their quantitative analysis by gas–liquid chromatography (GLC)

The ME and DMA obtained from different fractions of alkenyl acyl- and diacyl choline phosphatides were separated by preparative TLC, using Silica Gel G/toluene system. The ME and DMA were extracted from silica gel with diethyl ether and, after concentrating, were analyzed by GLC. The analyses were made with a Beckman GC-2A instrument equipped with a hydrogen flame ionization detector containing an aluminum column (6 ft long, 1/8 in. outer diameter) packed with Gas Chrom P, 80–100 mesh, impregnated with 20 % ethylene glycol succinate and 2 % phosphoric

acid. The temperature of the column was 190°, and helium flow rate was 30 ml/min. Peaks of ME and DMA were identified by comparing their retention values with authentic and synthetic standards, and the chain lengths of the unsaturated components were verified by GLC after hydrogenation. The peaks were quantitated by triangulation. The fatty acid methyl ester standards were supplied by The Hormel Institute Lipids Preparation Laboratory. The dimethyl acetals used as standards were synthesized and their purity confirmed by TLC, GLC and I.R. analysis as reported earlier from this laboratory[12].

Infrared analysis

The I.R. spectra of the samples, as liquid films, were obtained with a Perkin-Elmer Model 21 double beam spectrophotometer equipped with sodium chloride optics.

Determination of the plasmalogen content in different fractions

The percent plasmalogen content of the original mixture of alkenyl acyl- and diacyl choline phosphatides and the fractions thereof obtained by argentation TLC was determined by the use of the formula 2(amounts of DMA) × 100/(amounts of DMA + amounts of ME) after estimating the amounts of DMA and ME in methanolysis products of lipids by either densitometry or by quantitative weighing after preparative TLC separation and extraction. The densitometry was carried out as described by BLANK, SCHMIT AND PRIVETT[13] after running the products of methanolysis of lipids in the TLC system, Silica Gel G/toluene. The quantitative weighing of methyl esters and dimethyl acetals required larger amounts and hence the original mixture and fraction B (on argentation TLC) were subjected to methanolysis in five 20 mg batches.

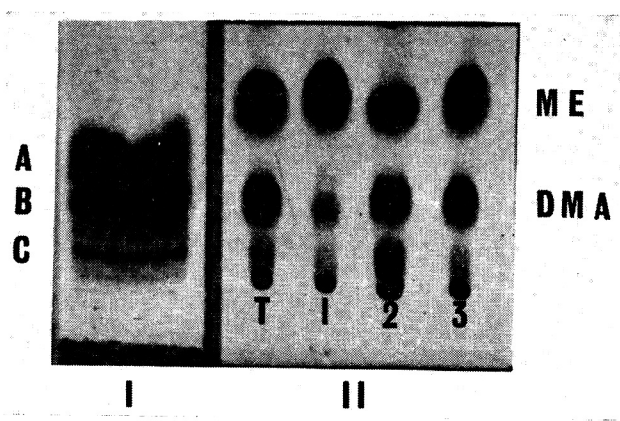


Fig. 1. (I) Fractionation of a mixture of alkenyl acyl- and diacyl choline phosphatides from beef heart. Adsorbent: Silica Gel G impregnated with silver nitrate. Activation: 2 h at 100°. Solvent $CHCl_3$–CH_3OH–H_2O (70:25:3). Spray reagent: 50% aqueous sulfuric acid (plate charred for 10 min at 160°). (II) Methanolysis products of original mixture and different fractions thereof obtained by argentation adsorption TLC of the original mixture. (T) Methanolysis products of original mixture of alkenyl acyl- and diacyl choline phosphatides. (1) Methanolysis products of fraction A. (2) Methanolysis products of fraction B. (3) Methanolysis products of fraction C.

RESULTS

The argentation fractionation of mixtures of alkenyl acyl- and diacyl choline phosphatides is demonstrated in Fig. 1 (I). Three major fractions were collected, designated A, B and C. Fractions A and B showed a tendency to separate into two parts but, under the experimental conditions employed, it was not possible to separate them completely. There were two other fractions in trace amounts, one above and one below fraction C, but their small amounts precluded their further investigation. The products of methanolysis of the original mixture (T) and those obtained from fractions A, B and C (designated as 1, 2 and 3, respectively) separated into ME and DMA (Fig. 1 (II)), indicating that the different fractions, as well as the original mixture, had alkenyl acyl choline phosphatides, though in different amounts (Table I). The original

TABLE I

THE PERCENT PLASMALOGEN CONTENT OF AN ORIGINAL MIXTURE OF ALKENYL ACYL- AND DIACYL CHOLINE PHOSPHATIDES AND THE FRACTIONS THEREOF OBTAINED BY ARGENTATION TLC

Estimation	*Percent plasmalogen content*			
	Original mixture	*Fraction A*	*Fraction B*	*Fraction C*
Densitometry	55.6	8.0	84.4	65.0
Quantitative weighing	59.8	—	88.6	—

mixture had a plasmalogen content of 55 to 60 % which agrees fairly well with the values reported in literature[1,2]. Fraction A had only 8 % of plasmalogen content, whereas fraction B had a plasmalogen content of 80 to 90 %, a very concentrated plasmalogen fraction. Fraction C had a plasmalogen content of 65 %.

The high concentration of plasmalogen in fraction B was confirmed further by I.R. studies. The ratio of absorbance of vinyl ether (at 1668 cm^{-1}) to ester (at 1735 cm^{-1}) for the original mixture was 0.27, and for fraction B it was 0.34, which confirmed our earlier findings that fraction B was a plasmalogen concentrate.

The composition of fatty acids and aldehydes in the original mixture and different fractions thereof are given in Table II. There was a steady increase in unsaturation of the fatty acids with a steady decrease in R_F value of the fraction. The composition of aldehydes in all the three fractions was nearly the same.

DISCUSSION

The technique described in this communication fractionated a mixture of alkenyl acyl- and diacyl choline phosphatides into different species, due to varied unsaturation in the fatty acid moieties and the additional unsaturation due to vinyl linkage present in the alkenyl acyl choline phosphatides. Unsaturation due to vinyl linkage helped further the concentration of alkenyl acyl choline phosphatides in fractions B and C (Fig. 1 (I)). The technique described by GRAY AND MACFARLANE[1] fractionated a similar mixture on the basis of unsaturation and chain length of the fatty acid moieties. Though they did not mention the effect of vinyl unsaturation on their separation, it must have had some effect on their separations. Their technique, however, will lead

TABLE II

FATTY ACID AND FATTY ALDEHYDE COMPOSITION OF BEEF HEART ALKENYL ACYL- AND DIACYL CHOLINE PHOSPHATIDES

	Percent fatty acid composition				*Percent fatty aldehyde composition*			
	Original sample	*Fraction A*	*Fraction B*	*Fraction C*	*Original sample*	*Fraction A*	*Fraction B*	*Fraction C*
13:0	—	—	0.5	—	2.4	1.5	—	—
14:0	tr	0.5	—	0.8	2.6	—	2.6	—
14:1	tr	tr	0.6	1.2	2.1	2.4	4.1	—
15:0	tr	0.7	tr	tr	2.6	8.3	3.7	12.3
15:1	tr	tr	1.1	1.2	2.1	1.8	2.7	1.3
16:0	30.8	38.8	6.7	14.6	69.4	64.6	66.3	55.0
16:1	1.7	3.1	0.5	1.3	3.1	4.2	3.8	3.5
17:0	tr	0.8	1.5	tr	1.2	2.2	2.5	6.8
17:1	tr	1.4	—	—	—	—	—	—
18:0	5.8	7.5	14.8	5.9	12.1	11.8	11.4	19.8
18:1	19.1	23.4	12.8	3.1	2.4	3.1	3.0	1.2
18:2	32.3	24.0	57.6	8.6	—	—	—	—
20:0	—	—	—	0.9	—	—	—	—
20:1	tr	—	tr	3.9	—	—	—	—
20:3	4.2	—	4.0	14.8	—	—	—	—
20:4	6.2	—	—	32.1	—	—	—	—
Unidentified	—	—	—	1.0	—	—	—	—
20:5	—	—	—	2.1	—	—	—	—
24:0	—	—	—	2.5	—	—	—	—
Unidentified C22?	—	—	—	1.4	—	—	—	—
Unidentified C22?	—	—	—	4.7	—	—	—	—

to the formation of "critical pairs" since the unsaturation and chain length have opposite effects as far as their partitioning properties between two phases are concerned. Thus, a fractionation by combination of argentation TLC, followed by partition chromatography (normal or reversed-phase) will give the maximum number of fractions as indicated by ARVIDSON[3]. Another advantage of argentation adsorption TLC over partition chromatography is that the former technique requires less time and can fractionate larger amounts.

In a recent communication, OWENS[14] mentioned that slight structural differences between diacyl phosphatides and analogous alkenyl acyl phosphatides militated against the chromatographic separation of intact plasmalogens. However, the present technique seemed to overcome this problem. This technique resulted also in recovery of all the components of the original mixture in their native form.

Work is in progress to obtain different molecular species of phosphatidal cholines, -ethanolamines and -serines by a combination of argentation adsorption and partition TLC to investigate their role in blood coagulation.

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CHROM. 3349

RÉSOLUTION DES OLIGONUCLÉOTIDES DU DNA APURINIQUE MARQUÉ AU PHOSPHORE [^{32}P] PAR CHROMATOGRAPHIE BIDIMENSIONNELLE SUR COUCHES MINCES DE CELLULOSE

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SUMMARY

Two-dimensional chromatographic resolution of [^{32}P] apurinic DNA oligonucleotides, on cellulose thin layers

The resolution of a mixture of pyrimidine oligonucleotides obtained by acidic hydrolysis of a DNA previously labelled with [^{32}P] in tissue culture was performed by two dimensional chromatography on commercially available cellulose thin layers. Sixteen nucleotides were identified and their amounts determined. This method does not require the elimination of terminal phosphoryl groups. Combined with a previously described method involving chromatography on silica gel thin layers, it permits the evaluation of 20 oligonucleotides from sheep embryo kidney apurinic DNA.

Dans un travail précédent[1] nous avons décrit la résolution rapide d'un mélange d'oligonucléotides pyrimidiques, résultant de l'hydrolyse partielle du DNA, au moyen de la chromatographie bidimensionnelle sur couche mince de gel de silice.

Cette méthode de séparation exige l'élimination préalable des groupements phosphoryles terminaux sous l'action de la phosphatase alcaline d'*Escherichia coli*. Il en est ainsi également dans la méthode de BURTON ET PETERSEN[2–4] utilisant la chromatographie bidimensionnelle sur papier, ainsi que dans celle de HALL ET SINSHEIMER[5] qui réalisent la séparation des nucléotides isoplithes sur colonne de DEAE Sephadex. Un court article de PETERSEN ET REEVES[6] signale la possibilité d'obtenir une séparation d'isoplithes non déphosphorylés par chromatographie sur DEAE Séphadex en opérant l'élution au moyen d'un très grand volume d'un gradient de NaCl en présence d'urée 7 *M*. Cependant ici encore on n'obtient que des mélanges d'isoplithes et leur résolution ultérieure pose les mêmes problèmes liés à l'existence des phosphoryles terminaux.

La déphosphorylation est surtout gênante lorsqu'on opère sur du DNA marqué par [^{32}P], puisqu'on perd une fraction importante de la radioactivité et donc de la précision des dosages, et surtout parce que les mononucléotides échappent à l'analyse quantitative.

Nous avons donc cherché à éviter cette déphosphorylation préalable. Malgré

* Adresse actuelle: Institut Pasteur, Service des Virus, 25, Rue du Docteur Roux, Paris (15°).

bien des tentatives, les couches de silice ne nous ont pas permis d'aboutir parce qu'elles ne tolèrent pas les solvants aqueux. Nous avons par contre obtenu satisfaction en utilisant des couches minces de cellulose que l'on trouve depuis peu toutes préparées dans le commerce. Le présent travail décrit cette nouvelle méthode ainsi que les résultats que nous avons obtenus par la combinaison des deux techniques dans l'analyse du DNA de reins de foetus de mouton marqués par [^{32}P] en culture cellulaire.

MATÉRIEL ET MÉTHODES

Culture des cellules et marquage du DNA

Les reins de foetus de mouton prélevés à l'abattoir sont disséqués pour éliminer les parties fibreuses, hachés finement aux ciseaux et soumis à l'action d'une solution de trypsine Serpasteur à 0.20 p. 100 dans du PBS* de DULBECCO ET VOGT[7] suivant les méthodes habituelles.

Les cellules ainsi obtenues sont mises en suspension à 1 p. 1000 (v/v) dans un milieu de culture comprenant 5 g de TC Lactalbumin Hydrolysate Difco, 1 g de TC Yeastolate Difco et 50 ml de sérum de veau préalablement inactivé à 56° pendant 30 min, dans Q.S.P. 1 l de soluté isotonique tamponné de EARLE[8]. La suspension cellulaire est répartie dans deux boites pour culture cellulaire de 3.500 l de capacité, du modèle en usage au service des Virus de l'Institut Pasteur, à raison de 0.500 l par boite, et incubée à 37°.

Lorsque le 1/3 environ de la surface des boites est couvert par la nappe cellulaire (en moyenne après 48 h), le milieu est éliminé, la nappe lavée trois fois au PBS, et le milieu est remplacé par un volume égal de milieu frais de même composition sauf qu'il a été préparé avec du liquide de EARLE *sans phosphore* et que le sérum de veau qu'il contient a été dialysé au préalable 24 h contre du sérum salé isotonique pour en éliminer les phosphates. On ajoute à chaque boite 2 millicuries de [^{32}P] neutre sans entraîneur (phosphate de sodium en solution isotonique stérile, 2 mC/ml, pH 7, provenant du Département des Radioéléments du C.E.A., Saclay).

Les cellules sont soumises à l'incubation à 37° jusqu'à ce que la nappe cellulaire soit continue (2 à 3 jours).

Le milieu est alors éliminé et la nappe cellulaire est lavée à plusieurs reprises au PBS. On introduit dans chaque flacon de culture 50 ml de solution à 0.02 p. 100 d'ethylène diamine tétraacétate (EDTA) dans du PBS privé de Ca^{2+} et Mg^{2+} et on reporte à 37° jusqu'à ce que la nappe cellulaire soit détachée du verre. Les cellules sont recueillies par centrifugation à faible vitesse et lavées à plusieurs reprises dans du PBS.

Isolement du DNA

Le DNA marqué est isolé selon la méthode de MARMUR[9]. On ajoute avant l'extraction 8 mg de DNA de thymus** de veau non marqué comme entraîneur.

Préparation du DNA apurinique

Nous avons suivi la méthode de BURTON ET PETERSEN[2–4]. Après hydrolyse du DNA par l'acide formique et la diphénylamine, les purines sont éliminées sur une colonne de Dowex 50 H^+ et la désalification des nucléotides pyrimidiques s'effectue par traitement sur colonne de charbon, selon les indications précédemment publiées[1].

* PBS = "Phosphate buffered saline".
** DNA de thymus Worthington.

Chromatographie bidimensionnelle sur couches minces de cellulose

Nous avons utilisé des couches minces de cellulose du commerce prêtes à l'emploi, de dimensions 20 × 20 cm*.

On applique le solution de DNA apurinique avec un capillaire près d'un coin à 3 cm des côtés de la plaque. 500 μg sont nécessaires pour obtenir une très bonne séparation des divers nucléotides tout en gardant la possibilité de les localiser au moyen d'une lampe à rayonnement ultra violet.

La chromatographie est pratiquée dans la cuve sandwich Kodak.

Les solvants utilisés sont:

Dans le premier sens: Propane-2-ol–acide chlorhydrique 0.2 *N* (50:50, v/v). Le temps de développement est d'environ 8 h.

Dans le deuxième sens: Sulfate d'ammonium saturé–propane-2-ol–eau distillée (79:2:19, v/v/v). 3 à 4 h permettent la migration du solvant sur toute la surface de la couche.

(Ce second solvant a été recommandé par PATAKI[10].)

Chromatographie bidimensionnelle sur couches minces de gel de silice

Cette méthode exige la déphosphorylation préalable des nucléotides terminaux et a été décrite dans un travail précédent[1].

Lecture des radiochromatogrammes

Les taches, repérées sous une lampe à rayonnement ultraviolet *Chromatolux* Pleuger munie d'un filtre à 260 mμ, sont cernées par un trait de crayon. On les limite à l'aide d'un diaphragme fait de 4 pièces rectangulaires en acier inoxydable de 60 × 30 × 5 mm. Un compteur G.M. Philips 13.514, dont la fenêtre a un diamètre de 35 mm est posé sur l'ouverture ainsi ménagée et est raccordé à une echelle de comptage Philips PW 4.035. Ce dispositif très simple donne toute satisfaction et laisse les chromatogrammes intacts.

Autoradiographie des chromatogrammes

Elle est utile pour localiser très exactement les taches, plus particulièrement les taches faibles peu visibles en lumière U.V. en l'absence d'indicateur de fluorescence.

On met en contact dans une cassette la couche chromatographique et un film radiographique** pendant un temps variable selon la radioactivité des taches et l'intensité recherchée.

En pratique, le temps de pose doit être calculé pour fournir environ 10^6 coups par tache. Il peut être nécessaire d'utiliser des temps différents pour les taches faibles et pour les taches fortes.

Identification des taches

Les taches observées sous la lampe à rayonnement ultraviolet sont entourées d'un trait de crayon, découpées et éluées dans une solution d'acide chlorhydrique 0.03 *N* pendant 15 h à température du laboratoire. On centrifuge 10 min à 3,500 tours/min.

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* Couches de cellulose Kodak No. 6.064 (sans indicateur de fluorescence).

** Films radiographiques Regulix Kodak.

La densité optique du surnageant est mesurée à 260 mμ et à 280 mμ au spectrophotomètre Zeiss PMQ II.

Calculs

Les activités mesurées pour chaque oligonucléotide sont, après décomptage du bruit de fond, transformées en quantités proportionnelles aux nombres de moles de pyrimidines par 100 atome-gramme de phosphore en les multipliant par $n/(n+1)$ pour les expériences sans déphosphorylation et par $n/(n-1)$ pour celles précédées de déphosphorylation, n étant le nombre de mononucléotides de l'oligonucléotide considéré. Cette correction est négligée pour les taches non identifiées ou d'identification incertaine, l'erreur ainsi commise étant négligeable, d'une part parce que $n \geqslant 5$ et d'autre part parce que la radioactivité totale de ces taches est faible.

On fait la somme des activités ainsi corrigées pour toutes les taches visibles sur les autoradiogrammes, y compris la tache de départ et on effectue les pourcentages par rapport à cette somme égalée à 50 p. 100, puisqu'il manque les purines.

Toutefois pour établir les pourcentages des expériences sur gel de silice on a identifié les valeurs de T_2 avec celles obtenues par la séparation sur couche de cellulose et calculé les pourcentages de tous les nucléotides par rapport à T_2. Les résultats sont du reste identiques si on prend pour base la somme des taches identifiées à la fois dans les deux méthodes.

RÉSULTATS

Identification

Les résultats sont présentés dans le Tableau I où les rapports E 280/E 260 à pH

TABLEAU I

ANALYSE SPECTROPHOTOMÉTRIQUE DE DIVERS OLIGONUCLÉOTIDES PYRIMIDIQUES

Taches identifiées	$\frac{E\ 280}{E\ 260}$ *à pH 2.5*	*Mélanges étalons*
$T_4p_5 + T_5p_6 + \cdots$ *	0.69	0.69
T_3p_4	0.71	0.69
T_2p_3	0.69	0.69
Tp_2	0.71	0.69
$CT_4p_6 + CT_5p_7 \cdots$ **	0.89	0.88
CT_3p_5	0.97	0.95
CT_2p_4	1.04	1.07
CTp_3	1.23	1.25
Cp_2	2.10	2.10
$C_2T_3p_6 \cdots$	1.11	1.20
$C_2T_2p_5$	1.20	1.25
C_2Tp_4	1.42	1.46
C_2p_3	1.89	2.10
$C_3T_2p_6 + C_3T_3p_7$	1.21	1.25
C_3Tp_5	1.57	1.65
C_3p_4	1.93	2.10
C_4p_5 ***		2.10
C_4Tp_6 ***		1.75

* Cette tache est un mélange de T_4 et des oligonucléotides supérieurs T_n ($n > 4$).
** Cette tache est un mélange de CT_4 et des oligonucléotides supérieurs CT_n ($n > 4$).
*** Identification seulement probable.

2.5 des divers oligonucléotides sont comparés à ceux de mélanges étalons élués dans les mêmes conditions.

Les autoradiographies représentées sur les Fig. 1 et 2 permettent de comparer la séparation sur couche mince de cellulose à celle précédemment décrite[1] sur gel de silice. Dans celle-ci les mononucléotides ne sont pas visibles. Dans la première leur séparation et leur mise en évidence sont au contraire excellentes. En outre les nucléotides cytidyliques plus longs que C_2, situés dans une région étroite et compacte dans la méthode sur silice sont très bien isolés jusqu'à C_4 sur cellulose. Il en est de même pour C_2T, C_3T et même C_4T. Parmi les thymidyliques, T_2 et T_3 donnent les mêmes résultats

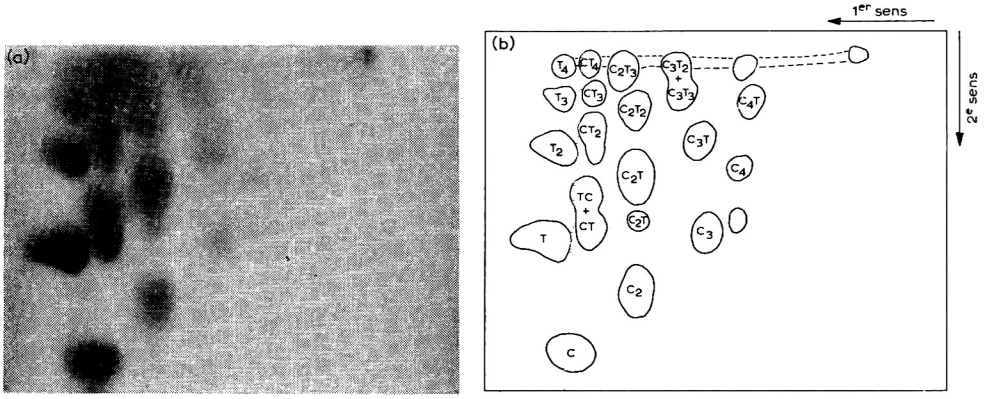


Fig. 1. Autoradiographie d'une chromatographie bidimensionnelle sur couche de cellulose des oligonucléotides pyrimidiques provenant de l'hydrolyse acide du DNA de mouton marqué par du phosphore [^{32}P]. Solvants: (1) Propane-2-ol–acide chlorhydrique 0.2 *N* (50:50, v/v); (2) Propane-2-ol–sulfate d'ammonium saturé–eau (2:79:19, v/v/v). Les taches sur la ligne horizontale supérieure sont en réalité des mélanges de nucléotides.

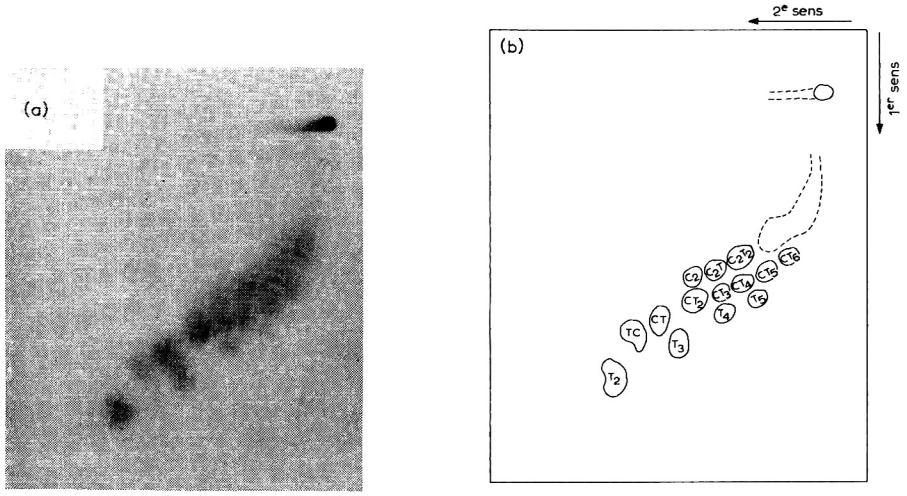


Fig. 2. Autoradiographie d'une chromatographie bidimensionnelle sur couche de gel de silice des oligonucléotides pyrimidiques provenant de l'hydrolyse acide du DNA de mouton marqué par du phosphore [^{32}P]. Solvants: (1) Propane-2-ol–eau (75:25, v/v); (2) *N* butanol–acide acétique–ammoniaque à 5%–eau (90:30:20:20, v/v/v/v).

dans les deux méthodes. Par contre, T_4 et T_5 très bien séparés sur silice (qui à la rigueur donnerait même T_6) sont mélangés sur cellulose avec les T_n supérieurs. De même CT_4, CT_5 et CT_6 bien visibles sur silice sont confondus avec les CT_n supérieurs sur cellulose. Enfin, C_2T_3 et C_3T_3 sont indiscernables des nucléotides supérieurs C_2T_n et C_3T_n.

On voit que les deux méthodes se complètent et se contrôlent mutuellement et qu'elles permettent l'isolement et l'identification de 20 oligonucléotides comportant notamment tous les mono-, di-, tri- et tétranucléotides et 3 pentanucléotides sur cinq.

Analyse quantitative

Le Tableau II donne la composition du DNA apurinique de rein de foetus de mouton marqué par [^{32}P] tel qu'il résulte de la combinaison des deux méthodes décrites plus haut. On peut y lire les pourcentages de 20 nucléotides représentant environ 84 p. 100 de l'ensemble des oligonucléotides pyrimidiques. Ces pourcentages ont été comparés à ceux que l'on pourrait attendre d'une distribution au hasard.

Les résultats obtenus sur gel de silice représentent la moyenne de six expériences, ceux obtenus sur cellulose la moyenne de trois expériences. Les déviations standards ont été inscrites à côté de chaque valeur. Dans ces conditions le test t de Student

TABLEAU II

COMPOSITION DU DNA APURINIQUE DE REIN DE FOETUS DE MOUTON
(Moles de pyrimidines pour 100 atomes-grammes de phosphore du DNA)

Nucléotides	*Sur gel de silice*	*Sur cellulose*	*Distribution au hasard**
C	—	4.68 ± 0.04	5.35
T	—	6.56 ± 0.12	7.07
T_2	3.33 ± 0.14	3.33 ± 0.14	4.00
CT	3.92 ± 0.60 } 6.42	5.98 ± 0.23	3.02 } 6.04
TC	2.50 ± 0.32 }		3.02 }
C_2	1.92 ± 0.59	1.69 ± 0.17	2.29
T_3	2.05 ± 0.16	2.04 ± 0.02	1.70
CT_2	3.54 ± 0.80	3.23 ± 0.22	3.85
C_2T	2.64 ± 0.28	3.77 ± 0.06	2.92
C_3	—	0.74 ± 0.09	0.76
T_4	0.73 ± 0.09	—	0.64
CT_3	1.56 ± 0.33	2.29 ± 0.35	1.94
C_3T	—	1.18 ± 0.13	1.11
C_2T_2	2.59 ± 0.57	2.63 ± 0.22	2.20
C_4	—	0.42 ± 0.05	0.21
T_5	0.27 ± 0.10	—	0.23
CT_4	0.94 ± 0.14	—	0.86
C_4T	—	0.33 ± 0.02	0.39**
$C_2T_3 + C_2T_4 \cdots$	—	2.96 ± 0.5***	1.30
CT_5	0.92 ± 0.20	—	0.35
$C_3T_2 + C_3T_3 \cdots$	—	1.91 ± 0.14***	0.68**
CT_6	0.37 ± 0.15	—	0.14**

* Valeurs de BURTON ET PETERSEN[3-4] pour le DNA de thymus de veau dont la composition en bases n'est pas significativement différente de celle du DNA de mouton (CHARGAFF ET DAVIDSON[15]).

** Valeurs calculées par la formule:

$$C_pT_{n-p} = \frac{n}{4} \frac{n!}{p!(n-p)!} c^p t^{n-p}$$

c et t = fractions molaires de C et T dans le DNA.

*** C_2T_3 et C_3T_2 sont probablement des mélanges de nucléotides.

montre que seules les répartitions de C, T, T_2, C_2T, C_4 et CT_5 sont significativement différentes de la répartition la plus probable, en admettant un intervalle de confiance 95 p. 100. Pour C_2 la probabilité de distribution aléatoire est inférieure à 1 p. 100. C, T et T_2 sont en défaut par rapport à la répartition la plus probable alors que C_2T, C_4 et CT_5 sont en excès.

Les activités totales observées sur une plaque ont été dans la méthode avec déphosphorylation de 50,000 à 100,000 c.p.m. et dans la méthode sans déphosphorylation de 100,000 à 150,000 c.p.m.

Les taches les plus radioactives ont fourni plus de 10^5 c.p.m. et les plus faibles environ 10^2 c.p.m.

DISCUSSION

Dans cet article essentiellement méthodologique nous ne ferons pas l'étude critique des résultats obtenus, qui seront l'objet d'une analyse statistique complète dans un travail ultérieur. Notons seulement que le DNA de rein de foetus d'agneau comme celui de thymus de veau étudié par BURTON ET PETERSEN[2-4] et par SPENCER ET CHARGAFF[11-13] montre une tendance à former des séquences pyrimidiques, et par suite puriques, plus longues que celles attendues dans le cas d'une distribution strictement aléatoire. En outre, le rapport CT/TC toujours significativement supérieur à 1 (moyenne 1.59; déviation standard $\pm$ 0.08) indique avec évidence une répartition non aléatoire. Il semble pourtant à première vue que la distribution des nucléotides dans ce DNA soit, dans l'ensemble, plus proche de la répartition la plus probable que celle de thymus de veau. Un nombre plus grand d'expériences est nécessaire pour l'affirmer et il n'est pas douteux que l'on puisse notablement réduire les fluctuations expérimentales.

A bien des égards le marquage par [^{32}P] qui évite l'élution nous parait avantageux. Les composés cytidyliques notamment s'éluent plus difficilement que les thymidyliques de la cellulose. L'élution est par contre rapide et quantitative à partir des plaques de silice.

Mais le grand avantage de l'emploi de [^{32}P] est le marquage du DNA des virus qui se multiplient dans le système cellulaire intéressé. Nous avons notamment appliqué cette méthode à l'analyse du DNA du virus de l'herpès en culture cellulaire de reins de foetus de mouton (sous presse).

Une variante intéressante consiste à marquer le DNA après chromatographie directement sur la plaque par activation neutronique (RUSHIZKY ET MILLER[14], MURRAY ET OFFORD[16]). De très petites quantités de DNA peuvent alors être utilisées mais on peut évidemment pas ajouter d'entraîneur.

RÉSUMÉ

La résolution d'un mélange d'oligonucléotides pyrimidiques provenant de l'hydrolyse acide du DNA marqué par [^{32}P] en culture cellulaire a été réalisée par chromatographie bi-dimensionnelle sur couches minces de cellulose. Seize nucléotides ont été identifiés et dosés. Cette méthode n'exige pas l'élimination préalable des groupements phosphoryles terminaux. Associée à une méthode précédemment publiée, utilisant des couches de gel de silice, elle a permis le dosage de 20 oligonucléotides pyrimidiques du DNA de rein de foetus de mouton.

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CHROM. 3343

CHROMATOGRAPHIC SEPARATION OF THE COMPONENTS OF THE GENTAMICIN COMPLEX

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SUMMARY

By means of paper and thin-layer chromatography using the lower phase of a solvent mixture consisting of chloroform–methanol–17 % ammonium hydroxide (2:1:1, v/v), the gentamicin antibiotic complex is shown to consist of three components, named C_1, C_{1a} and C_2. Methods are given for preparative separation of these antibiotics by use of cellulose and Chromosorb W chromatographic column procedures utilizing the solvent system described for paper and TLC.

INTRODUCTION

WEINSTEIN and co-workers[1] and ROSSELET and co-workers[2] had reported that the antibiotic gentamicin consisted of two closely related isomeric pseudo-oligosaccharides referred to as gentamicin C_1 and gentamicin C_2 which had essentially identical polarities. The two components were separable only after acetylation and partition chromatography on cellulose powder using a heptanol–pyridine–water system in a ratio of 6:4:3 and subsequent deacetylation of the separated N-acetyl derivatives by reflux for 70 h in 1.2 *N* sodium hydroxide.

Fig. 1 illustrates the chromatographic pattern of gentamicin N-acetyl C_1 and gentamicin N-acetyl C_2 on Whatman No. 1 paper developed in the heptanol–pyridine–water system. The compounds were detected by the starch-potassium iodide method

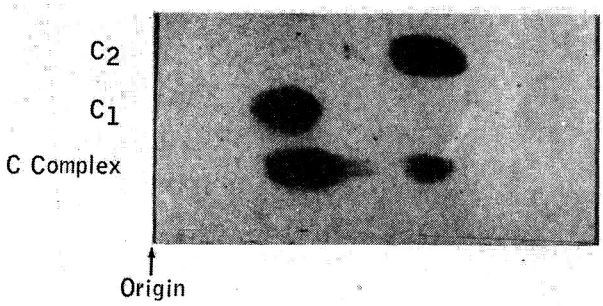


Fig. 1. Chromatogram of N-acetyl gentamicin C_1 and N-acetyl gentamicin C_2 on Whatman No. 1 paper using heptanol–pyridine–water (6:4:3). Compounds visualized by starch–potassium iodide spray.

of PAN AND DUTCHER[3], and the spot closest to the origin is C_1 and the faster moving one is C_2.

WEINSTEIN and co-workers[4] recently reported that the gentamicin complex produced by submerged fermentations of *Micromonospora purpurea* NRRL 2953 consists of three antibiotic components which have been designated as gentamicin C_1, gentamicin C_2, and gentamicin C_{1a} respectively. The data presented shows the biological properties of the individual components to be essentially the same.

This paper describes techniques for the separation of the three gentamicin components and it will also demonstrate that gentamicin C_1 referred to in the original paper was actually composed of a mixture of two antibiotics which are now designated as C_1 and C_{1a} and that C_2 was homogeneous.

EXPERIMENTAL AND RESULTS

Thin-layer chromatography

Separation of these antibiotic moieties has been accomplished with thin layer or paper chromatography using the lower phase of a solvent system composed of chloroform–methanol–17 % ammonium hydroxide in a ratio of 2:1:1. This system is a modification of the method reported by IKEKAWA *et al.*[5] in 1963.

Thin-layer chromatography of the gentamicin complex was carried out on plates coated with Silica Gel G. Plates were developed for 16–18 h.

Detection of spots was accomplished by two methods: (1) by the use of reagents; and (2) by a microbiological procedure. The first method was carried out by the usual technique of spraying the plates with 0.25 % ninhydrin in pyridine–acetone and consequent heating at 105° for several minutes. The zones appear as purple or blue spots against a white background. Also the starch–potassium iodide reagent spray can be used, with which the zones show up as dark blue spots against a white background.

Microbiological detection of the antibiotic zones was carried out by laying the plate face-down on a sheet of Whatman No. 1 paper in contact with agar seeded with *Staphylococcus aureus* ATCC 6538P. After 10 min the paper and plate were removed and after a suitable incubation period the zones of inhibition, representing the gentamicin components, were observed.

Paper chromatography

The method of separation utilizing the thin-layer plates is extremely slow compared to most thin-layer techniques. Increased speed of separation of the components was realized by use of paper chromatography utilizing the chloroform–methanol–ammonia system. Upper phase was placed in the bottom of the chromatographic jars several hours prior to use, and lower phase was used to develop the papergrams which were run descending on Whatman No. 1 paper in insulated chambers, for 5 h at 25°. The solvent was allowed to drip off the papers during development.

Detection of zones was accomplished by the previously described ninhydrin technique or by the bioautographic method. In the latter, the paper was placed directly on the agar, prepared as previously described, for a 10-min period prior to incubation.

The complex separated into three zones as seen by the microbiological method

illustrated in Fig. 2. These zones corresponded to those observed using the thin-layer chromatographic technique except that the paper chromatographic method was more sensitive and rapid. The zone of inhibition nearest to the front is identified as C_1, the zone following designated as C_2 and that closest to the origin, C_{1a}.

Fig. 3 shows a similar pattern with ninhydrin sprayed chromatograms. The R_F values of the ninhydrin spots are identical with those of the corresponding zones of inhibition.

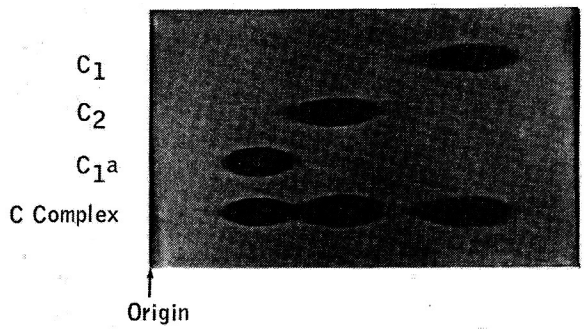


Fig. 2. Bioautograph of gentamicins. Chromatogram run on Whatman No. 1 paper using lower phase of chloroform–methanol–17% ammonia (2:1:1) and plated against *S. aureus* ATCC 6538P.

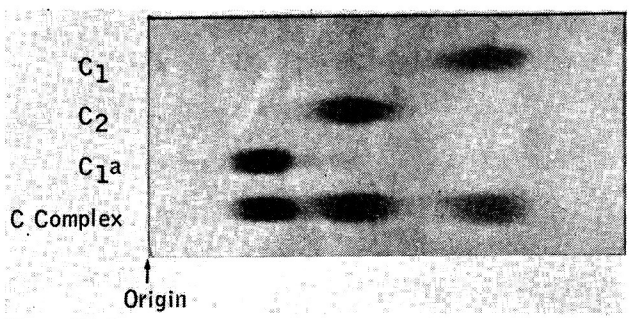


Fig. 3. Ninhydrin sprayed chromatogram of gentamicins run on Whatman No. 1 paper using lower phase of chloroform–methanol–17% ammonia (2:1:1).

Cellulose column chromatography

As a result of the success of the paper chromatographic separations of the gentamicin complex, it appeared feasible to attempt preparative column chromatography of the mixture using cellulose powder and the predescribed solvents.

Whatman No. 1 cellulose powder was packed in small segments in a column having an inner diameter of 2.4 cm, to a height of 30 cm. The upper phase of the solvent system comprised of chloroform–methanol–17% ammonia (2:1:1, v/v) was run through the column until a yellow band of impurities emerged and the column was allowed to drain.

A 200 mg quantity of gentamicin sulfate (equivalent to 129 mg of free base) was mixed with 2 g of cellulose powder, packed atop the cellulose in the column and wet with a small quantity of upper phase. Lower phase was allowed to run through the column at the rate of 2 ml per min; 16 ml fractions were collected every 8 min.

Aliquots of each fraction were spotted on filter paper and tested with ninhydrin

reagent to determine the presence or absence of antibiotic. All of the antibiotic (positive test) was located in fractions 12 through 49. Paper chromatography of each of the fractions determined that the three component peaks were located as seen in Table I. Like fractions, converted to their free bases on the column, were pooled and concentrated to dryness. Weights of peak fractions (Table I) indicated a 94 % recovery of starting material.

TABLE I

CHROMATOGRAPHIC SEPARATION OF THE GENTAMICIN COMPLEX USING A CELLULOSE COLUMN

Starting material: 200 mg of gentamicin complex sulfate (129 mg base equivalent). Total yield: 121.5 mg (94 %).

Component	*Fraction No.*	*Weight (mg) (base)*
C_1	12–19	58.2
C_2	23–33	50.8
C_{1a}	38–49	12.5

Paper chromatography of the bases and bioautography of the papergrams against *Staphylococcus aureus*, showed that the C_1 and C_2 components were free of impurities and that C_{1a} contained approximately 5 % of C_2. Rechromatography of the C_{1a} fraction using the described column resulted in isolation of this component free of the C_2 component.

Chromosorb chromatography

A major problem in separation of quantities of the gentamicin complex into its three components is scale-up of existing procedures. Major disadvantages of the above methods are that thin layer or paper chromatography can be used only for detection or preparation of minute quantities of material and cellulose powder chromatography is time-consuming because of the care that must be taken in packing the column.

An improved technique has been devised utilizing the solvent system previously described and Chromosorb W® an inert adsorbent. Although the mechanics of the separation are essentially similar to those described, this procedure is more easily carried out, does not require hand packing, can be scaled up readily and, because it uses a low ratio of adsorbent to gentamicin complex, can be used to separate large quantities of material.

Chromosorb W (60–100 mesh) manufactured by Johns-Manville, New York, N.Y. was slurried with the upper phase of chloroform–methanol–17 % ammonia (2:1:1,v/v) and filtered using suction on a Buchner funnel until excess solvent was removed. The Chromosorb was packed into a column having an I.D. of 3 cm to a height of 50 cm, in 5 cm segments (approximately 150 g as dry Chromosorb). An alternate method which has been used is to pour the slurry into the column and remove excess solvent by suction from the bottom of the column. One liter of lower phase was run through the column to wash the Chromosorb.

A 3 g portion of gentamicin base was dissolved in 10 ml of methanol, adsorbed to the smallest possible quantity of Chromosorb and dried under vacuum using a Rinco film evaporator. This mixture was packed atop the column and wet with a

small quantity of lower phase. The column was eluted with lower phase at the rate of 1 ml/min, collecting five fractions/h for a total of 231 fractions. Fractions were tested as described under cellulose column chromatography and the three major component peaks were located as shown in Table II, pooled, decolorized on IRA401S (OH^-) resin and concentrated to dryness. Paper chromatography of the bases and bioautography of the papergrams indicated that all components were free of microbiologically active impurities. Weights and bioassay data for the dried bases are presented in Table II.

TABLE II

CHROMATOGRAPHIC SEPARATION OF THE GENTAMICIN COMPLEX USING A CHROMOSORB W COLUMN
Starting material: 3 g gentamicin complex base.

Component	*Fraction Nos.*	*Purified fractions (weight, g)*	*Antibiotic activity* (μg/mg)*
C_1	35–60	1.01	833
C_2	95–140	0.74	1050
C_{1a}	165–231	0.31	1050

* Average of four assays against complex standard.

DISCUSSION

Thin-layer and paper chromatography were used to show that the gentamicin complex is made up of three components and not two as was previously reported. These components can now be separated by column chromatography on cellulose or Chromosorb using the chloroform–methanol–ammonia system.

Acetylation of each of the three gentamicin components and chromatography of the derivatives indicated that the N-acetyl gentamicins C_1 and C_{1a} moved as a single spot and differed from the R_F of the N-acetyl C_2 zone as illustrated in Fig. 4. This is in direct agreement with the original assumption that gentamicin was a mixture of only two components and would explain why three acetylated zones did not appear. The chromatographic behavior of the gentamicin components as evidenced by the

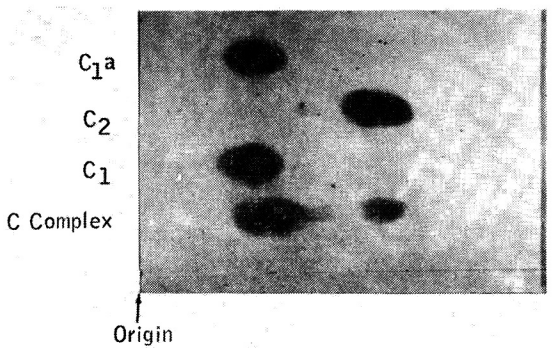


Fig. 4. Chromatogram of N-acetyl gentamicins showing identity of C_1 and C_{1a}. Whatman No. 1 paper; heptanol–pyridine–water (6:4:3). Spots visualized by starch–potassium iodide spray.

data presented in this paper, appears to confirm earlier views indicating very close structural relationship between these compounds.

This paper was presented in part at the 154th meeting of the American Chemical Society, Chicago, Illinois, September 12, 1967.

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CHROM. 3336

A NEW METHOD OF ISOLATION OF NATURAL FLAVINS USING PHENOL-TYPE RESINS

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SUMMARY

It was shown that riboflavin (as lumiflavin and lumichrome) is sorbed by all resins with free phenolic groups, *e.g.* phenolsulphonic and phenolcarboxylic ion exchangers or a simple resin of resorcinol and formaldehyde. FMN and FAD, in aqueous solutions, passed through these columns without sorption, but can be retained in salt solutions, especially by a resorcinol resin. Flavins can be eluted from phenol resins by a mixture of water and acetone, propanol or dioxane and saturated aqueous solutions of urea with ethanol or cellosolve. The ability of phenol resins to form complexes with flavins can be used both for analytical and preparative purposes.

The ability of phenol and its derivatives to form complexes with flavins has been demonstrated[1–4] and recently accounted for as charge-transfer complexes between electron deficient flavins and electron rich phenols. We have shown that such or similar complexes are also formed in case of flavins and resins with free phenolic groups (containing phenol or its derivatives). This fact was found to be very useful for the isolation of flavins from highly contaminated and strongly diluted extracts, both for analytical and preparative purposes.

EXPERIMENTAL

Materials and apparatus

Riboflavin (Merck), chromatographically pure.

Riboflavin 5′-phosphate (Sigma), purified on phenolsulphonic resin K-28 before use (riboflavin is sorbed by the resin, FMN* passed through the column with water).

Flavin-adenine dinucleotide (Fluka) on chromatograms shows two additional greenish-yellow fluorescent spots of FMN and probably YAGI's "fourth flavin compound"[5]; used without further purification.

Lumiflavin and lumichrome, prepared from riboflavin by prolonged photolysis of riboflavin solutions in 0.1 *N* NaOH, extracted with chloroform and dried in vacuum. Both were separated on columns of Whatman cellulose powder: lumiflavin

* Abbreviations used: Rfl = riboflavin; FMN = riboflavin 5′-phosphate; FAD = flavin-adenine dinucleotide; Lfl = lumiflavin; Lch = lumichrome.

by elution with water saturated with isoamyl alcohol and lumichrome by means of methanol.

Ion exchangers. K-28*, phenolsulphonic acid type and K-16*, phenolcarboxylic acid type. Analogous results were obtained when commercially available phenolsulphonic exchangers such as ZeoKarb 215, Wofatit F, P and KS, Lewatit PN and KSN, Staionit FN and F-extra, resin MSF or phenolcarboxylic resins such as Lewatit CNS and ZeoKarb 216 were tested.

Resin R-15, resorcinol type, was prepared in the following way: 15 g of sodium hydroxide and 33 g of resorcinol were dissolved in 60 ml of water and placed in an ice bath. To the resorcinol solution 45 ml of 40 % aqueous formaldehyde was added (in small portions to prevent the temperature rising above 50°). A further 30 ml of water was next added to the reaction mixture which was then placed on a water bath at 50°. After 25–30 min the mixture begins to form long threads when a glass rod is drawn through it (the beginning of gelatinisation) and from this time must be kept still at 50° for 15 min. This resin was then pressed through a sieve (1 mm mesh diameter) under a stream of tap water. After an hour the resin was pressed through another sieve (0.5 mm mesh diameter) and then washed successively with water, 3 *N* HCl, water, 0.5 *N* NaOH, water, 3 *N* HCl, water, acetone–water mixture (1:1, v/v), 0.5 *N* NaOH, water, 3 *N* HCl and water. Washing with the acetone–water mixture and NaOH solutions must be carried out till the effluents became non-fluorescent and non-coloured respectively.

The fluorimeter used was constructed in the laboratory and has great sensitivity and selectivity[6].

Procedure

Aqueous solutions containing 1–2 μg of flavins (as riboflavin) in one milliliter were passed through columns of resin (resin bed 1.0–1.2 cm in diameter and 3.5–7 cm in height; flow rate 1 ml/2–3 min); 2.5 or 5 ml fractions were collected.

The effectiveness of sorption was estimated on the basis of the resistance of the flavins to elution with pure water or aqueous buffer solutions. On the other hand, desorption was estimated as depending on the quantities of flavins eluted and the volumes of solutions used for this purpose.

The capacity of the resins in relation to riboflavin was established by passing solutions containing 15 μg of riboflavin/1 ml till the break-through point was reached.

Quantitative determinations were carried out based on the intensity of the yellow-green fluorescence of eluates. FAD was estimated according to CERLETTI's method[7] which depends on the increase of fluorescence after hydrolysis of FAD to FMN or Rfl. Qualitative analysis of flavins was accomplished by paper chromatography (paper Whatman No. 1, developing mixture: butanol–glacial acetic acid–water, 4:1:5; descending technique). Flavins were identified by the colour of fluorescence of the spots and their R_F values (Rfl = 0.30; FMN = 0.09; FAD = 0.03; Lfl = 0.42; Lch = 0.70).

As the flavins are highly photolabile, all operations have been carried out in a dark laboratory.

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* Ion exchangers K-28 and K-16 were prepared and kindly donated by Doc. Dr. H. WITKOWSKI of the Chair of General Chemistry of University A. M. in Poznań.

RESULTS AND DISCUSSION

Sorption of riboflavin, lumiflavin and lumichrome

All types of phenolic resins secured full, quantitative sorption of Rfl, Lfl and Lch from aqueous solutions, dilute acids (up to 0.5 *N*) and salt solutions (up to 2 *N*) over a pH range 1–8.

The capacity of resins as determined by the dynamic method (break-through point) was found to be practically unaffected by the resin form (H^+, Li^+, Na^+, K^+, NH_4^+) and closely related to the number of free phenolic —OH groups and was also probably affected by the presence of other functional groups (—COOH, —SO_3H) (see Table I and Fig. 1).

TABLE I

THE BREAK-THROUGH CAPACITY OF PHENOL TYPE RESINS* IN RELATION TO RIBOFLAVIN AND Na^+ (AQUEOUS SOLUTIONS)

Type of resin	*Rfl*	*Na+*
K-28: phenolsulphonic acid type	3×10^{-3}	2.1
K-16: phenolcarboxylic acid type	8×10^{-3}	1.0
R-15: resorcinol type	23×10^{-3}	0.27

* In mequiv. per g of air-dried resin.

Riboflavin, as well as lumiflavin and lumichrome, adsorbed on phenolic resins could not be eluted with acids (up to 2.5 *N*) or neutral salt solutions (up to 3 *N*); higher concentrations gave traces of riboflavin in the effluents. Better elution was obtained with solutions of alkaline character such as NaOH, KOH, NH_4OH, Na_2CO_3, $Na_2B_4O_7$, but here again the riboflavin content in the effluents was low and the elution was not quantitative[8]. Because of serious lability of flavins in alkaline media (especially photolability) such solutions could not be used for elution purposes. The most effective eluants appeared to be aqueous solutions of certain organic compounds[8,9].

From a series of mixtures that were examined the best results were obtained when mixtures of water with acetone, propanol or *p*-dioxane and saturated aqueous solutions of urea with ethanol or cellosolve were used (see Fig. 2).

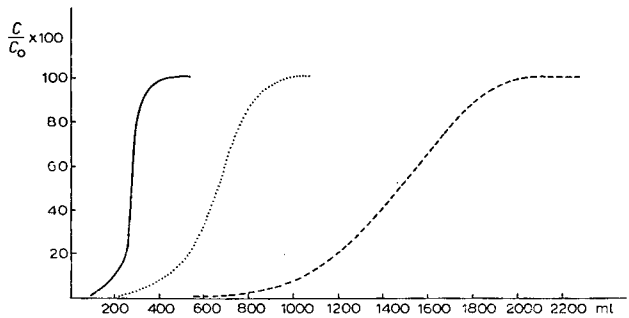


Fig. 1. The break-through curves (isoplanes) for aqueous solutions of riboflavin on phenol-formaldehyde resins: K-28, phenolsulphonic acid type (———); K-16, phenolcarboxylic acid type (· · ·); R-15, resorcinol type (– – –).

Sorption of FMN and FAD

Flavin mononucleotide and flavin-adenine dinucleotide from aqueous solutions are only partially bound by resorcinol type resins and are not sorbed on sulphonic and carboxylic resins. Both nucleotides undergo sorption under conditions exerting an inhibitory effect on their phosphate groups and probably other fragments of the molecules. In the case of resins of the phenolsulphonic acid type sorption of FMN and FAD takes place from saturated solutions of salts (for instance KCl, KH_2PO_4) as well as from solutions of pH below 1. In the case of resins of the phenolcarboxylic acid type lower concentrations of salts and acids are necessary, in both cases however,

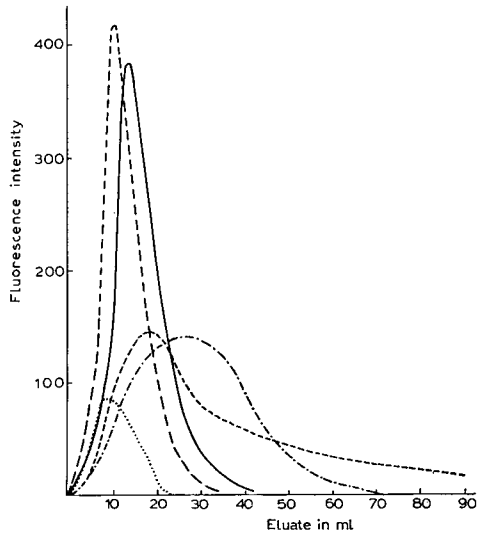


Fig. 2. Elution curves of riboflavin from K-28 resin for different solvents: acetone–water, *p*-dioxane–water, propanol–water (1:1) (– – –); cellosolve–water, ethanol–saturated aqueous solution of urea (1:1) (———); ethanol–water (1:1) (—·—·—·); absolute ethanol (- - -); acetone (···).

significant hydrolysis of nucleotides occurs. Using resorcinol resins traces of FMN and FAD are retained from aqueous solutions; complete sorption is effected from 0.1–0.05 *N* solutions of well dissociated salts. But it was found that many salts, especially salts of K^+, Na^+, Cl^-, PO_4^{3-}, can cause in the course of sorption and/or elution slight destruction of the FMN to riboflavin. This was not observed when, for instance, $(NH_4)_2SO_4$ was used. Both nucleotides can be eluted from resorcinol resin by a 10% aqueous solution of acetone.

Separation of riboflavin from FMN and FAD

The separation of riboflavin from flavin nucleotides was successful with all types of phenolic resins using two-step elution. From phenolsulphonic and phenolcarboxylic type resins FMN and FAD can be eluted with water and pure riboflavin in the next step when an acetone–water (1:1, v/v) mixture is poured through the column. In the case of a resorcinol type resin the nucleotides can be eluted with a 5–10% aqueous solution of acetone and riboflavin with a 50% acetone–water mixture.

Attempts were undertaken to separate all flavins sorbed on the resins. On phenolsulphonic and phenolcarboxylic acid type resins we could not separate FMN

from FAD. Using a gradient elution technique in the system: 0.1 *N* KCl–water–acetone, only partial separation of FMN from FAD was obtained; this is due to the tendency of FAD to remain on the resin as long as FMN (see Fig. 3).

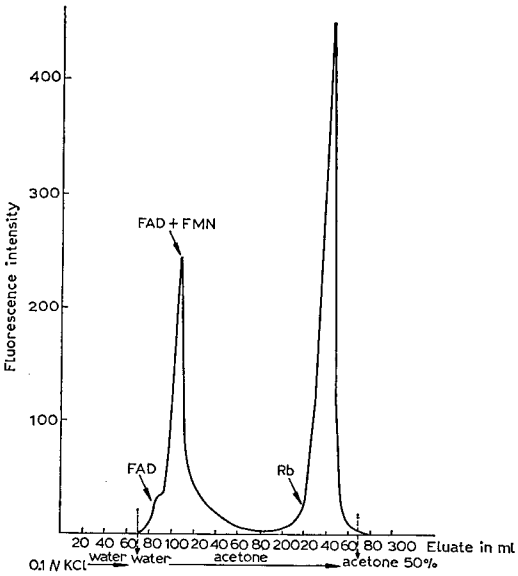


Fig. 3. Gradient elution on the R-15 resin, on which were sorbed equal quantities (25 μg) of riboflavin, flavin mononucleotide and flavin-adenine dinucleotide. Solvent system: aqueous 0.1 *N* KCl, water, acetone.

Use of phenol resins for estimation of flavins

The use of phenol resins allows the:

isolation of riboflavin from highly contaminated and strongly diluted extracts,

concentration of riboflavin solutions,

determination of individual forms of flavins occurring in natural products.

For the determination of flavins phenolsulphonic[9,10] as well as resorcinol type resins can be used.

For the determination of total flavins the crude extract obtained from samples of the analysed product (hydrolysis in 0.1 *N* H_2SO_4, neutralization by NH_4OH, enzymatic hydrolysis) was passed through the column of the resorcinol resin. The column was washed with 0.1 *N* $(NH_4)_2SO_4$ to remove the non-flavin compounds and then riboflavin and FMN were eluted with the 50 % acetone–water mixture (or the urea–water–ethanol mixture). Using the acetone–water mixture the fluorescence of the eluate was measured after acetone was removed under reduced pressure (acetone strongly enhances the fluorescence intensity of flavins[11]).

When Rfl, FMN and FAD were to be estimated, the crude extracts (obtained in 0.1 *N* $(NH_4)_2SO_4$ at 80°) were passed through the resorcinol resin. FMN and FAD were eluted with the 10 % acetone–water mixture and determined according to the method of CERLETTI[7]. Riboflavin was eluted from the column using the 50 % acetone–water mixture.

The efficacy of removal of the quenching substances and fluorescent non-flavin

compounds from extracts is of a high degree. The process of determination has been reduced, in this method, to few simple operations; the results obtained are accurate and of good reproducibility[10].

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CHROM. 3338

FAST SEPARATION OF Ti(IV), As(III), W(VI), Au(III) FROM NUMEROUS METAL IONS AND OF Fe–Al–Ti BY PAPER CHROMATOGRAPHY

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SUMMARY

Ti^{4+} has been successfully separated from its binary mixtures with 32 cations using formic acid–hydrochloric acid–acetone (3:5:2). The cations include Cu^{2+}, Al^{3+}, Fe^{3+}, V^{4+}, Mo^{6+}, W^{6+}, Zr^{4+}, Th^{4+}, Ni^{2+} and Co^{2+}. Ti^{4+} stays at the point of application while other ions have R_F values greater than 0.5. The separation is successful in the presence of acetate, tartrate, formate and citrate. Au^{3+} and W^{6+} have been separated from numerous metal ions using 70 % nitric acid–*n*-butanol (6:4). W^{6+} stays at the point of application while Au^{3+} goes to the solvent front. As^{3+} has been separated from its binary mixtures with 25 cations using pure formic acid as the eluent. The ions studied include Sb^{3+}, Cu^{2+}, and Sn^{2+}. The presence of common anions does not affect this separation. W^{6+} has been separated from its binary mixtures with 35 ions. The ions include Mo^{6+}, Ti^{4+}, Th^{4+}, UO_2^{2+}, V^{4+}, Co^{2+}, Ni^{2+} and Pd^{2+}. Separation of Fe^{3+}–Al^{3+}–Ti^{4+} has been achieved using formic acid–hydrochloric acid–acetone (3:4:3). The separation is successful in the presence of common anions and no special method for the preparation of the sample is necessary.

Paper chromatography offers a simple solution to many analytical problems. The aim is to achieve fast, reliable and selective separations of mixtures which are otherwise difficult to separate. These separations should be possible in the presence of anions and cations which are likely to be encountered in practical work. It should also be possible to separate one ion in trace quantities in the presence of large quantities of other ions. Some separations of this nature have been described earlier. Ti(IV) has been separated from iron, tin, arsenic, beryllium, thorium, cobalt and antimony in 22 h by ELBEIH AND ABOU-ELNAGA[1]. In this separation tartrate, citrate, oxalate, formate, acetate, and ammonium radicals interfere. MAGEE AND HEADRIDGE[2] have also separated 100 γ Ti(IV) from zirconium, aluminum, indium, zinc, thallium, gallium in 16 h. Fe–Al–Ti was separated by LACOURT[3] in only 240–330 min. In her separation the sample must be prepared in a definite manner and a special shape of paper is necessary, if a small quantity of one cation is to be separated from a large quantity of another cation. Au(III) has been separated from numerous metal ions by

WELLS[4]. In this separation sodium chlorate has to be added to the solvent system to prevent double spot formation. As(III)[5] has been separated by paper chromatography from numerous metal ions but the separation takes 20 h. As far as we are aware no separation of tungsten from numerous metal ions has been reported until now. A systematic study of different solvent systems was therefore made and the results achieved are summarized in this report.

EXPERIMENTAL

Apparatus

Development was performed in 20 × 5 cm glass jars, using the ascending method on 14.5 × 3 cm Whatman No. 1 paper strips.

Reagents

Only reagent grade chemicals were used.

Cation solutions

All test solutions were 0.1 *M* unless otherwise stated. Zirconium and thorium nitrates and thorium chloride were made in 0.1 *M* nitric acid and 0.1 *M* HCl, respectively. Zirconium oxychloride was dissolved in 3.5 *N* HCl. As(III) was prepared by dissolving arsenious oxide in 0.1 *M* nitric acid. Sn(II), Sn(IV), Sb(III) and Ti(IV) were dissolved as chlorides in 4 *M* hydrochloric acid. The rest of the cations were dissolved as nitrates in 0.1 *M* nitric acid. Molybdenum oxide was dissolved in dilute NaOH and neutralized with dilute HCl. Sodium tungstate was dissolved in distilled water.

Detectors

The following detectors were used for the detection of the cations noted against each:

Yellow ammonium sulphide. Ag(I), Hg(I), Hg(II), Bi(III), Pb(II).

1 % aluminon in 1 % ammonium acetate: Cr(III), Al(III), Ca(II), Sr(II), Ba(II), Mg(II).

10 % aqueous solution of potassium iodide. Tl(I), Tl(III).

0.1 % alcoholic solution of alizarin. Rare earths.

2 % dithizone in chloroform. As(III), Sb(III), Zn(II), Cd(II).

1–2 % aqueous solution of potassium ferrocyanide. Fe(III), Cu(II), UO_2^{2+}, V(IV).

Stannous chloride in 4 M HCl. Pt(IV), Pd(II).

Alcoholic solution of 2 % dimethyl-glyoxime. Ni(II), Co(II).

5 % aqueous chromatropic acid. Ti(IV).

Time of conditioning was always 10 min. All R_F values are the average of two replicates. Sample size was approximately 0.0014 ml. Valence states were those given in Table I. When an ion is chromatographed in two valence states only the lower valence state has been indicated. To indicate the compactness of zones, R_F values of leading and trailing edges are given. Solvent ascent was always 12.5 cm unless otherwise stated. Time of development and temperature are given in Table I when the solvent is mentioned first. In all later separations, the time of development and temperature were the same as earlier mentioned unless otherwise stated.

RESULTS

Separation of Ti(IV) from numerous metal ions

The following systems were found to be suitable for this separation:

(b) Formic acid–hydrochloric acid–acetone (3:3:4)

(c) Formic acid–hydrochloric acid–acetone (3:4:3)

(d) Formic acid–hydrochloric acid–acetone (3:5:2).

The R_F values of numerous metal ions in these systems are given in Table I. The actual separation of titanium from the more important ions was tried in all these systems (Table II). Since system (d) gave the best results for the separation of titanium

TABLE I

R_F VALUES OF CATIONS IN SOME IMPORTANT SOLVENTS

F = Formic acid; H = hydrochloric acid; A = acetone; N = 70% nitric acid; B = *n*-butanol.

Cations	*F*[a]	*F.H.A.*[b] (3:3:4)	*F.H.A.*[c] (3:4:3)	*F.H.A.*[d] (3:5:2)	*N.B.*[e] (6:4)
Ag(I)	0.00–0.60	0.00–0.68	0.40–0.77	0.60–0.81	0.00–0.33
Pb(II)	0.00–0.64	0.21–0.62	0.36–0.54	0.63–0.84	0.31–0.51
Hg(I)	0.00–0.16 0.61–0.70	0.00–0.72	0.00–0.80	0.00–0.74	0.00
Hg(II)	0.00–0.68	0.64–0.78	0.61–0.76	0.62–0.75	0.60–0.78
Cu(II)	0.58–0.74	0.56–0.68	0.62–0.75	0.64–0.78	0.42–0.52
Bi(III)	0.14–0.27	0.44–0.62	0.50–0.66	0.54–0.73	0.51–0.66
Cd(II)	0.26–0.71	0.64–0.76	0.74–0.85	0.67–0.78	0.40–0.51
As(III)	0.00–0.13	0.79–0.90	0.78–0.90	0.78–0.90	0.50–0.66
Sb(III)	0.39–0.56	0.00, 0.58–1.0	0.53–0.66	0.61–0.80	0.75–0.91
Sn(II)	0.46–0.55	0.68–0.75	0.55–0.72	0.54–0.68	0.70–1.0
Sn(IV)	0.46–0.59	0.61–0.78	0.55–0.79	0.53–0.70	0.60–0.91
Pd(II)	0.00–0.23	0.43–0.60	0.36–0.54	0.38–0.62	0.34–0.77
Tl(I)	0.53–0.72	0.00–0.59	0.00–0.59	0.28–0.80	0.43–0.59
Rb(I)	0.68–0.94	0.50–0.68	0.62–0.79	0.68–0.81	0.48–0.65
Fe(II)	0.69–0.89	0.75–1.0	0.80–0.92	0.72–0.87	0.29–0.48
Fe(III)	0.73–0.87	0.80–1.0	0.80–0.92	0.72–0.87	0.31–0.53
Cr(III)	0.73–0.92	0.21–0.45	0.44–0.67	0.60–0.75	0.28–0.51
Al(III)	0.68–0.88	0.21–0.45	0.37–0.53	0.57–0.76	0.30–0.52
Ni(II)	0.68–0.84	0.21–0.39	0.33–0.47	0.54–0.72	0.30–0.48
Co(II)	0.75–0.90	0.58–0.76	0.62–0.73	0.68–0.80	0.37–0.53
Mn(II)	0.69–0.89	0.46–0.66	0.54–0.68	0.59–0.74	0.34–0.50
Zn(II)	0.21–0.66	0.64–0.75	0.66–0.79	0.66–0.78	0.31–0.55
Ca(II)	0.71–0.83	0.23–0.38	0.28–0.44	0.41–0.54	0.35–0.54
Ba(II)	0.71–0.81	0.00–0.31	0.00–0.29	0.62–0.75	0.00–0.48
Sr(II)	0.73–0.85	0.23–0.32	0.20–0.31	0.33–0.50	0.27–0.38
Mg(II)	0.73–0.86	0.00–0.21	0.52–0.67	0.68–0.82	0.42–0.56
UO_2(II)	0.65–0.78	0.54–0.72	0.56–0.68	0.50–0.62	0.61–1.0
V(IV)	0.55–0.70	0.38–0.55	0.45–0.58	0.58–0.75	0.28–0.51
Zr(IV)	0.00–0.87	0.00–0.68	0.49–0.65	0.43–0.62	0.50–0.68
Th(IV)	0.00–0.80	0.16–0.44	0.31–0.48	0.40–0.65	0.47–0.71
Ce(III)	0.00–0.91	0.13–0.42	0.36–0.54	0.49–0.66	0.40–0.58
Ce(IV)	0.00–0.64	0.19–0.44	0.36–0.51	0.40–0.59	0.37–0.53
Ga(III)	0.39–0.63	0.90–1.0	0.80–1.0	0.76–1.0	0.35–0.67
In(III)	0.33–0.66	0.59–0.75	0.48–0.64	0.50–0.68	0.40–0.60
La(III)	0.00–0.90	0.22–0.43	0.38–0.53	0.48–0.65	0.35–0.57
Y(III)	0.68–0.90	0.21–0.51	0.50–0.63	0.59–0.77	0.33–0.54

(*continued on p. 225*)

TABLE I (*continued*)

Cations	*F*[a]	*F.H.A.*[b] (3:3:4)	*F.H.A.*[c] (3:4:3)	*F.H.A.*[d] (3:5:2)	*N.B.*[e] (6:4)
Ti(IV)	0.00–0.28	0.00–0.14	0.03–0.19	0.05–0.16	0.40–0.56
Mo(VI)	0.00–0.17	0.00	0.50–0.70	0.54–0.68	0.23–0.43
		0.62–0.80			
W(VI)	0.00	0.00	0.62–0.75	0.54–0.64	0.00
		0.64–0.75			
Pt(IV)	0.00–0.58	0.31–0.55	0.50–0.58	0.50–0.62	0.48–0.71
Se(IV)	0.00–0.26	0.84–0.96	0.00–0.12	0.38–1.0	0.66–0.89
			0.79–0.93		
Te(IV)	0.00–0.13	0.53–0.75	0.53–0.74	0.44–0.66	0.36–0.55
K(I)	0.73–0.84	0.48–0.66	0.58–0.77	0.70–0.89	
Cs(I)	0.88–1.0	0.54–0.68	0.67–0.81	0.77–1.0	0.60–0.75
NH_4(I)	0.76–0.87	0.62–0.75	0.62–0.73	0.60–0.84	0.46–0.63
Au(III)	0.23–0.57	0.00	0.00	0.00	0.94–1.0
		0.58–0.77	0.58–0.77	0.75–0.89	
Nb(V)		0.00	0.00	0.00	
		0.62–0.75	0.75–0.88	0.75–0.89	

[a] Separation of arsenic from numerous metal ions; time of development 1 h; temperature 31°.
[b] Separation of Ga–Al–Zn; time of development 1 h 30 min; temperature 29°.
[c] Separation of Fe–Al–Ti; time of development 2 h; temperature 30°.
[d] Separation of titanium from numerous metal ions; time of development 1 h; temperature 29°.
[e] Separation of gold and tungsten from numerous metal ions; time of development 2 h 30 min; temperature 22–23°.

in binary mixtures it was studied for the separation of titanium in the presence of impurities and anions (Tables III and IV). In binary mixtures Fe and Ni were used most owing to their obvious importance. Titanium was also successfully separated from Fe, Ni or Mo in the ratios of (1:100) and (100:1) (Table V).

Separation of mixtures containing titanium

Separation of numerous mixtures containing titanium was practical using hydrochloric acid–formic acid–acetone (5:3:2). The more important results are given below:

R_F values are given in parentheses and the mixtures separated were:

Ti (0.03–0.14), Pt (0.41–0.58), Fe (0.71–0.83)
Ti (0.06–0.25), Zr (0.41–0.63), Fe (0.73–0.89)
Ti (0.00–0.16), Th (0.39–0.66), Fe (0.61–0.83)
Ti (0.02–0.20), Sr (0.26–0.56), Mg (0.78–0.92)
Ti (0.06–0.18), UO_2^{2+} (0.43–0.63), Fe (0.69–0.89).

Specific chromatographic detection of titanium

Titanium is easily detected with chromotropic acid after prior elution with hydrochloric acid–formic acid–acetone (5:3:2). None of the cations and anions which interfere in the chromotropic acid test affect the detection. The results are given in Table VI.

TABLE II

SEPARATION OF BINARY MIXTURES OF TITANIUM

Mixtures	R_F *values*					
	F.H.A. (3:3:4)		*F.H.A.* (3:4:3)		*F.H.A.* (3:5:2)	
	Ti	*Other ion*	*Ti*	*Other ion*	*Ti*	*Other ion*
Ti–Cu	0.00–0.12	0.54–0.68	0.00–0.12	0.54–0.68	0.00–0.16	0.62–0.77
Ti–Bi	0.00–0.12	0.38–0.56	0.00–0.11	0.42–0.62	0.04–0.16	0.53–0.73
Ti–Cd	0.00–0.14	0.64–0.76	0.00–0.11	0.73–0.84	0.05–0.16	0.67–0.78
Ti–Hg	0.00–0.11	0.63–0.77	0.03–0.19	0.61–0.76	0.04–0.16	0.62–0.75
Ti–Fe(II)	0.00–0.11	0.74–1.0	0.02–0.20	0.80–0.92	0.04–0.16	0.72–0.37
Ti–Al	0.00–0.12	0.21–0.43	0.03–0.20	0.36–0.53	0.04–0.16	0.57–0.76
Ti–Fe	0.00–0.12	0.74–1.0	0.03–0.19	0.78–0.93	0.04–0.18	0.68–0.85
Ti–Ni	0.00–0.11	0.21–0.39	0.04–0.20	0.32–0.47	0.04–0.18	0.54–0.72
Ti–Co	0.00–0.11	0.56–0.76	0.04–0.19	0.62–0.72	0.04–0.18	0.68–0.80
Ti–Mn	0.00–0.12	0.43–0.65	0.03–0.20	0.53–0.67	0.04–0.16	0.59–0.74
Ti–Cr	0.00–0.18	0.44–0.67	0.03–0.19	0.20–0.45	0.07–0.19	0.56–0.68
Ti–Mg	0.00–0.12	0.64–0.77	0.03–0.20	0.51–0.66	0.04–0.18	0.68–0.82
Ti–UO_2^{2+}	0.00–0.12	0.54–0.72	0.02–0.19	0.54–0.68	0.03–0.20	0.50–0.62
Ti–V	0.00–0.12	0.37–0.55	0.02–0.19	0.44–0.57	0.04–0.18	0.58–0.75
Ti–Sn(II)	0.00–0.12	0.67–0.75	0.02–0.18	0.54–0.71	0.04–0.20	0.54–0.68
Ti–La			0.03–0.20	0.36–0.52	0.04–0.18	0.48–0.65
Ti–Th			0.02–0.20	0.30–0.48	0.04–0.18	0.40–0.65
Ti–Y			0.02–0.18	0.50–0.63	0.04–0.20	0.59–0.77
Ti–Ce(III)			0.02–0.20	0.34–0.54	0.04–0.20	0.49–0.66
Ti–Te	0.00–0.11	0.53–0.74	0.02–0.18	0.53–0.74	0.04–0.20	0.44–0.66
Ti–Ce			0.02–0.20	0.34–0.51	0.03–0.20	0.40–0.59
Ti–Pt	0.00–0.11	0.31–0.54	0.02–0.18	0.50–0.58	0.04–0.20	0.50–0.62
Ti–Sn	0.00–0.12	0.60–0.77	0.02–0.20	0.55–0.70	0.04–0.20	0.53–0.70
Ti–Zr			0.03–0.20	0.48–0.64	0.03–0.20	0.42–0.62
Ti–Mo			0.02–0.20	0.48–0.70	0.02–0.18	0.54–0.68
Ti–W			0.02–0.18	0.61–0.74	0.04–0.20	0.54–0.64
Ti–Ga			0.03–0.18	0.80–1.0	0.02–0.20	0.76–1.0
Ti–In			0.02–0.18	0.47–0.63	0.04–0.20	0.50–0.68
Ti–K					0.03–0.20	0.70–0.89
Ti–Cs					0.03–0.18	0.77–1.0
Ti–Rb					0.02–0.18	0.60–0.80
Ti–BO_3					0.02–0.18	0.77–1.0

Separation of Fe–Cu–Ni–Ti

This separation is very neatly achieved using the system formic acid–HCl–acetone (3:4:3) (see Table VII).

Separation of Fe–Al–Ti

These ions are best separated from one another using the system formic acid–hydrochloric acid–acetone (3:4:3). This separation is also successful in the presence of various anions (Table VIII) and also in varying ratios (Table IX). The effect of sample preparation on the separation of Fe–Al–Ti was also studied. This system also proved very helpful for the separation of traces of Ti(IV) from large quantities of Fe and Al.

TABLE III

SEPARATION OF TITANIUM FROM OTHER CATIONS IN THE PRESENCE OF IMPURITIES

Titanium: cation: impurity (1:1:3)

Mixture separated	*Impurity present*											
	Ammonium		*Oxalate*		*Tartrate*		*Formate*		*Acetate*		*Citrate*	
	Ti	*Other ion*	*Ti*	*Other ion*	*Ti*	*Other ion*	*Ti*	*Other ion*	*Ti*	*Other ion*	*Ti*	*Other ion*
Ti–Mo	0.08–0.20	0.58–0.75	0.03–0.17	0.50–0.75	0.05–0.19	0.60–0.75	0.06–0.24	0.50–0.68	0.00–0.19	0.50–0.68	0.00–0.14	0.43–0.66
Ti–Cr	0.05–0.19	0.78–0.88	0.03–0.17	0.77–0.87	0.06–0.19	0.54–0.66	0.06–0.19	0.56–0.68	0.07–0.18	0.52–0.63	0.06–0.15	0.66–0.81
Ti–Fe	0.06–0.17	0.71–0.85	0.08–0.19	0.62–0.78	0.04–0.15	0.73–0.84	0.07–0.18	0.68–0.82	0.04–0.14	0.68–0.81	0.06–0.18	0.69–0.85
Ti–Al	0.07–0.19	0.60–0.75	0.05–0.19	0.52–0.68	0.02–0.13	0.59–0.70	0.06–0.19	0.46–0.58	0.03–0.19	0.58–0.74	0.02–0.14	0.52–0.64
Ti–Ni	0.04–0.19	0.58–0.74	0.11–0.27	0.55–0.71	0.06–0.19	0.55–0.68	0.09–0.28	0.56–0.68	0.08–0.25	0.57–0.68	0.06–0.18	0.53–0.68
Ti–V	0.06–0.17	0.58–0.74	0.00–0.09	0.55–0.71	0.09–0.13	0.56–0.68	0.07–0.19	0.50–0.64	0.06–0.17	0.53–0.68	0.00–0.07	0.53–0.71

TABLE IV

EFFECT OF ANIONS ON THE SEPARATION OF TITANIUM FROM IRON OR NICKEL
Ti:Fe or Ni:anions (1:1:3).

Anion present	*R_F in Ti–Fe mixtures*		*R_F in Ti–Ni mixtures*	
	Ti	*Fe*	*Ti*	*Ni*
Chlorate	0.06–0.19	0.74–0.86	0.07–0.19	0.54–0.66
Sulphate	0.06–0.19	0.74–0.84	0.00–0.12	0.54–0.66
Fluoride	0.12–0.25	0.68–0.81	0.04–0.14	0.49–0.62
Nitrate	0.04–0.13	0.70–0.89	0.05–0.16	0.53–0.66
Bromide	0.00–0.12	0.66–0.78	0.00–0.08	0.46–0.56
Sulphide	0.07–0.19	0.71–0.81		
Chloride	0.12–0.25	0.68–0.81	0.06–0.19	0.56–0.66
Iodide	0.06–0.18	0.68–0.81	0.05–0.17	0.50–0.62
Tellurite	0.09–0.19	0.66–0.81	0.04–0.17	0.53–0.65
Arsenite	0.09–0.20	0.68–0.81	0.06–0.22	0.57–0.75
Iodate	0.06–0.19	0.68–0.81	0.04–0.19	0.50–0.63
Chromate	0.06–0.19	0.68–0.81	0.06–0.19	0.53–0.66
Bromate	0.07–0.19	0.68–0.81	0.05–0.22	0.53–0.65
Thiocyanate	0.06–0.17	0.66–0.81	0.06–0.19	0.53–0.65
Dichromate	0.07–0.17	0.68–0.81	0.06–0.22	0.53–0.68
Molybdate	0.07–0.17	0.68–0.81	0.06–0.23	0.56–0.68
Selenite	0.05–0.19	0.68–0.81	0.06–0.24	0.53–0.68
Arsenate	0.06–0.23	0.75–0.85	0.08–0.25	0.54–0.68

Separation of As(III) from numerous metal ions

Pure formic acid proved best for this separation (Table I). Numerous binary mixtures of arsenic could be separated using this solvent system (Table X). Some important binary separations were studied in greater detail (Tables XI and XII).

Separation of tungsten from numerous metal ions

The best system for this separation was found to be 70 % nitric acid–*n*-butanol (6:4). The results are given in Table I. Binary mixtures of tungsten were also successfully separated as shown in Table XIV. Successful separation of tungsten and molybdenum was also achieved under varying conditions (Tables XV and XVI). Some other solvents for the separation of tungsten and molybdenum are given in Table XIII.

Separation of gold from numerous metal ions

70 % nitric acid–*n*-butanol (6:4) solvent proved very efficient for the separation of gold from numerous metal ions (Table I). Binary mixtures containing gold are easily separated as shown in Table XVII.

Miscellaneous important separations

Other important separations which were achieved practically are given in Table VII.

DISCUSSION

The separation of titanium from numerous metal ions described here is probably the best yet reported. The separation is fast and unaffected by the presence of com-

TABLE V

SEPARATION OF TITANIUM FROM Fe, Ni OR Mo IN DIFFERENT RATIOS

Mixture separated	*Ratio of Ti:other ion*							
	1:10		*1:100*		*10:1*		*100:1*	
	R_F value Ti	*R_F value other ion*	*R_F value Ti*	*R_F value other ion*	*R_F value Ti*	*R_F value other ion*	*R_F value Ti*	*R_F value other ion*
Ti–Fe	0.10–0.20	0.66–0.81	0.12–0.24	0.68–0.82	0.12–0.25	0.69–0.82	0.08–0.23	0.69–0.81
Ti–Ni	0.06–0.19	0.54–0.68	0.05–0.18	0.52–0.74	0.08–0.25	0.56–0.67		
Ti–Mo	0.09–0.19	0.55–0.75	0.06–0.19	0.53–0.73	0.06–0.19	0.56–0.68		

plexing anions like tartrate and citrate. This is because of the high hydrochloric acid concentration in the solvent system. The separation does not need any special preparation of the sample and is unaffected by the presence of various ions which are likely to be encountered in practice. The R_F values of Fe–Al–Ti are evenly spaced from 0–1 and therefore it is possible to handle mixtures of varying ratios more easily. Thus it is possible to separate Ti–Al–Fe (1:1:1000) without using any special shape of paper.

TABLE VI

DETECTION OF TITANIUM IN MIXTURES

Mixture	*Microgram of Ti*	*Microgram of other ion*	R_F *value of Ti*	R_F *value of other ion*
Ti–UO_2^{2+}	0.26	94.50	0.02–0.20	0.43–0.72
Ti–Co	0.26	42.0	0.02–0.12	0.52–0.84
Ti–Mn	0.26	39.90	0.02–0.14	0.54–0.86
Ti–Ni	0.26	42.0	0.01–0.14	0.42–0.77
Ti–Cr	0.26	21.0	0.00–0.12	0.41–0.72
Ti–Cu	0.26	43.10	0.06–0.17	0.53–0.77
Ti–Fe	0.26	27.30	0.03–0.17	0.64–0.90
Ti–(Fe + Cu + Mn + Al + Ca + Mg + Zn)	26	26	0.01–0.09	Fe 0.69–0.90 Cu 0.53–0.77 Mn 0.54–0.86 Al 0.52–0.69 Ca 0.41–0.54 Mg 0.78–0.92 Zn 0.66–0.78

TABLE VII

SOME OTHER IMPORTANT SEPARATIONS

Solvent system	R_F *values and mixture separated*				
Formic acid–hydrochloric acid–acetone (3:3:4)	Zn	(0.63–0.83)	Al	(0.17–0.47)	
	UO_2^{2+}	(0.56–0.68)	V	(0.41–0.54)	
	Th	(0.12–0.31)	V	(0.33–0.57)	
	Ni	(0.19–0.39)	Cu	(0.52–0.71)	Fe (0.87–1.0)
	Ni	(0.21–0.41)	Co	(0.60–0.75)	Fe (0.81–1.0)
	Al	(0.28–0.43)	Zn	(0.60–0.77)	Ga (0.83–1.0)
Formic acid–hydrochloric acid–acetone (3:4:3)	Fe	(0.86–0.94)	Al	(0.20–0.40)	Ti (0.00–0.12)
	Fe	(0.93–1.0)	Cu	(0.60–0.70)	Ni (0.30–0.41)
	Ti	(0.00–0.15)			
Formic acid–hydrochloric acid–acetone (3:5:2)	Ti	(0.03–0.14)	Pt	(0.41–0.58)	Fe (0.71–0.83)
	Ti	(0.06–0.25)	Zr	(0.41–0.63)	Fe (0.73–0.89)
	Ti	(0.00–0.16)	Th	(0.39–0.66)	Fe (0.61–0.83)
	Ti	(0.02–0.20)	Sr	(0.26–0.50)	Mg (0.78–0.92)
	Ti	(0.06–0.18)	UO_2^{2+}	(0.43–0.63)	Fe (0.69–0.89)
n-Butanol–70% nitric acid (6:4)	Te	(0.37–0.55)	Se	(0.67–0.83)	
	Au	(0.90–1.00)	Pd	(0.52–0.68)	Ru (0.14–0.30)
	Au	(0.90–1.0)	Pt	(0.52–0.65)	Ag (0.00)

TABLE VIII

SEPARATION OF Fe–Al–Ti IN THE PRESENCE OF ANIONS

Fe–Al–Ti:anions (1:1:1:3).

Anion present	R_F *values*		
	Fe	*Al*	*Ti*
Chromate	0.82–0.94	0.41–0.62	0.00–0.19
Iodide	0.87–1.0	0.43–0.60	0.08–0.25
Dichromate	0.83–0.93	0.44–0.62	0.08–0.20
Bromate	0.79–0.93	0.38–0.54	0.08–0.20
Oxalate	0.75–1.0	0.33–0.50	0.03–0.14
Thiocyanate	0.81–0.95	0.37–0.54	0.04–0.19
Nitrate	0.83–0.95	0.37–0.54	0.00–0.15
Arsenite	0.83–0.94	0.40–0.56	0.04–0.18
Acetate	0.85–1.0	0.41–0.57	0.04–0.19
Selenite	0.83–0.93	0.38–0.55	0.03–0.18
Tellurite	0.81–0.96	0.33–0.51	0.00–0.15
Iodate	0.86–0.96	0.41–0.56	0.04–0.18
Arsenate	0.87–0.95	0.41–0.56	0.05–0.19
Sulphate	0.81–0.95	0.37–0.56	0.02–0.19
Citrate	0.85–0.98	0.40–0.60	0.05–0.20
Sulphide	0.81–0.96	0.37–0.52	0.05–0.19
Fluoride	0.85–0.98	0.43–0.59	0.05–0.19
Chlorate	0.85–0.97	0.39–0.54	0.04–0.18
Molybdate	0.87–0.99	0.39–0.55	0.03–0.19
Tartrate	0.82–0.98	0.41–0.56	0.06–0.20
Phosphate	0.89–0.99	0.40–0.54	0.04–0.19
Bromide	0.81–0.93	0.40–0.56	0.03–0.19
Formate	0.79–0.93	0.37–0.56	0.03–0.17

TABLE IX

SEPARATION OF Fe–Al–Ti IN DIFFERENT RATIOS

Ratio of Fe:Al:Ti in the mixture	R_F *values*		
	Fe	*Al*	*Ti*
10:10:1	0.81–0.96	0.37–0.57	0.05–0.17
1:10:1	0.81–0.93	0.37–0.56	0.03–0.19
10:1:1	0.78–0.92	0.43–0.56	0.04–0.17
10:1:10	0.73–0.92	0.36–0.50	0.00–0.14
1:10:10	0.85–0.96	0.37–0.55	0.00–0.19
1:1:10	0.87–1.0	0.40–0.54	0.00–0.21
1000:1:1	0.87–1.0	0.43–0.58	0.08–0.23

TABLE X

SEPARATION OF BINARY MIXTURES OF ARSENIC WITH NUMEROUS METAL IONS

Temperature: 22°; solvent: formic acid.

Mixture	R_F *values*	
	Arsenic	*Other ion*
As–Sb	0.00–0.08	0.40–0.52
As–Tl	0.00–0.02	0.61–0.79
As–V	0.00–0.02	0.62–0.80
As–Co	0.00–0.02	0.62–0.85
As–Sn	0.00–0.03	0.28–0.44
As–NH_4	0.04–0.21	0.66–0.74
As–Mg	0.00–0.03	0.80–1.0
As–Mn	0.00–0.09	0.64–0.88
As–Zn	0.00–0.02	0.21–0.66
As–Ga	0.02–0.14	0.39–0.63
As–In	0.00–0.02	0.03–0.66
As–Be	0.00–0.03	0.82–0.94
As–Sr	0.00–0.02	0.70–0.85
As–Ba	0.00–0.04	0.70–0.81
As–Ca	0.00	0.64–0.80
As–UO_2^{2+}	0.00–0.02	0.62–0.73
As–Fe	0.00–0.02	0.58–0.74
As–Cr	0.00–0.04	0.69–0.93
As–K	0.00–0.03	0.73–0.84
As–Rb	0.02–0.12	0.68–0.94
As–Cs	0.02–0.14	0.88–1.0
As–Al	0.04–0.14	0.71–0.89
As–Fe(II)	0.00–0.04	0.36–0.77
As–Cu	0.00–0.09	0.39–0.67
As–Ni	0.00–0.03	0.65–0.87

TABLE XI

SEPARATION OF ARSENIC FROM OTHER CATIONS IN VARYING RATIOS

Temperature: 23–24°.

Mixture	R_F *values*		
	Ratio	*Arsenic*	*Other ion*
As–Sb	10:1	0.00–0.09	0.45–0.54
	100:1	0.00–0.07	0.38–0.51
	1:10	0.01–0.09	0.45–0.54
	1:100	0.02–0.16	0.41–0.60
As–Sn(II)	1:10	0.00–0.11	0.34–0.54
	1:100	0.00–0.04	0.32–0.52
	10:1	0.00–0.09	0.35–0.55
	100:1	0.00–0.10	0.37–0.52
As–Sn	10:1	0.00–0.12	0.35–0.42
	100:1	0.00–0.07	0.26–0.42
	1:10	0.00–0.07	0.19–0.43
	1:100	0.00–1.0	

TABLE XII

EFFECT OF FOREIGN IONS ON THE SEPARATION OF ARSENIC–ANTIMONY

As–Sb–foreign ion (1:1:3).
Temperature: 24–25°; solvent: formic acid.

Foreign ion	R_F *values*	
	Arsenic	*Antimony*
Fluoride	0.00–0.12	0.45–0.60
Thiocyanate	0.00–0.12	0.43–0.60
Sulphate	0.00–0.06	0.24–0.38
Oxalate	0.00–0.09	0.26–0.38
Phosphate	0.00–0.08	0.24–0.38
Formate	0.00–0.06	0.22–0.34
Sulphite	0.00–0.14	0.45–0.60
S lphide	0.00–0.07	0.45–0.55
Citrate	0.00–0.04	0.27–0.39
Tartrate	0.00–0.09	0.23–0.36
Nitrate	0.00–0.05	0.45–0.55
Iodate	0.00–0.05	0.43–0.53
Chloride	0.00–0.05	0.43–0.53
Iodide	0.00–0.10	0.42–0.52
Bromide	0.00–0.07	0.48–0.58

TABLE XIII

SOME USEFUL SYSTEMS FOR THE SEPARATION OF TUNGSTEN AND MOLYBDENUM

Solvent system		R_F *values*	
		Mo	*W*
70 % nitric acid–40 % sodium tartrate	(8:2)	0.44–0.72	0.00–0.12
70 % nitric acid–*n*-butanol	(2:1)	0.24–0.36	0.00
	(6:4)	0.23–0.43	0.00

Various other mixtures containing Ti are also easily separated *e.g.*, Fe, Ti, Ni and Cu. The separation of arsenic developed here is much faster than the separation reported earlier and almost all important ions are easily separated. The separation of gold is attained in 70 % HNO_3–*n*-butanol (6:4). The high concentration of nitric acid prevents reduction of gold and no oxidizing agent has to be incorporated in the sample solution. Gold is easily separated from Pt, Pd, Ir, Ru and other metal ions in one hour. This is therefore probably the best separation of gold from numerous metal ions. Sometimes

TABLE XIV

SEPARATION OF BINARY MIXTURES OF TUNGSTEN

Solvent: 70% nitric acid–*n*-butanol (6:4); temperature: 22–23°; time of development: 2 h 30 min.

Mixture	R_F *values*	
	W	*Other ion*
W–Mo	0.00	0.23–0.43
W–Ti	0.00–0.13	0.31–0.47
W–NH_4	0.00	0.44–0.61
W–Th	0.00	0.20–0.72
W–Mg	0.00	0.33–0.46
W–UO_2^{2+}	0.00	0.33–0.46
W–Sr	0.00	0.27–0.38
W–Ca	0.00	0.36–0.46
W–Fe	0.00	0.47–0.58
W–Mn	0.00	0.34–0.50
W–V	0.00	0.35–0.51
W–Co	0.00	0.38–0.51
W–Ni	0.00	0.31–0.46
W–In	0.00	0.34–0.46
W–Ga	0.00	0.33–0.63
W–Y	0.00	0.32–0.48
W–La	0.00	0.36–0.48
W–Ce(III)	0.00	0.30–0.42
W–K	0.00	0.40–0.50
W–Rb	0.00	0.54–0.65
W–Pb	0.00	0.13–0.43
W–Cs	0.00	0.54–0.64
W–Se	0.00	0.64–0.84
W–Cr	0.00	0.32–0.48
W–Zn	0.00	0.33–0.48
W–Al	0.00	0.38–0.48
W–Pt	0.00	0.48–0.71
W–As	0.00	0.54–0.67
W–Pd	0.00	0.39–0.77
W–Sb	0.00	0.76–0.94
W–Hg	0.00	0.60–0.78
W–Te	0.00	0.39–0.56
W–Bi	0.00	0.47–0.66
W–Tl(I)	0.00	0.50–0.63
W–Be	0.00	0.52–0.63

TABLE XV

SEPARATION OF TUNGSTEN FROM IMPORTANT IONS IN DIFFERENT RATIOS

Mixture	*Ratio*	R_F *values*	
		W	*Other ion*
Mo–W	1:10	0.00	0.21–0.42
	1:100	0.00	0.18–0.32
	10:1	0.00	0.26–0.44
	100:1	0.00	0.23–0.41
W–Co	1:100	0.00	0.34–0.51
W–V	1:100	0.00	0.31–0.43
W–Ni	1:100	0.00	0.31–0.47
W–Ti	1:100	0.00	0.31–0.50
W–Fe	1:100	0.00	0.19–0.50

TABLE XVI

SEPARATION OF MOLYBDENUM AND TUNGSTEN IN THE PRESENCE OF FOREIGN ION

W: Mo:foreign ion (1:1:3).
Temperature: 29–30°.

Foreign ion	R_F *values*	
	W	*Mo*
Chromium	0.00	0.27–0.40
Iron	0.00	0.27–0.37
Cobalt	0.00	0.27–0.39
Vanadium	0.00	0.36–0.48
Nickel	0.00	0.27–0.37
Phosphate	0.00	0.30–0.45
Borate	0.00	0.29–0.44
Citrate	0.00	0.27–0.40
Tartrate	0.00	0.25–0.38

TABLE XVII

SEPARATION OF GOLD FROM OTHER IONS IN DIFFERENT RATIOS

Solvent: 70% nitric acid–*n*-butanol (6:4); time of development: 2 h 30 min; temperature: 26–27°.

Mixture	*Ratio*	*Au*	*Other ion*
Au–Ga	1:10	0.86–1.0	0.29–0.50
Au–Se	1:10	0.90–1.0	0.67–0.81
Au–Hg	1:10	0.90–1.0	0.67–0.75
Au–V	1:10	0.89–1.0	0.39–0.54
Au–Te	1:10	0.90-1.0	0.35–0.54
Au–Cr	1:10	0.91–1.0	0.25–0.37
Au–Pd	1:10	0.90–1.0	0.53–0.67
Au–As	1:10	0.90–1.0	0.50–0.62
Au–Fe	1:10	0.90–1.0	0.36–0.51
Au–Ni	1:10	0.89–1.0	0.31–0.45
Au–Cu	1:10	0.90–1.0	0.42–0.53
Au–Ir	1:10	0.92–1.0	0.00–0.12
Au–Fe	1:1000	0.90–1.0	0.27–0.62
Au–Ni	1:1000	0.86–1.0	0.31–0.58

when binary mixtures of tungstate were chromatographed a precipitate appeared owing to the formation of an insoluble tungstate. The precipitate was dissolved by adding the least quantity of tartaric acid. This however did not interfere in the separations. Tungsten is probably precipitated as oxide and remains at the point of application while most metal ions migrate in the highly acidic medium.

The separation of arsenic from numerous metal ions utilizes pure formic acid as an eluent. In order to understand the chemistry of the test formic acid was added to the cation solutions. The following gave precipitates; Ag(I), Hg(I), Hg(II), Pd(II), W(VI) and Ru(III). All these show tailing when chromatographed with formic acid except W(VI) which is probably precipitated very rapidly and remains on the point of application. Arsenic did not give a precipitate with formic acid. Therefore, it is not

a case of precipitation even though As remains on the point of application but probably because the paper is more polar than formic acid and arsenic does not form a complex with this acid. Cations which form complexes with formic acid show greater migration, *i.e.*, Ni(II), Co(II), Mn(II) etc.

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CHROM. 3339

QUANTITATIVE SEPARATION OF TITANIUM FROM NUMEROUS METAL IONS AND OF IRON, ALUMINUM AND TITANIUM BY PAPER CHROMATOGRAPHY

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SUMMARY

Titanium has been quantitatively separated from numerous metal ions using formic acid–hydrochloric acid–acetone (3:5:2). The method has been found useful for the quantitative separation of about 200 μg of titanium from a synthetic mixture of 17 metal ions including Mo^{6+}, W^{6+}, Cu^{2+}, Fe^{3+}, Al^{3+}, Cr^{3+}, Ni^{2+}, Co^{2+}, Mn^{2+}, UO_2^{2+} and V^{4+}. Quantitative separation of Fe^{3+}–Al^{3+}–Ti^{4+} has been achieved using formic acid–hydrochloric acid–acetone (3:4:3). Both separations are fast and selective.

Titanium is an important metal which is used in pigments and in alloys. Its separation from numerous metal ions is therefore important. SCHEFFER has pointed out in the treatise on Analytical Chemistry that "The present application of paper chromatography is restricted to qualitative identification of titanium but this separation technique is worthy of serious consideration"[1]. Iron, aluminum and titanium are also difficult to separate by classical means; LACOURT therefore developed a quantitative separation of iron, aluminum and titanium[2]. Her separation suffers from many limitations: (i) Fe and Ti are separated only if the sample is prepared in a particular manner; (ii) the method cannot be used for varying ratios of iron, titanium and aluminum, and according to MACEK[3] "when the samples are applied as solutions in 10 % citric acid or in acetate buffer, all three cations are separated. When the cations are dissolved in dil. HCl or in dil. HNO_3, Ti and Fe migrate as a single zone". As a result her method has not gained wide acceptance. Paper chromatography was therefore undertaken: (i) to quantitatively separate titanium from numerous metal ions; (ii) to quantitatively separate iron, aluminum and titanium. The present report summarizes the results of such a study.

EXPERIMENTAL

Apparatus

Development was performed in 25 by 5.5 cm glass jars employing the ascending technique. The dimensions of the paper strip were 16 by 4 cm. Estimation was done on a Bausch and Lomb spectronic-20 colorimeter. All pH measurements were made on pH Meter Model LI-10 (ELICO, Hyderabad, India).

Reagents

Whatman No. 1 paper was used for all quantitative work. All chemicals were of reagent grade.

Standard titanium solution (containing 40 mg Ti per ml) was prepared by diluting Analar grade titanic chloride solution with HCl and water and was standardized against potassium dichromate[4].

The stock solution of ferric ammonium sulfate (containing 34.85 mg of Fe/ml) was prepared in 5 % H_2SO_4.

The stock solution of aluminum (containing 8.537 mg Al/ml) was prepared in 1 % H_2SO_4 and was standardized against EDTA[4].

4 % gallium chloride and 0.1 *M* indium chloride were prepared in 4 *M* HCl. 0.1 *M* sodium molybdate and 1 % sodium tungstate were prepared in water.

Solutions of other metal ions were prepared by taking their chlorides, nitrates or sulfates in 15 % HCl, HNO_3 and H_2SO_4, respectively.

The developer used for the separation of titanium from numerous metal ions was a mixture of formic acid–hydrochloric acid–acetone in the ratio of (3:5:2) and the developer for the separation of Fe–Al–Ti was formic acid–hydrochloric acid–acetone in the ratio of (3:4:3). Titanium, iron and aluminum were detected by 0.1 % alcoholic alizarin Red-S and exposing over ammonia.

Procedure

(*a*) *Quantitative separation of titanium from numerous metal ions.* The solution containing titanium with other metal ions as impurities was streaked on the line of application with the help of a λ-pipette. The strips were dried in air and saturated for 15 min with the vapours of the developer and the developer was allowed to ascend 12.5 cm. Blank paper was run simultaneously and detected; 15 % HCl solution was streaked as a blank. Zones were cut into small pieces and were eluted thrice with 5 % H_2SO_4 by taking 10 ml each time and heating on a water bath for 1 h. Papers were washed twice by heating with 10 ml of distilled water for 15 min. The volume was reduced to 2–3 ml by evaporation on a water bath and the paper pulp was oxidized with 2 ml of a mixture of HNO_3 + $HClO_4$ (1:3) + 2 ml of H_2SO_4. The solution was evaporated to 0.5–1 ml. It was taken into a standard 50 ml flask and made up to volume with water and titanium was determined by sulphosalicylic acid[6] at 410 mμ.

(*b*) *Separation and determination of titanium, iron and aluminum.* A mixture of titanium, iron and aluminum was streaked and developed in the given developer. Elutions were made as in procedure (a). The eluting reagent for titanium and aluminum was also 5 % H_2SO_4, while for iron it was 1 % HCl. The determination of titanium, iron and aluminum was done with sulphosalicylic acid[6]; 1,10-phenanthroline[7]; and triochrome cyanine-R[8], respectively.

RESULTS

Determination of titanium in presence of numerous metal ions

Titanium in presence of common impurities was studied and the quantitative separation of titanium from these ions was studied. The sample contained Ti^{4+} and the impurities were in the ratio of (1:1).

A (1:1) synthetic mixture of titanium with 17 metal ions: Ga^{3+}, In^{3+}, K^+, Cs^+,

NH_4^+, Cu^{2+}, Cd^{2+}, Fe^{3+}, Cr^{3+}, Al^{3+}, Ni^{2+}, Co^{2+}, Mn^{2+}, Zn^{2+}, Mg^{2+}, UO_2^{2+} and V^{4+}, was studied and found quantitative. The results are given in Table I.

Quantitative separation of Ti^{4+}, Fe^{3+} and Al^{3+}

0.01 ml of a mixture of Ti^{4+}, Fe^{3+} and Al^{3+} was applied. Zones of Ti^{4+}, Fe^{3+} and Al^{3+} were cut out and determined by the procedure described earlier. The results are summarized in Table II.

TABLE I

DETERMINATION OF Ti^{4+} IN THE PRESENCE OF IMPURITIES

200 γ Ti^{4+} applied.

Impurity added	*Ti^{4+} recovered (γ)*	*% Error*
Ga^{3+}	210	+5.0
	204	+2.0
In^{3+}	204	+2.0
	204	+2.0
Mo^{6+}	200	±0.0
	204	+2.0
W^{6+}	204	+2.0
	204	+2.0
K^+	210	+5.0
	204	+2.0
Cs^+	196	−2.0
	200	±0.0
NH_4^+	204	+2.0
Cu^{2+}	210	+5.0
Cd^{2+}	204	+2.0
	192	−4.0
Fe^{3+}	200	±0.0
	204	+2.0
Cr^{3+}	192	−4.0
	200	±0.0
Al^{3+}	196	−2.0
	200	±0.0
Ni^{2+}	200	±0.0
	210	+5.0
Co^{2+}	192	−4.0
	200	±0.0
Mn^{2+}	196	−2.0
	204	+2.0
Zn^{2+}	200	±0.0
Ba^{2+}	196	−2.0
Mg^{2+}	200	±0.0
	200	±0.0
UO_2^{2+}	192	−4.0
	192	−4.0
V^{4+}	204	+2.0
	196	−2.0
17 metal ions	208	+4.0
	204	+2.0
	204	+2.0
	204	+2.0

TABLE II

DETERMINATION OF Fe^{3+}, Al^{3+} AND Ti^{4+}

Ti^{4+} applied (γ)	Ti^{4+} recovered (γ)	% Error	Fe^{3+} applied (γ)	Fe^{3+} recovered (γ)	% Error	Al^{3+} applied (γ)	Al^{3+} recovered (γ)	% Error
133.3	133.3	0.0	23.2	23.1	—0.4	28.0	27.0	—3.0
133.3	133.3	0.0	23.2	23.1	—0.4	28.0	29.0	+3.0
133.3	133.3	0.0	11.46	11.5	+0.3	28.0	27.0	—3.0
133.3	133.3	0.0	11.46	11.5	+0.3	28.0	26.5	—4.5
133.3	133.3	0.0	11.46	11.5	+0.3	28.0	28.0	±0.0

DISCUSSION

The results show that the methods of separation are fast, selective and quantitative. Titanium is easily separated from most of the ions which are likely to be found in actual analytical work. Using paper chromatography iron, aluminum and titanium have been separated only by LACOURT[2]. The method of separation developed by us is however faster, more selective and is not subject to common interferences.

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Short Communication

CHROM. 3411

Reaction thin-layer chromatography in the analysis of mixtures of alkenyl acyl- and diacyl-phosphatides

Recently KAUFMANN, RADWAN AND AHMAD[1] reported quantitative alkaline methanolysis of monoacyl-(lyso-) and diacyl-phosphatides directly on thin-layer plates. By this technique they calculated the phosphatide content of mixed lipids by multiplying the weight of fatty acid methyl esters obtained from phosphatides by appropriate factors. These values agreed well with those obtained by phosphorus determinations. However, they obtained low values for tissue lipids that contained substantial amounts of either alkenyl acyl-phosphatides, or sphingolipids, or both. The explanation for this discrepancy was the resistance of the vinyl ether linkage of the plasmalogens (alkenyl acyl-phosphatides) or the acid-amide linkage of the sphingolipids to alkaline methanolysis.

In the present communication we describe a suitable modification of the method of KAUFMANN *et al.*[1] by which the alkenyl content, and the fatty acid and aldehyde composition of mixtures of alkenyl acyl- and diacyl-ethanolamine phosphatides may be determined.

Experimental

A mixture of alkenyl acyl- and diacyl-ethanolamine phosphatides from hog brain and hog spinal cord were prepared by the method of FOLCH[2] and purified by preparative thin-layer chromatography (TLC) on Silica Gel G plates using chloroform–methanol–concentrated ammonia (70:30:5) as the developing solvent. The Silica Gel G plates were prepared as described previously[3].

Reaction thin-layer chromatography (*plate method*) was carried out as follows: 1 ml of solution (50 mg/ml) of a mixture of alkenyl acyl- and diacyl-ethanolamine phosphatides in chloroform was deposited as a 16 cm long strip, 2 cm above the lower edge of the plate and parallel to it. Margins of 2 cm were left at both ends of the strip for reference standards. The strip was sprayed with a solution containing 12 % hydrochloric acid in methanol, and the plate was left undisturbed in an atmosphere of nitrogen for 2 min. An aldehyde reference (50 μg of palmitaldehyde) was spotted at the left end of the strip and then the entire strip was exposed to a stream of dry nitrogen to remove the residual hydrochloric acid and methanol. The plate was developed in toluene until the solvent front reached the top of the plate. The plate was removed, and, after drying with a stream of nitrogen, the strip containing monoacyl-(lyso-) and diacyl-ethanolamine phosphatides was subjected to a spray of solution containing 12 % potassium hydroxide in methanol and left undisturbed in an atmosphere of nitrogen for 5 min. A reference methyl ester standard (50 μg of methyl linoleate) was spotted at the right end of the strip, and the entire strip exposed to a stream of dry nitrogen to remove the residual methanol from the plate. The plate was

then redeveloped in the same direction with toluene until the solvent front reached half the height of the plate. The plate was dried with a stream of nitrogen and only the two 2 cm strips containing the reference standards were sprayed with 0.2 % solution of 2,7-dichlorofluorescein. The location of the reference palmitaldehyde (R_F 0.5) and methyl linoleate (R_F 0.23) was determined under ultraviolet light. The unsprayed portions corresponding to the aldehydes and methyl esters liberated from the mixture of alkenyl acyl- and diacyl-ethanolamine phosphatides were scraped off into separate containers and quantitatively extracted with diethyl ether. After distilling off the diethyl ether, the residual aldehydes and methyl esters were weighed, and from these weights the alkenyl content of the mixture was calculated using the following formula:

$$\text{Percent alkenyl} = \frac{2(\text{amount of DMA, calculated from weight of aldehyde})}{(\text{calculated amount of DMA} + \text{amount of ME})} \times 100$$

Methanolysis of the phosphatide mixture was carried out at —30° (*cold method*) as described previously[4]. Up to 100 mg of a mixture of alkenyl acyl- and diacyl-ethanolamine phosphatides was dissolved in 20 ml of diethyl ether contained in a 125 ml Erlenmeyer flask. This flask was immersed in a dry ice–acetone bath maintained at —30°. While the contents of the flask were being agitated with a magnetic stirrer, 2 ml of 100 % sulfuric acid was added and the stirring continued for 10 min during which time the temperature of the bath rose to 0°. The bath temperature was lowered again to —30°, 15 ml of absolute methanol added and the stirring continued for 10 min. The reaction mixture was then carefully neutralized with 20 ml of 35 % potassium hydroxide in methanol, followed by continuous swirling until the jelly-like mixture turned into a white milky solution. After 10 min, the contents of the flask were transferred to a separatory funnel containing 200 ml of cold distilled water, and the Erlenmeyer flask was rinsed twice with 20 ml of diethyl ether. The products of methanolysis were extracted with 150 ml of diethyl ether, and the ether extract was washed free of alkali with distilled water and dried over anhydrous sodium sulfate. The mixture of methyl esters and dimethyl acetals resulting from cold methanolysis was separated quantitatively by preparative TLC on Silica Gel G using toluene as the developing solvent, and the two fractions, after scraping off, were extracted and weighed. The alkenyl content of the mixture was calculated as follows:

$$\text{Percent alkenyl} = \frac{2(\text{amount of DMA})}{(\text{amount of DMA} + \text{amount of ME})} \times 100$$

The gas–liquid chromatography (GLC) analysis of aldehydes and methyl esters obtained by *plate method* and dimethyl acetals and methyl esters obtained by *cold method* were made with a Beckman GC-2A instrument equipped with a hydrogen flame ionization detector containing an aluminum column (6 ft. long, 1/8 in. outer diameter) packed with Gas Chrom P, 80–100 mesh impregnated with 20 % ethylene glycol succinate and 2 % phosphoric acid. The temperature of the column was 160° and helium flow rate was 30 ml/min. Peaks of the aldehydes, dimethyl acetals and methyl esters were identified by comparing their retention times with authentic and synthetic standards. The chain lengths of unsaturated dimethyl acetals, methyl esters and aldehydes (after converting them to dimethyl acetals) were verified by GLC after hydrogenation. The peak areas were quantitated by triangulation.

TABLE I

FATTY ACID AND ALDEHYDE COMPOSITION OF A MIXTURE OF ALKENYL ACYL- AND DIACYL-ETHANOLAMINE PHOSPHATIDES FROM HOG SPINAL CORD (SAMPLE 1)

Carbon number	*Percent of total fatty acids*		*Percent of total fatty aldehydes*	
	Plate method	*Cold method*	*Plate method*	*Cold method*
C15:0	—	—	—	—
C16:0	7.0	6.9	29.1	28.7
C16:1	0.8	0.7	—	—
C17:0	—	—	—	—
Unidentified	—	—	—	—
Unidentified	—	—	—	—
C18:0	9.0	8.8	25.5	25.3
C18:1	59.1	58.6	45.4	46.0
C18:2	—	—	—	—
C18:3	—	—	—	—
C19:0	Traces	Traces	—	—
C20:0	0.3	0.5	—	—
C20:1	19.0	19.2	—	—
C20:4	3.4	3.5	—	—
C22:1	1.4	1.8	—	—

TABLE II

FATTY ACID AND ALDEHYDE COMPOSITION OF A MIXTURE OF ALKENYL ACYL- AND DIACYL-ETHANOLAMINE PHOSPHATIDES FROM HOG SPINAL CORD (SAMPLE 2)

Carbon number	*Percent of total fatty acids*		*Percent of total fatty aldehydes*	
	Plate method	*Cold method*	*Plate method*	*Cold method*
C15:0	—	—	—	—
C16:0	5.3	5.4	33.6	33.9
C16:1	0.6	0.7	—	—
C17:0	—	—	—	—
Unidentified	—	—	—	—
Unidentified	—	—	—	—
C18:0	9.3	9.2	23.4	23.2
C18:1	56.7	55.9	43.0	42.9
C18:2	—	—	—	—
C18:3	—	—	—	—
C19:0	—	—	—	—
C20:0	0.8	0.7	—	—
C20:1	21.1	20.8	—	—
C20:4	2.1	2.5	—	—
C22:1	4.1	4.8	—	—

TABLE III

FATTY ACID AND ALDEHYDE COMPOSITION OF A MIXTURE OF ALKENYL ACYL- AND DIACYL-ETHANOLAMINE PHOSPHATIDES FROM HOG BRAIN

Carbon number	*Percent of total fatty acids*		*Percent of total fatty aldehydes*	
	Plate method	*Cold method*	*Plate method*	*Cold method*
C15:0	0.8	0.9	—	—
C16:0	7.4	7.6	33.4	33.1
C16:1	1.3	1.7	—	—
C17:0	Traces	Traces	—	—
Unidentified	1.2	1.6	—	—
Unidentified	Traces	Traces	—	—
C18:0	21.2	21.0	27.8	28.3
C18:1	49.8	49.2	37.7	37.2
C18:2	1.1	0.6	1.1	1.4
C18:3	1.7	1.7	—	—
C19:0	—	—	—	—
C20:0	—	—	—	—
C20:1	10.6	10.7	—	—
C20:4	4.9	5.0	—	—
C22:1	—	—	—	—

TABLE IV

THE PERCENT ALKENYL CONTENT OF A MIXTURE OF ALKENYL ACYL- AND DIACYL-ETHANOLAMINE PHOSPHATIDES

Hog spinal cord (Sample 1)		*Hog spinal cord (Sample 2)*		*Hog brain*	
Plate method	*Cold method*	*Plate method*	*Cold method*	*Plate method*	*Cold method*
92.2	90.4	91.9	89.6	Not determined	

Results

The analyses reported in Tables I–IV by the *plate method* (reaction thin-layer chromatography) and the *cold method*, for three different mixtures of alkenyl acyl- and diacyl-phosphatides from hog tissues, indicate good agreement for their fatty acid and aldehyde composition as well as for their alkenyl content. Thus the *plate method* described above is a quick alternative for the analysis of mixtures of alkenyl acyl- and diacyl-phosphatides.

Discussion

This modification of the method of KAUFMANN *et al*[1] has resulted in a useful technique for characterizing mixtures of alkenyl acyl- and diacyl-phosphatides. The hydrolysis of vinyl ether bonds present in phosphatides either by methanolic hydrochloric acid as described here or aqueous mercuric chloride solutions[5] on TLC plates can partly overcome the difficulties previously encountered by KAUFMANN *et al.*[1] in

analyzing phosphatide mixtures containing alkenyl acyl-phosphatides. The hydrolysis of acid-amide bonds of sphingolipids on thin-layer plates still remains as a problem.

The hydrolysis of vinyl ether bond by hydrochloric acid solution in methanol is more advantageous than by aqueous mercuric chloride solution[5] because the reactivation of plates after using aqueous mercuric chloride solution spray required appreciably longer time and more rigorous conditions[5].

Reaction thin-layer chromatography, as described in this communication, was used on a preparative TLC scale and in this way sufficient amounts of methyl esters and aldehydes can be obtained for their structural analysis. When only small amounts of mixtures of alkenyl acyl- and diacyl-phosphatides are available, the same procedure can still be used to determine their fatty acid and aldehyde composition. The alkenyl content of the mixture can then be determined by incorporating internal standards of an aldehyde and a methyl ester into the mixture which were not originally present.

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Notes

CHROM. 3362

Calculation of the retention time of the "air peak" in gas chromatograms

On gas chromatograms determined by an ionization detector the "air peak" does not normally show up due to the fact that oxygen and nitrogen are not ionized under the conditions used. Knowledge of the retention time corresponding to the "air peak" is necessary when chromatograms are to be compared, when the adjusted retention time is to be calculated, or when certain parameters, such as the partition ratio, are to be calculated.

It is possible to inject a low molecular weight hydrocarbon, preferably methane, and then take the retention time for methane as the retention time for the "air peak". However, methane is more or less soluble in almost all stationary phases, and the method is therefore only applicable if the retention times to be corrected are large in comparison with the retention time of the "air peak".

PETERSON AND HIRSCH[1] have published a method for the calculation of the retention time or distance of the "air peak" utilizing the fact that a straight line relationship exists between the logarithm of the adjusted retention time and the number of carbon atoms in the members of a homologous series, which have to be equally spaced with respect to carbon numbers. The method does not give the retention time of the "air peak" directly, but the difference between this and an ancillary line put arbitrarily in the chromatogram. GOLD[2] has published a method without the above-mentioned restriction with respect to carbon numbers, but his equations for the determination of the "air peak" can only be solved by successive approximation.

Without knowledge of the above-mentioned methods, we have developed a method for the calculation based on the fact that a straight line relationship exists between the logarithm of the adjusted retention time and the Kovats index of the members of a homologous series. The method is similar to that of PETERSON AND HIRSCH[1], but it gives the position of the "air peak" directly and utilizes the retention times of the hydrocarbons otherwise used for the calibration with regard to Kovats index, we therefore believe our method to be of interest.

Theory

The Kovats index for a normal alkane is defined as the number of carbon atoms in the alkane multiplied by 100, and the linear relationship between the logarithm of the adjusted retention time or distance and the Kovats indices for three normal alkanes with equally spaced numbers of carbon atoms might therefore be represented by the following equations:

$$\log (t_1 - t_a) = m \cdot z \cdot 100 + q \quad (1)$$

$$\log (t_2 - t_a) = m \cdot (z + b) \cdot 100 + q \quad (2)$$

$$\log (t_3 - t_a) = m \cdot (z + 2b) \cdot 100 + q \quad (3)$$

where:

t_1 = the unadjusted retention time or distance for the n-alkane with z carbon atoms;
t_2 = the unadjusted retention time or distance for the n-alkane with $z + b$ carbon atoms;
t_3 = the unadjusted retention time or distance for the n-alkane with $z + 2b$ carbon atoms;
t_a = the retention time or distance for the "air peak".
m = constant;
q = constant;
b = a whole positive number.

If we subtract eqn. (1), from eqn. (2), we obtain:

$$\log (t_2 - t_a) - \log (t_1 - t_a) = m \cdot (z + b) \cdot 100 - m \cdot z \cdot 100 + q - q$$

or:

$$\log \frac{t_2 - ta}{t_1 - t_a} = m \cdot b \cdot 100 \tag{4}$$

If we subtract eqn. (2) from eqn. (3), we obtain:

$$\log (t_3 - t_a) - \log (t_2 - t_a) = m \cdot (z + 2b) \cdot 100 - m \cdot (z + b) \cdot 100 + q - q$$

or:

$$\log \frac{t_3 - t_a}{t_2 - t_a} = m \cdot b \cdot 100 \tag{5}$$

Eliminating m·b·100 from eqn. (4) and eqn. (5), we obtain:

$$\log \frac{t_2 - t_a}{t_1 - t_a} = \log \frac{t_3 - t_a}{t_2 - t_a}$$

or:

$$\frac{t_2 - t_a}{t_1 - t_a} = \frac{t_3 - t_a}{t_2 - t_a} \tag{6}$$

From eqn. (6) we obtain:

$$t_a = \frac{t_2^2 - t_1 t_3}{2t_2 - t_1 - t_3} \tag{7}$$

Examples

Four determinations on a 2 m 1/8 in. diameter stainless steel column with 10 % squalane on Gas Chrom Z, mesh 80–100, mounted in a Perkin Elmer F-11 with a pyrolysis attachment at an oven temperature of 50°, have given the results shown in Table I.

Two determinations on a 3 m 1/8 in. diameter stainless steel column with 10 % Carbowax 20 M terephthalic acid on Gas Chrom Z, 80–100 mesh, mounted in an Aerograph 204 at an oven temperature of 130°, have given the results shown in Table II.

TABLE I

DETERMINATIONS ON THE SQUALANE COLUMN

Run No.	*Retention distance (cm)*				
	n-propane t_1	*n-butane* t_2	*n-pentane* t_3	*"air peak"* t_a	*methane*
1	0.61	0.80	1.29	0.49	0.52
2	0.65	0.82	1.29	0.55	0.58
3	0.64	0.80	1.32	0.57	0.60
4	0.63	0.81	1.32	0.53	0.56

TABLE II

DETERMINATIONS ON THE CARBOWAX COLUMN

Run No.	*Retention distances (cm)*				
	n-decane t_1	*n-dodecane* t_2	*n-tetradecane* t_3	*"air peak"* t_a	*methane*
1	1.78	2.91	5.88	1.09	1.14
2	1.77	2.94	5.96	1.03	1.08

Conclusions

It is believed that the method presented here for the determination of the retention time or distance of the "air peak" might prove to be an easy and accurate way of obtaining this information, if the results are to be expressed in Kovats indices. Furthermore, determinations of this kind might prove to be of value in comparing different gas chromatographs with regard to "dead volumes".

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CHROM. 3341

The application of repeated wide-range zero shifting in gas chromatography*

Gas chromatography has repeatedly proved to be a powerful tool in trace analysis[1]. Trace constituents that are present in some mixtures, however, frequently exhibit a retention behavior similar to that of the matrix component of these products. This problem is frequently encountered in the analysis of flavors, solvents and impurities in monomers.

Even when the retention time of a trace constituent is 30 % longer than that of the matrix component, its peak appears only on the shoulder of the matrix component peak; under high-sensitivity conditions, the shoulder extends over many times the full-scale range of the recorder. Under such conditions most of the shoulder of the matrix-component peak remains unrevealed in the "off-scale" portion. As a consequence trace constituents appearing on the shoulder of the "off-scale" portion of the matrix peak remain undetected. If the sample size or the sensitivity of the detection system is decreased so that a greater portion of the matrix peak appears on the chart, the detection of the trace peaks will again not be possible: the size of these trace peaks also decreases, and they frequently become smaller than the minimum needed for detection. The problem of detecting trace constituents appearing on the shoulders of large gas chromatographic peaks was solved by use of a method which can be called "wide-range zero shifting". A high-sensitivity flame-ionization system consisting of a Keithley 410 electrometer and a modified flame-ionization detector[2] was used. The electrometer was operated at the 3×10^{-10} A range; only about 1 % of the output was fed to a 1-mV Honeywell recorder.

The zero shifting method involves the following procedure: slightly after the matrix peak reaches "off-scale", the baseline position is shifted from + 0.1 mV (which is the original position of the baseline) to about —10 mV. This shift brings into the range of the 1-mV recorder the range which without the shift would be ten times "off-scale". A 1-mV portion of the matrix peak shoulder is then recorded. The baseline position is immediately shifted to —9 mV, and the portion of the peak which without the shift would be nine times "off-scale" is then recorded. This procedure is repeated until the baseline reaches its original position.

Samples of alcoholic beverages in which ethanol is the matrix component were analyzed by gas chromatography, with the zero shifting method. A typical chromatogram is shown in Fig. 1. Peaks 11, 12, and 13 remained in the "off-scale" of the chromatogram when the sample was run without zero shifting. A large peak (No. 10) remained undetected even with zero shifting, because its retention time was too close to that of ethanol. When the sensitivity of the system was decreased 10 times so that the greater portion of the matrix peak shoulder was recorded on the chart, peaks 11, 12, and 13 remained undetected in most of the samples. In later experiments application of a Keithley model 261 picoampère source proved to be superior for wide-range zero shifting to the zero shifting achieved through the use of the original zero control knob of the electrometer. The suppression currents are delivered by the picoampère source

* Part of this work was carried out at the Department of Plant Science, University of Manitoba, Canada.

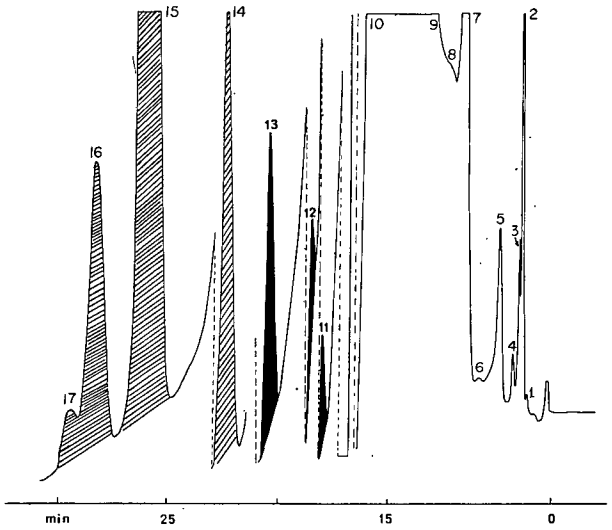


Fig. 1. Chromatogram of an alcoholic beverage which reveals trace flavor constituents on the shoulder of the ethanol peak by high sensitivity and repeated zero shifting.

in a stepwise mode; in turn, this permits reproducible shifting of the zero position to be achieved by switching rather than by rotation.

The zero shifting procedure permits the detection of trace constituents by exposing on the recorder chart a greater portion of the matrix peak. It was successfully applied in the analysis of trace flavor constituents in alcoholic beverages.

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CHROM. 3397

Characterization of cholestanols and cholestanones by thin-layer and gas chromatography

Steroid boranes. II.

The thermal isomerization of organoboranes derived from steroid olefins, followed by oxidation with alkaline hydrogen peroxide yields a complex mixture of 5α- and 5β-cholestanols, differing only in the position and stereochemistry of the hydroxyl groups[1].

In this paper we report the retention times and R_F values of a series of isomeric cholestanols and cholestanones, as well as those of some of their derivatives, which were used to identify the components of a mixture of stanols resulting from the isomerization and hydroboration of cholest-5-ene.

Experimental

Steroids. The steroids used in the investigation were prepared by well-known published methods[2].

Thin-layer chromatography. All chromatograms were run on Silica Gel G (E. Merck). The plates were stored in a desiccator after activation at 110°. The plates were prepared on a Shandon plate spreader and coated to a thickness of 0.25 mm.

The solvent systems used were: cholestanoles: two developments with benzene (Matheson, Coleman and Bell, Spectrograde); cholestanones: one development with benzene.

Detection: vanillin–sulphuric acid–ethanol spray; the plate is heated to 110° for 5 min after spraying. Cholestanols give blue-violet spots, whereas the cholestanones give colours from yellow to violet[3].

Vapour phase chromatography. A Pye Panchromatograph with argon ionization detector and 7 ft. × 1/4 in. glass columns was used. The solid support was Gas Chrom Q 100/120 mesh (Applied Science Laboratories, Inc.). The liquid phase used was 3 % HI-EFF 8BP (cyclohexane–dimethanol succinate, Applied Science Laboratories, Inc.). The carrier gas was argon (Agamex). The conditions are given in Table I.

Trimethylsilyl ethers of the alcohols were prepared with BSA reagent (Pierce Chemical, Inc.) according to instructions supplied by the manufacturer.

TABLE I

CONDITIONS FOR VAPOUR PHASE CHROMATOGRAPHY

	Temperature (°C)		*Flow (ml argon/ min)*
	Column	*Detector*	
Free alcohols	225	235	68
Trimethylsilyl ethers Trifluoroacetates	200	225	91
Ketones O-Methyloximes	225	235	68

Trifluoroacetates were prepared by allowing the alcohols to react with trifluoroacetic anhydride (Distillation Products, Inc.) at room temperature and evaporating to dryness with a stream of dry nitrogen. The residues were dissolved in chloroform for injection into the column.

O-Methyloximes were prepared by treating the ketone with O-methoxyamine (Applied Science Laboratories, Inc.)[4].

1-Microlitre injections of 5 mg/ml chloroform solutions of the steroids and their derivatives were made.

Results

The retention times (5α-cholestane = 1.0) and R_F factors are listed in Table II.

TABLE II

RETENTION TIMES AND R_F VALUES OF STEROID ALCOHOLS AND STEROID KETONES

Steroid alcohols	*Retention time (5α-cholestane = 1.0)*			R_F *value of free alcohol*
	Free alcohol	*TMS ether*	*Trifluoro-acetate*	
5α-Cholestan-1α-ol	—	0.77	—	0.80
5α-Cholestan-1β-ol	—	1.18	—	0.74
5α-Cholestan-2α-ol	6.81	1.81	1.68	0.32
5α-Cholestan-2β-ol	—	1.50	—	0.50
5α-Cholestan-3α-ol	5.75	1.38	1.49	0.43
5α-Cholestan-3β-ol	6.52	2.24	2.08	0.25
5β-Cholestan-3α-ol	7.12	1.83	1.69	0.39
5α-Cholestan-4α-ol	3.69	1.96	1.62	0.39
5β-Cholestan-4β-ol	4.56	1.41	1.21	0.48
5α-Cholestan-6α-ol	5.45	1.40	1.38	0.53
5β-Cholestan-6β-ol	—	1.03	—	0.64
5α-Cholestan-7α-ol	—	1.41	1.45	0.83
5α-Cholestan-7β-ol	—	0.698	0.71	0.69

Steroid ketones	*Retention time*		R_F *value of free ketone*
	Free ketone	*O-Methyl oxime*	
5α-Cholestan-2-one	5.47		0.25
5α-Cholestan-3-one	6.48	4.82	0.20
5β-Cholestan-3-one	5.64	4.08	0.23
5α-Cholestan-4-one	4.8	3.29	0.27
5β-Cholestan-4-one	3.8	2.35	0.41
5α-Cholestan-6-one	4.88	2.61	0.31
5β-Cholestan-6-one	4.04	2.56	0.24
5α-Cholestan-7-one	4.2	2.59	0.53

Discussion

Under the conditions of vapour phase chromatography used, the retention times of the free stanols were rather long and the peaks broad, resulting in considerable overlapping and poor resolution. The sharpest peaks and best resolution were obtained with the TMS ethers of the cholestanols. In cases where mixtures could not

be resolved using TMS ethers, trifluoroacetates were tried, as well as conversion of the stanols to ketones. The comparison of the R_F values of the cholestanols and cholestanones helped resolve the last doubts on the identity of the components of almost any mixture of the above-tabulated cholestanols.

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CHROM 3323

Gas chromatographic separation of 4'-nitroazobenzene-4-carboxylic acid esters of alcohols

The basic reagent 4'-nitroazobenzene-4-carboxylic acid chloride was first prepared by HECKER[1] in 1955, and later on used as a reagent for hydroxyl compounds as reported by BUTENANDT[2,3] during investigations on 'bombicol'. The separation of amines as 4'-nitroazobenzene-4-carboxamides has been described earlier[4]. In the same way the gas chromatographic separation of esters can be helpful in the identification of saturated and unsaturated aliphatic and aromatic alcohols as well as phenols.

Experimental

Apparatus. For chromatography, 1-m and 2-m stainless steel tubes (4-mm I.D.) packed with 2.5 % w/w silicone grease (E. Merck AG., Darmstadt, Germany) on 60 to 80 mesh Chromosorb G, acid washed and DMCS treated, were used in fractometer type F6 (Perkin Elmer Bodenseewerk, Überlingen, Germany). The fractometer was equipped with a flame-ionization detector and a 2.5-mV recorder (Siemens-Kompensograph L 288 × 288) with a paper feed of 0.5 cm/min.

Procedure. The 4'-nitroazobenzene-4-carboxylic acid esters were prepared according to HECKER[1] and purified to the melting points listed there. Previously unrecorded derivatives were crystallized to a constant melting point. Samples of 1 to 2 μg in about 5 μl of benzene were injected into the chromatograph.

TABLE I

RETENTION TIMES OF 4′-NITROAZOBENZENE-4-CARBOXYLIC ACID ESTERS OF ALCOHOLS

Parent alcohol	*Retention time (min)*
2-m column	
Methanol	4.0
Ethanol	4.8
1-Propanol	6.0
1-Butanol	7.6
1-Pentanol	9.0
2-Methyl-3-butanol	8.0
2,2-Dimethyl-1-propanol	7.4
1-Hexanol	12.6
1-Heptanol	16.2
3-Octanol	13.4
4-Octanol	12.8
2-Ethyl-1-hexanol	15.2
1-Nonanol	23.5
1-Dodecanol	47.8
1-Propen-2-ol (allyl alcohol)	5.8
2-Buten-1-ol	7.6
3-Hexen-1-ol	12.4
3,7-Dimethyl-2,6-octadien-1-ol (geraniol)	28.8
Lavandulol	28.2
1,2-Propanediol	8.4
2-Methyl-2,4-pentanediol	9.3
3-Hydroxy-2-butanone (acetoin)	8.3
Cyclolavandulol	29.2
Cyclopentanol	11.8
Cyclohexanol	15.6
Cycloheptanol	11.6
2,5-Dimethyl-cyclohexanol	20.0
3,4-Dimethyl-cyclohexanol	22.0
3,5-Dimethyl-cyclohexanol	17.2
Menthol	27.4
3-Thujanol	24.8
1,3,3-Trimethyl-2-norbornanol (fenchyl alcohol)	24.6 + 31.0 (two isomers)
Borneol	30.2
Phenol	16.0
o-Cresol	19.2
m-Cresol	21.0
p-Cresol	21.8
Benzyl alcohol	21.6
o-Ethyl-phenol	22.2
p-Ethyl-phenol	27.6
β-Phenylethyl alcohol	26.0
2,3-Xylenol	28.0
3,4-Xylenol	30.6
2,6-Xylenol	21.4
2,4-Xylenol	25.6
3,5-Xylenol	28.4
2,5-Xylenol	24.8
3,4,5-Trimethyl-phenol	42.0
2,3,5-Trimethyl-phenol	33.8
2,4,6-Trimethyl-phenol (mesitol)	28.2
Carvacrol	30.8
Thymol	28.0
Guaiacol	24.2

(*continued on p. 255*)

TABLE I (*continued*)

Parent alcohol	*Retention time (min)*
2-m column	
2-Allyl-phenol	25.7
1,2,3,4-Tetrahydro-2-naphthol	62.0
1-Naphthol	60.4
2-Naphthol	67.6
2-Methoxy-*p*-cresol (creosol)	29.2
o-Methoxy-benzyl alcohol	31.0
m-Methoxy-benzyl alcohol	31.0
4-Ethyl-guaiacol	36.4
4-Vinyl-guaiacol	39.2
5-Vinyl-guaiacol	43.6
2-Methoxy-4-propenyl-phenol (isoeugenol)	60.6
4-Allyl-2-methoxy-phenol (eugenol)	46.4
2,6-Dimethoxy-phenol	36.6
4-(2,6,6-Trimethyl-2-cyclohexen-1-yl)-butan-2-ol	46.4
4-(2,6,6-Trimethyl-1-cyclohexen-1-yl)-butan-2-ol	∞
p-Hydroxy-benzaldehyde	38.6
Vanillin	27.0
Isovanillin	58.2
5-Allylvanillin	66.0
2-(N-ethylanilino)-ethanol	44.6
Furfuryl alcohol	11.4
5-(Hydroxymethyl)-furfurol	28.8
3-Pyridinol	16.8
4-Pyridinol	19.4
1-m column	
1-Heptanol	4.2
3-Octanol	4.4
4-Octanol	4.0
2-Ethyl-1-hexanol	4.6
1-Nonanol	7.6
1-Undecanol	12.0
1-Dodecanol	14.6
1-Octadecanol	56.8
3-Hexen-1-ol	3.8
Nerol	9.0
1,3,3-Trimethyl-2-norbornanol (fenchyl alcohol)	9.4
o-Cresol	5.8
3,5-Xylenol	8.0
3,4,5-Trimethyl-phenol	12.0
Santalol	28.6
4-Methyl-1,2,3,4-tetrahydro-1-naphthol	19.8
1-Naphthol	18.6
2-Naphthol	20.4
o-Methoxy-benzyl alcohol	9.6
4-Ethyl-guaiacol	11.0
Isovanillin	16.7
Vanillylacetone	24.6
Furfuryl alcohol	3.8
3,4-(Methylenedioxy)-phenol (sesamol)	11.6
3-Pyridinol	4.8

A slight peak distortion caused by some bleeding of silicone grease from the column was overcome by using a relatively high hydrogen flow rate to the flame-ionization detector (30 ml/min).

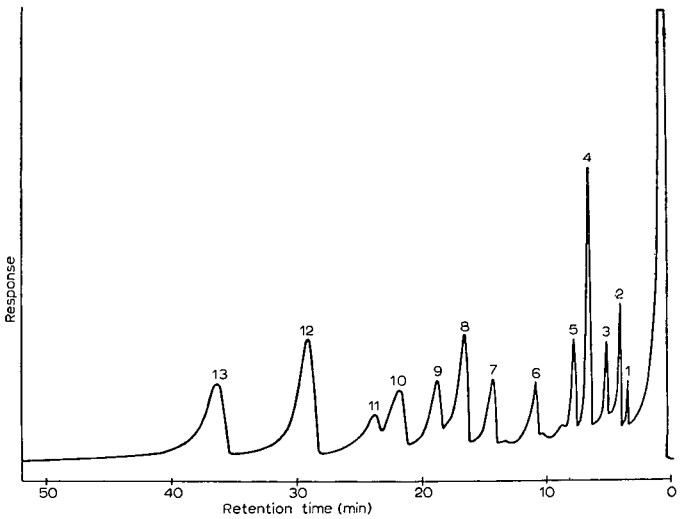


Fig. 1. Gas chromatographic separation of 4′-nitroazobenzene-4-carboxylic acid esters. Sample size: 1 to 2 μg of each derivative in 5 μl benzene. Constant column temperature: 270°; injection temperature: 300°; carrier gas: helium; flow rate: 30 ml/min. 1 = Methanol; 2 = ethanol; 3 = 1-propanol; 4 = 1-butanol; 5 = 1-pentanol; 6 = 1-hexanol; 7 = phenol; 8 = *o*-cresol; 9 = *p*-cresol; 10 = 2,3-xylenol; 11 = 3,4-xylenol; 12 = 2,3,5-trimethyl-phenol; 13= 3,4,5-trimethyl-phenol.

Results

Fig. 1 shows the gas chromatographic separation of a mixture of 4′-nitroazobenzene-4-carboxylic acid esters. Table I lists the retention times of all derivatives tested on the 2-m and 1-m columns under the conditions indicated in the legend of Fig. 1. The retention time of the reagent, 4′-nitroazobenzene-4-carboxylic acid chloride, is about that of the solvent, thus excluding any interference with the derivatives.

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CHROM. 3324

Gas chromatographic separation of 5-nitro-2-hydroxybenzal derivatives of unsymmetric hydrazines

5-Nitro-2-hydroxybenzal derivatives of unsymmetric hydrazines have been prepared and used for the separation and identification of hydrazines as well as N-nitroso compounds, the latter *via* reduction to hydrazines and formation of 5-nitro-2-hydroxybenzal derivatives. The method has been successful in the isolation of trace amounts of these compounds in complex mixtures, on a micro scale[1-5]. The separation shown in this paper was performed with derivatives of unsymmetric dialkyl-, aralkyl-, diaryl-, and cyclic hydrazines up to C_{12} as well as amino derivatives of tobacco alkaloids.

Experimental

Apparatus. For chromatography, a 2-m stainless steel tube (4-mm I.D.) packed with 2.5% w/w silicone grease (E. Merck AG., Darmstadt, Germany) on 60 to 80 mesh Chromosorb G, acid washed and DMCS treated, was used in fractometer type F6 (Perkin Elmer Bodenseewerk, Überlingen, Germany). The fractometer was equipped with a flame-ionization detector and a 2.5-mV recorder (Siemens-Kompensograph L 288 × 288) with a paper feed of 0.5 cm/min.

Procedure. The 5-nitro-2-hydroxybenzal derivatives were prepared as described earlier[2] and showed the melting points listed there.

Results

Fig. 1 shows the gas chromatographic separation of a mixture of 12 different 5-nitro-2-hydroxybenzal derivatives. Table I lists the retention times of all the derivatives tested on the 2-m column under conditions indicated in the legend of

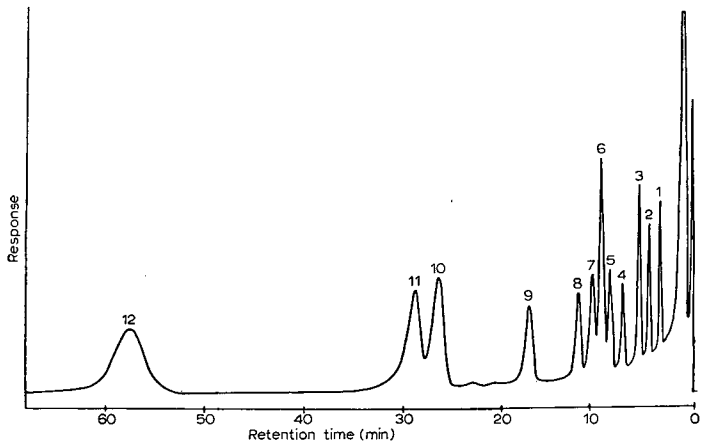


Fig. 1. Gas chromatographic separation of 5-nitro-2-hydroxybenzal derivatives of unsymmetric hydrazines. Sample size: 1 to 5 μg of each derivative in 5 μl acetic ester. Constant column temperature: 220°; injection temperature: 300°; carrier gas: helium; flow rate: 30 ml/min. 1 = 1,1-Dimethyl-hydrazine; 2 = 1-methyl-1-ethyl-hydrazine; 3 = 1,1-diethyl-hydrazine; 4 = 1-methyl-1-isobutyl-hydrazine; 5 = 1-methyl-1-butyl-hydrazine; 6 = 1,1-dipropyl-hydrazine; 7 = 1-methyl-1-pentyl-hydrazine; 8 = 1,1-diisobutyl-hydrazine; 9 = 1,1-dibutyl-hydrazine; 10 = 1-Ethyl-1-heptyl-hydrazine; 11 = 1-methyl-1-benzyl-hydrazine; 12 = 1,1-diphenyl-hydrazine.

TABLE I

RETENTION TIMES OF 5-NITRO-2-HYDROXYBENZAL DERIVATIVES OF UNSYMMETRIC HYDRAZINES

Parent hydrazine	*Retention time (min)*
1,1-Dimethyl-hydrazine	3.8
1-Methyl-1-ethyl-hydrazine	4.9
1,1-Diethyl-hydrazine	5.9
1-Methyl-1-butyl-hydrazine	8.5
1-Methyl-1-isobutyl-hydrazine	7.5
1,1-Dipropyl-hydrazine	9.3
1,1-Diisopropyl-hydrazine	8.5
1-Methyl-1-pentyl-hydrazine	10.2
1-Methyl-1-isopentyl-hydrazine	10.2
1-Ethyl-1-*sec.*-butyl-hydrazine	8.9
1-Ethyl-1-*tert.*-butyl-hydrazine	8.2
1,1-Dibutyl-hydrazine	16.0
1,1-Diisobutyl-hydrazine	11.5
1-Ethyl-1-heptyl-hydrazine	23.8
1,1-Dihexyl-hydrazine	51.8
1-Methyl-1-allyl-hydrazine	6.0
1-Ethyl-1-phenyl-hydrazine	23.4
1,1-Diphenyl-hydrazine	50.8
1-Methyl-1-benzyl-hydrazine	26.1
1-Amino-pyrrolidine	10.6
1-Amino-piperidine	12.0
1-Amino-2-methyl-pyrrolidine	11.1
1-Amino-2,5-dimethyl-pyrrolidine	11.0
1-Amino-4-methyl-piperazine	15.0
4-Amino-morpholine	11.5

Fig. 1. The retention time of the reagent 5-nitro-2-hydroxy-benzaldehyde is about 1 min, thus excluding any interference with the derivatives. The logarithm of the relative retention values was a linear function of the number of C-atoms.

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A sensitive method to detect traces of peptidase activity

During the course of investigations on the possible presence of an ε-peptide bond in bovine growth hormone (BGH)[1,2] by means of ε-peptidase[3] and reduction of the disulfide bonds, there was evidence for the breakdown of the hormone both in the presence and absence of enzyme. The breakdown could be a result of digestion by α-peptidase activity present in the preparation of growth hormone. No peptidase activity was detected by the method of casein digestion[4] even when the incubation period was extended to 3 h. Two distinct protein peaks were obtained upon gel filtration of BGH (NIH-GH-8) from the Endocrinology Study Section, National Institutes of Health, Bethesda, Md., on a Sephadex G-100 column[5,6]; the second peak contained most of the biological activity[6]. A sensitive method for detection of the peptidase activity was developed which gave evidence for the presence of peptidase activity associated with the first peak[7]. This study has now been extended for assay of proteolytic enzymes at very low concentrations and to look for proteolytic contamination of commercial enzyme preparations.

Bovine serum albumin (BSA) (Pentex, Inc., Kankakee, Ill.) was acetylated with ^{14}C-acetic anhydride[8] (New England Nuclear Corp., Boston, Mass.). The unreacted ^{14}C-acetate was removed by extensive dialysis and gel filtration on Sephadex G-25. Peptidase activity was detected by using ^{14}C-acetyl BSA as substrate; the hydrolysis of peptide bonds was ascertained by following changes in the elution profile of the radioactivity during gel filtration on Sephadex G-75.

By casein digestion an increase of absorbance of 0.05 at 280 mμ was observed at a concentration of 1 μg of trypsin per ml of the incubation mixture[9]. Tryptic activity was better detected by using ^{14}C-acetyl BSA as the substrate as illustrated in Fig. 1. There were two peaks of radioactivity: the first one (unretarded) represented unhydrolyzed ^{14}C-acetyl BSA and the second one (retarded) was peptide from hydrolysis of labelled protein. The extent of hydrolysis is directly proportional to the total

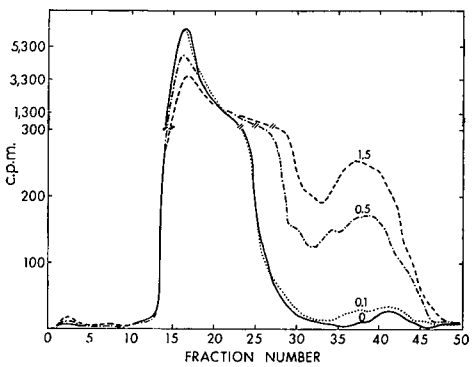


Fig. 1. Assay of trypsin using ^{14}C-acetyl bovine serum albumin. ^{14}C-Acetyl BSA (500 μg) and varying amounts of DFP treated trypsin (Worthington Biochemicals, Freehold, N.J.) in μg as indicated to a total volume of 0.2 ml in 1% NH_4HCO_3 were incubated at 37° for 1 h. The incubation mixture was subjected to gel filtration on a Sephadex G-75 column (31.5 × 0.8 cm) with 0.05 *M* NaCl as eluent. Fractions of 8 drops were collected and 5 ml of aqueous scintillation fluid[10] was added and counted in a Liquid Scintillation Spectrometer (Nuclear Chicago Mark I).

radioactivity of the second peak. The amount of radioactivity under the second peak was calculated by finding the area under the curve. There was a linear relationship between the amount of radioactivity of the second peak and the amount of trypsin up to 0.75 μg. Linearity was lost beyond 0.75 μg, probably because a significant portion of enzymatic hydrolysis involves further hydrolysis of peptides.

Using ^{14}C-acetyl BSA as the substrate and increments of chymotrypsin up to 1 μg, the enzyme was assayed. There were also two peaks of radioactivity, the second peak represented the extent of hydrolysis by chymotrypsin. Linearity was not observed between the amount of radioactivity of the retarded peak and concentration of enzyme. This may be due to the limited amounts of enzyme used or the broad specificity of hydrolysis of peptide bonds[11].

With ^{14}C-acetyl BSA as a substrate, low levels of trypsin and chymotrypsin which could not be detected by other methods were detected in a fast and easy way. In this method trypsin was not fully active on ^{14}C-acetyl BSA because many of the ε-amino groups were modified.

Pronase was assayed by casein digestion[12]. At a concentration of 2.75 μg of pronase per ml of the incubation mixture, an increase in absorbance of about 0.05 at 280 mμ was observed. Pronase (less than 2.75 μg) was assayed by using ^{14}C-acetyl BSA as the substrate. The second radioactive peak indicated the peptidase activity of the enzyme as in the case of trypsin and chymotrypsin.

Low concentrations of papain were also detected by using ^{14}C-acetyl BSA as substrate. Since ^{14}C-acetyl BSA was precipitated at low pH, it could not be used to detect pepsin, which acts best at about pH 2[13].

Peptidase activity was detected in samples of ribonuclease in 0.1 *M* tris-HCl, 0.05 *M* KCl, pH 7.4 (Fig. 2). It was absent in electrophoretically pure deoxyribonuclease in 0.1 *M* sodium acetate 0.015 *M* Mg^{2+}, pH 6.5 at which DNase was most active[15].

Peptidase activity at low concentrations of trypsin, chymotrypsin, pronase and papain was detected by using ^{14}C-acetyl BSA as substrate and observing changes in the radioactive profile upon gel filtration on a Sephadex G-75 column. Peptidase

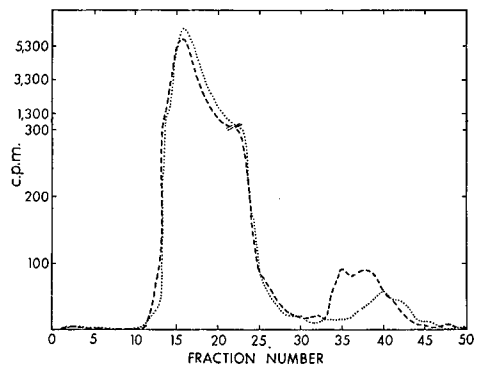


Fig. 2. Gel filtration of ^{14}C-acetyl bovine serum albumin treated with ribonuclease. ^{14}C-Acetyl BSA (500 μg) and ribonuclease (100 μg) to a total volume 0.2 ml in 0.1 *M* tris-HCl, 0.05 *M* KCl, pH 7.4[14] were incubated at 37° for 1 h with stirring and then subjected to gel filtration on Sephadex G-75 column (31.5 × 0.8 cm). Fractions of 8 drops were collected and counted for radioactivity. (- - -) ^{14}C-acetyl BSA treated with RNase; (· · · · ·) ^{14}C-acetyl BSA without RNase.

activity was found in samples of pancreatic ribonuclease. This method could be employed to detect traces of peptidase in biological preparations.

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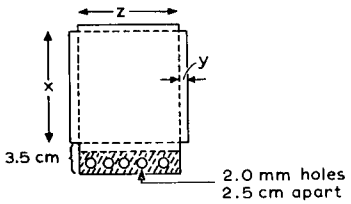
CHROM. 3381

Inexpensive preparative thin-layer and thick-layer chromatography

Preparative thin and thick-layer chromatography is a valuable tool for the organic chemist. But, in many cases, the cost of commercial equipment makes its use prohibitive. The following describes a durable, inexpensive, and highly flexible preparative thin and thick-layer system.

Description of the plates

The plates are constructed from sheets of 18 gauge stainless steel cut to the pattern shown in Fig. 1. The 0.50 cm edges are crimped and the corners welded. The bottom flap is crimped again and welded to the side edges (see Fig. 2).



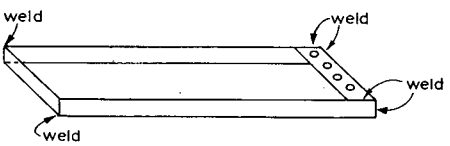


Fig. 1. Pattern for plates. $y = 0.50$ cm; x and z = variable dimensions.

Fig. 2. Side view of assembled plate.

Preparation of layers

The preparative thin layers (less than 1 mm in thickness) are prepared by slurring the adsorbent with chloroform or water to the consistency of a mobile syrup which is then poured onto the center of the plate. The adsorbent is uniformly distributed by tilting the plate until the surface is coated evenly, then the plate is dropped on a level surface several times to compact the adsorbent.

When chloroform is used as the liquid medium, the plates are dried by evaporation of the solvent at room temperature. They may be used without further activation. When water is used, the plates are air dried at room temperature, then activated at 120° for 1 h in an oven.

In the preparation of the thick layers (1 mm up to 5 mm), the same procedure is used except that water is used exclusively.

Developing tank

The developing tank was constructed of light weight stainless steel with the following dimensions: height 50 cm, length 75 cm, width 20 cm. A thin sheet of plywood is used for a cover. Solvent volumes are minimized by tilting the tank (Fig. 3).

Sample application and visualization

Samples are applied and visualized using the usual methods and reagents[1,2]. I_2 vapor may be used if a cup-like container is constructed from aluminium foil (Fig. 4).

Discussion

In our work, the stainless steel chromatographic system described has proved to be as good as the commercial equipment or better. The plates are unbreakable and therefore do not require special storage facilities. In addition, they can be constructed in any dimensions depending on the specific need. At present, we have been using two

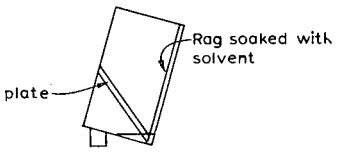


Fig. 3. Developing tank.

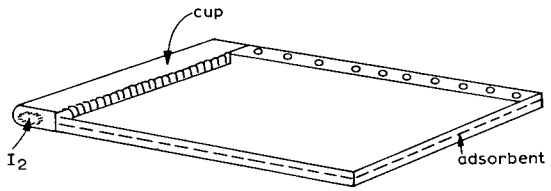


Fig. 4. Cup for staining edges with I_2 vapor.

sizes (30 cm × 30 cm × 0.50 cm and 30 cm × 60 cm × 0.50 cm) with excellent results in separating quantities from 50 mg up to approximately 10 g.

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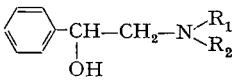
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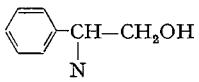
CHROM. 3391

Zum Nachweis isomerer Phenyläthanolamine

Die analytische Erfassung isomerer Phenyläthanolamine des Typs A und B, insbesondere in Gemischen, ist bislang nur in einzelnen Fällen untersucht worden[1].



A



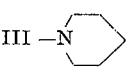
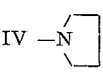
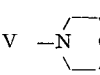
B

Dies resultiert daraus, dass die Isomere B vergleichsweise schwerer zugänglich und daher zum Teil noch nicht dargestellt waren und zum andern, weil rein methodisch die Voraussetzungen früher nicht günstig schienen. Papierchromatographisch wurden, wenn überhaupt, nur sehr geringe R_F-Differenzen festgestellt und auch I.R.-spektroskopisch[2] war nur in einem Einzelfall eine grobe Schätzung möglich. Überraschenderweise lagen auch gaschromatographisch nur negative Ergebnisse vor[2].

Nach Reindarstellung einer Reihe von Isomeren entwickelten wir zur qualitativen Bestimmung dünnschichtchromatographische Verfahren.

TABELLE I

DÜNNSCHICHTCHROMATOGRAPHISCHE TRENNUNG

$-N\langle^{R_1}_{R_2}$	*Fliessmittel α*		*Fliessmittel β*	
	Typ A	*Typ B*	*Typ A*	*Typ B*
I $-N(CH_3)_2$	0.40	0.57	0.38	0.31
II $-N(C_2H_5)_2$	0.32	0.58	0.60	0.59
III 	0.42	0.63	0.64	0.58
IV 	0.30	0.58	0.42	0.38
V 	0.80	0.78	0.59	0.46

Im Fliessmittelsystem α gelingt eine gute Auftrennung der Komponenten I–IV und auch in Mischungen ist infolge der relativ grossen R_F-Unterschiede—selbst bei ungünstigen Mengenverhältnissen— eine eindeutige Differenzierung möglich. Für die Morpholinderivate V ist diese Voraussetzung erst bei Anwendung des Fliessmittelsystems β gegeben. (Siehe Tabelle I.)

Noch eleganter ist die gaschromatographische Trennung an einer alkalisch unterlegten K-Säule. Hier kann eine Mischung aller zehn Aminoalkohole bei 200° recht gut getrennt werden (vgl. Fig. 1).

Zur optimalen Trennung der entsprechenden Isomere A und B empfehlen sich die in der Tabelle II genannten Bedingungen.

Auf die Weise ist auch eine quantitative Bestimmung einwandfrei möglich.

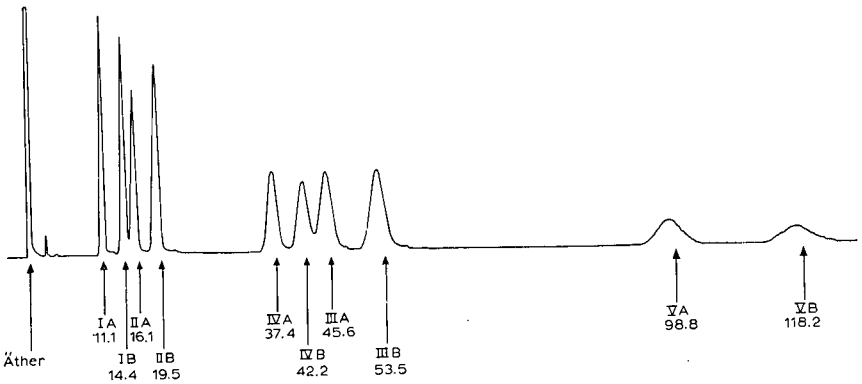


Fig. 1. Gaschromatogramm isomerer Phenyläthanolamine. Die Retentionszeit versteht sich vom Ätherpeak bis zum Maximum des Substanzpeaks und ist in Minuten angegeben.

TABELLE II

GASCHROMATOGRAPHISCHE TRENNUNG

Isomere	*Temperatur* (°C)	*N_2-Durchfluss*	*Retentionszeit (Min.)*	
			A	*B*
I	200	100 ml/Min.	11.1	14.4
II	200	100 ml/Min.	16.1	19.5
III	200	100 ml/Min.	45.6	53.5
IV	190	100 ml/Min.	45.3	50.8
V	220	100 ml/Min.	33.3	38.8

Experimentelles

Dünnschichtchromatographie. Sorptionsmittel HF_{254} (Merck); Aktivierung 1 Std. 110°. Schichtdicke 0.3–0.4 mm. Entwicklungslänge 10 cm bei 19–20°. Detektion: (a) U.V.-Fluoreszenzlöschung; (b) Dragendorff-Reagens modifiziert[3].

Fliessmittelsystem α: 96 % Äthanol–Wasser–konz. Ammoniak (40:60:2).

Fliessmittelsystem β: Benzol–Aceton–konz. Ammoniak (70:30:2).

Gaschromatographie. Gerät Hewlett-Packard, Modell 5754, Flammenionisationdetektor. Säule: Länge 3 m, Durchmesser 1/4 in.; Chromosorb 30/60 5 % mit KOH und dann 10 % mit Polyäthylenglykol 20000 belegt.

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CHROM. 3358

An improved method for the detection and radioassay of monosaccharides on thin-layer or paper chromatograms

Separation and quantitative determination of carbohydrates by thin-layer or paper chromatography usually requires the co-chromatography of these compounds with known standards. After visualizing the standards, corresponding areas are cut out of the paper or scraped off the thin-layer plate. The material is then extracted and the sugars are quantitatively determined. This technique may lead to erroneous results particularly if mixtures contain compounds with only slightly different R_F values.

It would therefore be advantageous if sugars could be visualized and then sharply separated from neighbouring compounds. However, most of the spray reagents in use at the present time cause the sugars to be washed out in the procedure or destroyed by heating during the development of color. These reagents may also form water-insoluble pigments. The strongly colored extracts may lower the efficiency of the detection of such labeled materials by liquid scintillation spectrometry because of quenching.

The present work describes techniques in which modified spray reagents have been developed which eliminate potential dilution of the saccharides. Furthermore for each of these reagents, a method of decoloration was devised, thus eliminating quenching in the subsequent liquid scintillation radioassay.

Experimental

D-Glucose-^{14}C was obtained from New England Nuclear Corp. Benzoic acid- ^{14}C in toluene standard was obtained from National Bureau of Standards. Silica Gel G was a Merck A.G. product. Other reagents used in the course of this work were analytical grade materials. The solvents were U.S.P. standard quality.

Silver nitrate spray reagent. The procedure by TREVELYAN *et al.*[1] was modified in the following fashion. A solution of 1 g of silver nitrate in 4 ml of water was added to 125 ml of acetone. Thin-layer plates or paper chromatograms were sprayed with this solution and air dried. Monosaccharides appear as dark spots on yellow background when the chromatograms were sprayed with a solution of 1.2 g of sodium hydroxide in 1.2 ml of water to which 98.8 ml of absolute ethanol had been added.

2,3,5-Triphenyl-2H-tetrazolium chloride spray reagent. This reagent was also originally described by TREVELYAN *et al.*[1]. In the present work, a 0.2 % (w/v) solution of triphenyltetrazolium chloride in 100 ml of chloroform was used. To this, 5 ml of triethylamine was added in order to replace the inorganic base used previously. The sugars were visualized by heating the plates or the paper chromatograms for 10 min at 110°.

p-Aminobenzoic acid spray reagent. The reagent used in the present experiments is a modification of a mixture described by ROY[2]. Oxalic acid (2 H_2O), 75 mg, was dissolved in 15 ml of ethanol. A solution of 150 mg of *p*-aminobenzoic acid in 25 ml of chloroform and 2 ml of acetic acid was prepared. The two solutions were mixed just prior to use. The plates were sprayed with the mixture and heated for 15 min at 110°. With this reagent, as little as 0.5 μg of glucose could be detected when viewed under ultraviolet illumination at 254 mμ.

Development of the chromatograms. Glucose-1-^{14}C, 5 to 25 μg, in an 0.1 % (w/v) aqueous solution corresponding to 10–50,000 d.p.m. was spotted on a Whatman No. 1 chromatography paper or on a Silica Gel G (250 μ thick) thin-layer plate. The papers were developed with ethyl acetate–pyridine–water (3.6:1.0:1.15, v/v/v), as described by COLOMBO *et al.*[3]. Thin-layer chromatography was carried out using *n*-propanol–water (7:1, v/v) as the developing solvent as described in a previous publication[4]. Glucose migrated with an R_F 0.62 in this thin-layer system. After the chromatograms were developed, the migration of glucose was visualized with one of the spray reagents. The thin-layer plates or the papers were dried and heated as required by the various techniques. The sharply delineated glycose-containing areas were cut out of the paper chromatograms or scraped from the thin-layer plates with a scalpel.

Decolorization of the spots and liquid scintillation radioassay. The paper discs or the colored silica gel scrapings were placed in low background liquid scintillation vials. When the spots were visualized with silver nitrate or triphenyltetrazolium chloride, 10 μl of a 4 *N* nitric acid solution were applied to the samples. Decolorization was usually complete in one hour. If necessary this reaction could be accelerated by heating the vials moderately with a hot air blower. To the decolorized samples was added 0.5 ml of water and the suspension was allowed to stand for 16 h. The brown spots obtained by the *p*-aminobenzoic acid spray were decolorized and extracted by treating the samples with 0.5 ml of an aqueous solution of 5 mg of sodium borohydride[5] overnight. The aqueous extracts were then mixed with 10 ml of a scintillation solvent containing 250 g of naphthalene, 24 g of PPO (2,5-diphenyloxazole) and 0.6 g of POPOP (*p*-bis[2-(5-phenyloxazolyl)]benzene) in 2 l of dioxane.

The recovery of labeled glucose from thin-layer plates was also studied by using a thixotropic gel counting solution. The plates were sprayed with the silver nitrate reagent, the carbohydrate containing areas were identified and were decolorized with 10 μl of a 4 *N* nitric acid solution as described above. The colorless scrapings were suspended in 10 ml of a 4 % *Cab-O-Sil* (w/v)scintillation solution containing 5 g of PPO and 0.3 g of POPOP per liter of toluene according to SNYDER AND STEPHENS[6].

Radioactive measurements. The counting was carried out in a *Packard Tricarb* model 3003 liquid scintillation spectrometer equipped with an external radium source for standardization. The efficiency for ^{14}C was 82 % when the dioxane scintillation solution was used. The average background count was 15 c.p.m.

Results and discussion

The recovery of glucose-^{14}C from the two types of chromatographic procedures ranged from 75 to 82 % of the theoretical (Table I). Control experiments were performed in which the recovery of labeled glucose was determined without irrigating the chromatograms. The sugar was spotted, visualized and decolorized. The radioactive counts obtained were about 10 % higher than those found after development with the respective solvent systems. With the use of the procedures outlined in this paper no quenching occurred. The lower recovery obtained by using the *Cab-O-Sil* suspension is not due to chemical or color quenching. The radioactive material is partly concealed by the silica gel absorbent. Estimation of quenching by the external source is therefore valueless under such condition.

These values represent the average of three or more determinations. Quenching was negligible when the quantity of nitric acid used to decolorize the spots obtained

TABLE I

RECOVERY OF GLUCOSE-^{14}C

Analytical procedure	*Spray reagent*			
	Tetrazolium (%)	*Silver nitrate* (%)	*p-Amino-benzoic acid* (%)	*Silver nitrate and Cab-O-Sil* (%)
Paper chromatography	79	82	77	
Thin-layer chromatography	81	81	75	64

by spraying with tetrazolium or silver nitrate was restricted to 10–20 μl of a 4 *N* solution. However, 0.1 ml of an 8 *N* nitric acid solution caused a 20 % decrease in counts. No quenching was observed with sodium borohydride which was used to decolorize the spots produced with the *p*-aminobenzoic acid solution.

All three spray reagents are useful for the detection of reducing sugars except for aminosugars. For the determination of amino- or acylaminosugars, the silver nitrate spray solution should be chosen since it gives dark spots with these monosaccharides as well. Of the reagents described, the *p*-aminobenzoic acid mixture is the most sensitive if the chromatograms are viewed under ultraviolet light.

The data presented in this paper are based on radioassays with glucose-^{14}C. Radioactivity of other reducing sugars can also be determined by the use of the same techniques. For accurate quantitative results, radioactive standards should be co-chromatographed.

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CHROM. 3376

Eine modifizierte Nachweisreaktion für Östrogene auf Papier- und Dünnschichtchromatogrammen

Mehrere spezifischere Farbreaktionen auf phenolische Steroide sind in der Literatur beschrieben[1-5]. Die hier vorgeschlagene *in situ* Nachweisreaktion ist leicht durchzuführen und spezifisch für Steroide mit einer freien phenolischen Gruppe. Sie basiert auf einer Abwandlung der Phosphormolybdänsäure-Farbreaktion. Phosphormolybdänsäure wird allgemein als ein unspezifisches Farbreagens für Steroide mit reduzierenden Eigenschaften verwendet[6-9]. Die von uns vorgeschlagene Modifikation beruht auf einer zweiten Behandlung des Chromatogrammes mit "Alkali".

Die Farbreaktion wurde durchgeführt, indem das Papierchromatogramm (nach einwandfreier Trocknung) zunächst durch eine alkoholische 8 %-ige Phosphormolybdänsäurelösung gezogen bzw. die Dünnschichtplatten damit besprüht wurden. In gleicher Weise erfolgte nun die Behandlung mit einem basischen Reagens (wässrige 2.5 %-ige KOH- oder eine 3 %-ige NaOH-Lösung, bei Verwendung von Ammoniak genügt es, die Chromatogramme in die Dämpfe einer 25 %-igen NH_3-Lösung zu halten).

Während der Behandlung mit dem basischen Reagens erscheinen die Steroide mit einer freien phenolischen Gruppe spontan als dunkelblaue Flecken.

Folgende Steroide wurden in einer Menge von *ca.* 10 μg getestet und ergaben eine positive Reaktion: Östron, Östradiol-17β, Östradiol-17α, Östriol, Equilin, Δ^{16}-Östratetraen, 2-Hydroxyöstron und 2-Methoxyöstron. Die beiden letztgenannten sehr reaktiven Verbindungen ergaben allein mit Phosphormolybdänsäure eine positive Farbreaktion. Die Farbintensität erhöhte sich jedoch wesentlich nach der Behandlung mit dem basischen Reagens. Bei allen anderen in gleicher Konzentration getesteten Steroiden, die keine freie phenolische Gruppe aufweisen (Östronsulfat, Östronmethylester, Östronacetat, Testosteron, Androstendion, Progesteron, 17α-Hydroxyprogesteron, 17α-Hydroxypregnenolon, Corticosteron und Cortisol), war die Reaktion negativ.

Die Empfindlichkeit der Methode wurde an Hand der Verbindungen Östron, Östradiol-17β und Östradiol-17α auf Dünnschichtplatten (Kieselgel nach Stahl, Fa. Merck, 0.1 mm Schichttiefe) erprobt. Die Platten wurden in einem Äthanol–Benzol (1:9)-System entwickelt. Bei allen drei Steroiden lag die untere Nachweisgrenze bei 1 μg/cm^2.

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CHROM. 3372

A quantitative indicator for the paper chromatographic or electrophoretic determination of polyaminocarboxylic acids

The increasing use of polyaminocarboxylic acids as complexing agents have necessitated the development of better methods of analysis. Paper chromatography and electrophoresis are potentially useful tools for the analysis of chelating agents in biological systems. For example, HILL-COTTINGHAM AND LLOYD-JONES[1] have separated EDTA, CDTA, and iron chelates by paper chromatography. The chelating agents were observed by staining with nickel dimethylglyoxime (Ni-DMG) producing white spots, and iron chelates observed by viewing under ultra-violet light. HILL-COTTINGHAM[2] found that the DARBEY reaction[3], utilizing Ni-DMG, produced white and pale-yellow spots with many complexing agents, while ninhydrin indicated relatively few. A note by BLASS AND VICAIGNE[4] reports the use of the Folin reagent for the detection of phenols.

With the exception of Ni-DMG, the detection methods are limited and restricted to only a small number of complexing agents. Although Ni-DMG is a general indicator for polyaminocarboxylic acids, the white or pale-yellow color produced on paper cannot be used for quantitative determinations requiring densitometry or other light-absorption methods. A general reagent which would enable the ready quantitative detection of polyaminocarboxylic acids is yet to be reported.

We found that phosphomolybdotungstic acid (the Folin–Ciocalteau reagent) possessed stable color characteristics with certain polyaminocarboxylic acids. Since these color reactions have not been characterized, quantitative applications of this reagent were tested. Polyaminocarboxylic acids in an Arizona soil were resolved with paper electrophoresis using the Folin reagent as an indicator to illustrate the application of this reagent to a biological system.

Experimental

Paper. Beckman R paper strips No. 320046 measuring 3.0 × 30.6 cm made for use with the Beckman Model R Durrum type paper electrophoresis system were used in testing the detection reagent for the polyaminocarboxylic acids and in the electrophoresis study of the complexing agents.

Detection reagent. Phosphomolybdotungstic acid 1 *N* (the Folin–Ciocalteau reagent), obtained from the Fisher Scientific Co., Fair Lawn, N. J., U.S.A. was used as the indicator for the polyaminocarboxylic acids. The reagent may be easily prepared according to the method described by COLOWICK AND KAPLAN[5].

Detection on paper. The detection procedure developed is as follows:

(1) The moist paper strips from the electrophoresis cell are removed and allowed to dry.

(2) The paper is then sprayed with 1 *N* Folin–Ciocalteau reagent until moist and yellow in color.

(3) This is followed by a second spraying with 2.8 *N* Na_2CO_3 (under a ventilating hood). A blue color indicating the chelating agent will begin to appear within a minute or two. Spraying of Na_2CO_3 is continued until the yellow color of the Folin reagent has nearly disappeared.

(4) The paper is then suspended in a closed container for about 15 min to allow the blue color of the chelate to fully develop and the yellow background color of the Folin reagent to fade away before being air-dried.

Polyaminocarboxylic acids and complexing agents not containing amino-carboxylic acid groups listed in Tables I and II, respectively, were applied to the paper and allowed to dry before testing for color reaction by the procedure above.

Electrophoresis of polyaminocarboxylic acids in soil solution extracts. Columns of 2.03 kg of Mohave sandy loam, a semiarid calcareous soil from southern Arizona, were wetted with 1.25 l of 8×10^{-2} *M* amounts of EDTA, EGTA, DCTA, HEEDTA, and DTPA in five separate treatments. Soil solution extracts were obtained from the five separate treatments and 30 λ from each were applied to five separate electrophoretic paper strips. Soil solution extract from untreated soil was applied to a sixth strip as a control. The electrophoresis buffer media having a pH and ionic strength of 8.2 and

TABLE I

POLYAMINOCARBOXYLIC ACIDS STUDIED

No.	*Chelating agents*	*Abbreviations*	*Amount tested* (μg)	*Purity* (%)
1	Ethylenediaminetetraacetic acid	EDTA	1.46	99.0
2	Ethyleneglycol bis(β-aminoethyl ether) N,N′-tetraacetic acid	EGTA	1.90	> 98
3	1,2-Diaminocyclohexanetetraacetic acid	DCTA	1.73	> 93
4	N-2-Hydroxyethyl-ethylenediamine-N,N′,N′-triacetic acid	HEEDTA	1.39	> 98
5	Diethylenetriaminepentaacetic acid	DTPA	1.97	> 98
6	Uramil-N,N-diacetic acid	UDA	1.39	> 98
7	β-Alanine-N,N-diacetic acid	ADA	1.04	—
8	N,N′-Ethylenebis[2-(*o*-hydroxyphenyl)]-glycine	EDDHA	1.71	94.8
9	N,N-Bis(carboxymethyl)anthranilic acid	CMA	1.26	—
10	N,N-Di-2-hydroxyethyl-glycine	DHEG	0.82	—
11	Diaminoethyl ether tetraacetic acid	chel Me[a]	1.68	—
12	Ethylenediaminedi-*o*-hydroxyphenylacetic acid	EDHPA	1.66	> 95
13	N-Methyliminodiacetic acid	MeIDA	1.47	> 95
14	N-2-Hydroxyethyl-iminodiacetic acid	HEIDA	1.77	> 95
15	Nitrilotriacetic acid	NTA	15.28	—
16	Diaminopropanetetraacetic acid	DPTA	12.24	—
17	Ethylenediamine-N,N′-diacetic acid	EDDA	3.52	> 95

[a] Trademark of Geigy Chemical Company.

TABLE II

COMPLEXONES GIVING NO COLOR REACTION WITH THE FOLIN REAGENT

No.	*Chelating agent*	*Abbreviation*	*Amount tested* (μg)	*Purity* (%)
1	5-Sulfosalicylic acid	SSA	50.84	99.7
2	DL-Malic acid	—	13.41	> 99
3	D-Tartaric acid	—	15.01	99.0
4	Adipic acid	—	14.61	—
5	Itaconic acid	—	13.01	—
6	Adenosine-5′-phosphate	AMP	36.42	99
7	Adenosine-5′-triphosphate	ATP	60.50	99

0.30, respectively, consisted of 13.00 ml of 0.20 *M* $NaH_2PO_4 \cdot H_2O$ and 488.5 ml of 0.20 *M* $Na_2HPO_4 \cdot 7H_2O$ diluted to 1 l with deionized water. A potential difference and current intensity of 75 V and 5.3 mA, respectively, was applied for a duration of 8.5 h after which the strips were air-dried and sprayed with the Folin reagent and Na_2CO_3 as described.

Densitometric determination of DTPA. Applications of 10, 20, 30, 40, 50, 60, and 70 mμ moles of DTPA were spotted on a paper strip, dried and color-developed. A standard extinction curve was drawn by use of a Spinco Model RB "Analytrol" Densitometer (Spinco Division, Beckman Instruments, Inc., Palo Alto, Calif. U.S.A.) which measures and records the light absorbed by colored material distributed on a paper strip placed before a measuring photocell according to the Lambert-Beer Law:

$$E = \log \frac{I}{I_0} = K\frac{a}{f} = kcd$$

where E is the extinction or light absorbed; I_0 and I, the light intensity transmitted through the blank paper and through the colored spot, respectively; K and k, proportionality constants; a, the weight of material on the paper; f, the area of the strip perpendicular to the transmitted light beam; d, the thickness of the layer; and c, the color concentration.

The instrument contains an integrator which automatically produces saw-tooth patterns representing the areas or extinctions under the densitometric curves. These objective values of the area were plotted against concentration of the separate DTPA fractions to yield a standard extinction curve.

Results and discussion

A blue spot showing the presence of the chelating agents on paper was produced when sprayed with the Folin reagent, with all polyaminocarboxylic acids listed in Table I. The color reaction occurred with all polyaminocarboxylic acids examined. Table II lists complexing agents examined which did not give any color with the Folin reagent.

Fig. 1 illustrates the result of electrophoresis of soil extracts and spraying with the Folin reagent. Strips numbered 1 through 5 represent electrophoresis of soil extracts from soils treated with EDTA, EGTA, DCTA, HEEDTA, and DTPA, respectively. Strip No. 6 represents a control, the electrophoresis of extract obtained from untreated soil.

The Folin reagent served as an excellent indicator in the above case, showing the movement of complexones toward the positive pole due to the negative charge on the chelate complexes.

Three separate blue bands were observed on strip No. 5. Since only DTPA was added, we suggest that the three color bands represent a separation of three distinct metal-chelate complexes of DTPA. (These were not identified.) No colored regions were observed with the control strip No. 6.

Fig. 2 shows the absorption peaks plotted by the "Analytrol" densitometer corresponding to blue spots of 10, 20, 30, 40, 50, 60, and 70 mμ moles of DTPA developed by the Folin reagent. The saw-tooth patterns below each spot represent the areas under the absorption peaks determined by the "Analytrol" integrator. Other complexones were not tested quantitatively so far.

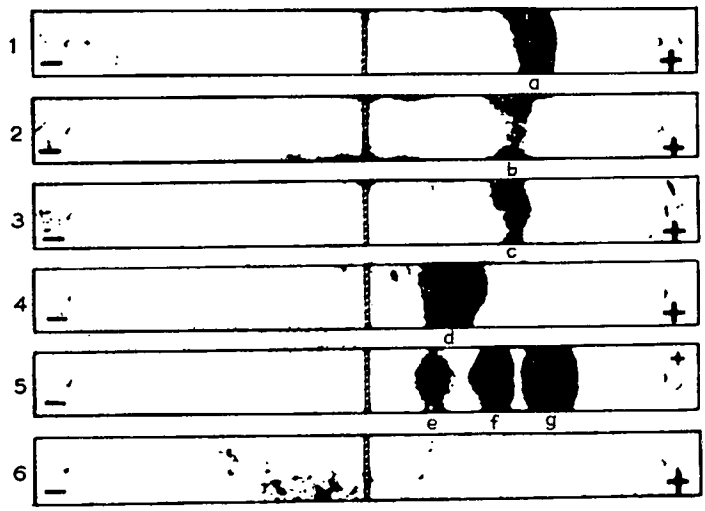


Fig. 1. Electrophoretic movement of polyaminocarboxylic acids from soil solution extracts after treatment with the Folin reagent. Strips numbered 1 through 6 represent soils containing EDTA, EGTA, DCTA, HEEDTA, DTPA, and control, respectively.

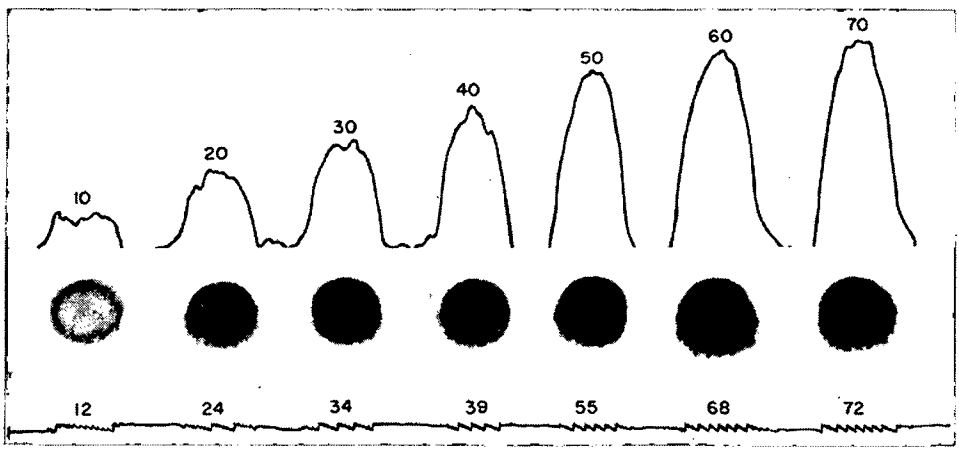


Fig. 2. Absorption peaks of blue DTPA spots plotted by a densitometer with a wavelength of 6000 Å. The figures above each peak from left to right represent increasing amounts of DTPA in mμmoles. Below each spot is given the total number of saw-tooth patterns or area under each curve.

The extinction of area under each of the above peaks plotted against amount of DTPA produced the standard curve shown in Fig. 3. The points were linear from 0–70 mμ moles of DTPA. A definite negative deviation from linearity occurred with amounts > 70 mμ moles.

The color reactions for the quantitative detection of certain complexones, which

utilizes phosphomolybdotungstic acid as an indicator for paper chromatographic and electrophoretic techniques, were found to be stable and quantitatively measurable with a densitometer.

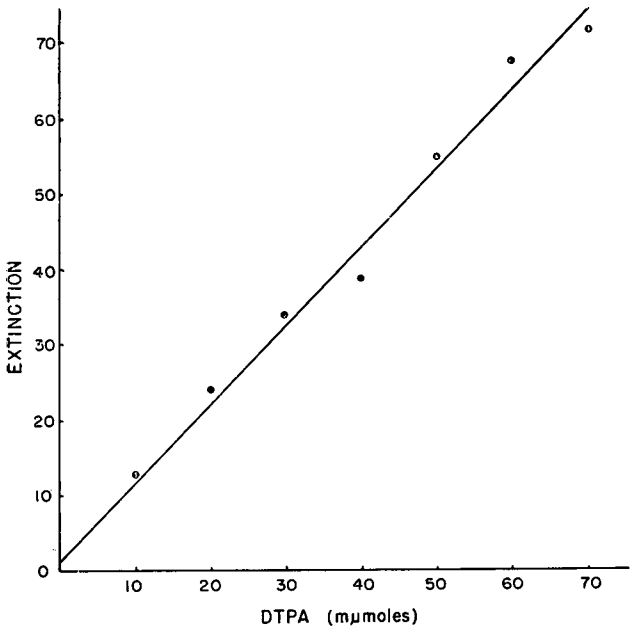


Fig. 3. A standard absorption curve for DTPA with extinction (area under absorption curve) plotted against increasing amounts of DTPA. The values γ and y represent the product-moment coefficient of linear correlation and the least-square equation of the line, respectively. $\gamma = 0.9991$; $y = 1.044x + 1.333$.

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Comparative electrophoresis of cytidines, adenines, uracils and guanines*

The separation of the individual compounds of the four ribobase families is difficult and time consuming. Each of the four groups is composed of five common members ranging in complexity from ribobase to riboside triphosphate. A relatively simple and extremely rapid electrophoretic resolution appeared possible by means of a technique previously described for the separation of the adenines[1], RNA nucleotides[2], nucleosides[3] and nucleobases[4]. The uncomplicated characteristics of the separatory system along with the simple tools required for the electrophoretic step could make this a useful analytical adjunct to either chromatographic determinations or other separation procedures. The present study will illustrate the following:

(1) The separation of the five members of the cytosines, adenines, guanines and uracils at both alkaline and acid pHs.

(2) The relationship of the mobilities of the separated compounds to those of the adenines as a baseline of study.

(3) The position of uric acid in the electrophoretic separation of the adenines to see if it is a possible source of interference in the analytical scheme for free nucleotides.

Materials and methods

Reagents

Nucleobase families. The five adenines, cytosines, guanines and uracils representing the bases, nucleosides and mono-, di- and triphospho-nucleosides were prepared as four mixtures of five compounds. Ten milligrams of each of the five compounds containing the same base were dissolved in 1 ml of H_2O. Guanine represented a special problem in the study. For alkaline electrophoresis 10 mg of guanine were dissolved in 1 ml of 0.5 *N* NaOH with gentle heating. The remaining members of the guanine family were dissolved in a separate solution as described above. A sliver of Whatman No. 3MM paper was dipped into the guanine solution, dried and then 2–3 μl of the second solution were pipetted onto the sliver. Alternatively, the dried sliver containing the guanine was quickly dipped into the second solution before electrophoresis. This wetting step did not cause the guanine to dissolve off.

For acid electrophoresis, 10 mg of guanine were dissolved in 1 ml of 0.05 *N* HCl and the procedure described for alkaline electrophoresis was followed.

Alkaline buffer (4X), pH 9.6. Prepare a buffer solution to contain 3.728 g of KCl, 3.092 g of boric acid and 43.7 ml of 0.5 *M* NaOH per liter.

Electrophoresis

Agarose gel (0.3 %) was prepared with the acid buffer as previously described. Agar gel (0.3 % Ion Agar No. 2) was prepared with the alkaline buffer in the same manner as described for the agarose. Each gel when used was pipetted onto a lantern slide and allowed to solidify. The semi-solid gel was connected to the buffer in the

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baffle boxes with filter paper wicks (Schleicher and Schuell No. 900). Solutions of each of the four 5-membered families were transferred to slivers of Whatman 3MM filter paper by dipping (the slivers) into the respective solutions and then blotting them free of excess liquid.

The slivers were placed in the center of the 4 inch axis for the pH 3.1 gel and 1.25 in. from the cathode in the pH 9.6 gel, and 250 V was applied to the entire system for 15 min. At the end of this time the voltage was disconnected, the positions of the different compounds were observed under a 256 nm ultraviolet lamp and their distances from the starting point were recorded.

Results and discussion

The resolution characteristics of the four groups of five compounds are shown in Figs. 1 and 2 for both the acid and alkaline mediums of agar and agarose gel respectively. The uracil–uridine pair migrate at the same speed in acid mediums during the 15 min time period and represent the only coincidental mobilities shown for an individual family. As a single advantage, in alkaline medium (Fig. 2) there is no set in which the same mobility was obtained for any two compounds of a family. In general, the acid resolution was better than the alkaline in terms of reproducibility of mobility. The order of separation of nucleotides varied with the pH for it can be seen that the

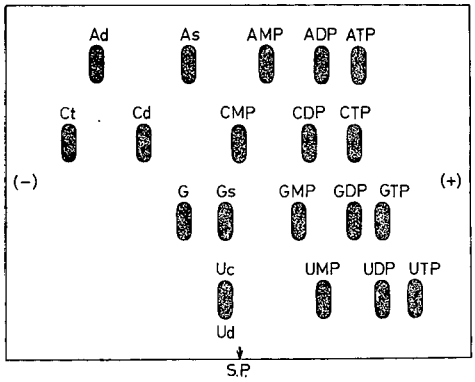


Fig. 1. Separation of the families of compounds at pH 3.1. Potential applied: 250 V; time: 15 min; 0.3% agarose.

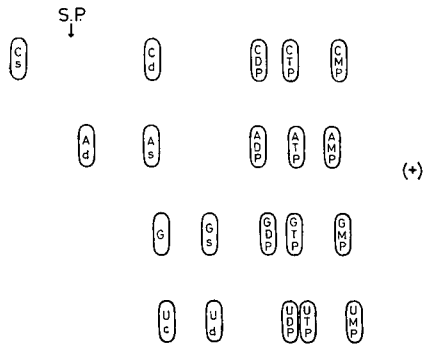


Fig. 2. Separation of the families of compounds at pH 9.6. Potential applied: 250 V; time: 15 min; 0.3% agarose.

triphosphate, and monophosphate of the pH 3.1 system change places in the alkaline system in every group while the others maintain their same relative positions.

A practical probability for the described procedure is the separation of free nucleotides such as adenine compounds in biological systems. But a potential interference could be uric acid, a fact which has been previously reported[5]. Therefore, an experiment was carried out to test this possibility. Solutions of the individual adenine compounds and uric acid were prepared as well as the mixture which was contaminated with uric acid. The results are shown in Fig. 3.The individually electrophoresed

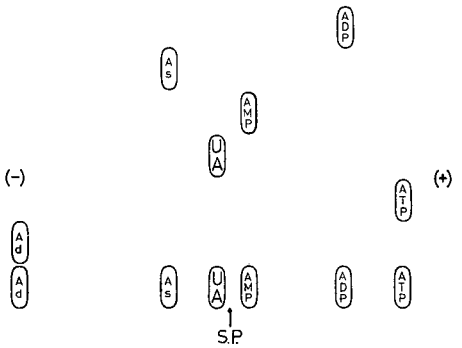


Fig. 3. Position of uric acid in the electrophoresis schematic of the pH 3.1 separation of the adenine family. Potential applied: 250 V; time: 15 min; agarose 300 mg/100 ml.

compounds had the same mobility characteristics as did the same compounds in a mixture and the uric acid moved very slightly toward the cathode. There is obviously no interference from uric acid in this particular separatory scheme.

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CHROM. 3382

Purification of antidiuretic hormone by high voltage electrophoresis*

During the course of a project involving antidiuretic hormone, it was realized that milligram quantities of this substance would be required. Commercial sources indicated that antidiuretic hormone from animals was electrophoretically, and therefore chemically, impure. Further investigation revealed that the hormone is most difficult to prepare in large quantity by a synthetic route[1]. Hence, it seemed most logical to attempt to purify the commercially available product.

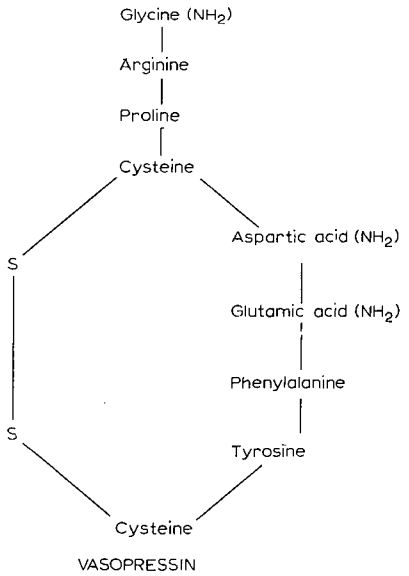


Fig. 1. Structure of arginine-8-vasopressin (human antidiuretic hormone).

Human antidiuretic hormone is arginine-8-vasopressin (Fig. 1)[2]. If arginine is replaced by leucine, and phenylalanine by isoleucine, the structure becomes oxytocin, a hormone with uterus-contracting and milk-ejecting properties. Oxytocin is the most physiologically active contaminant of vasopressin preparations from animal sources; both vasopressin and oxytocin are stored in the posterior lobe of the pituitary gland, the most common commercial source of these hormones[3]. As early as 1941, duVigneaud and associates suggested that these two compounds could be separated by electrophoresis[4]; later work showed that they have sufficiently dissimilar isoelectric points (pH 10.9 for vasopressin and pH 7.7 for oxytocin[5]) for good separation by this technique. At a pH of 9.4, then, vasopressin would migrate toward the cathode and oxytocin toward the anode. Accordingly, preparative electrophoresis seemed to be a feasible method for large-scale preparation and purification of antidiuretic hormone.

High-voltage electrophoresis was chosen over the orthovoltage technique since

* The mentioning of trade names and manufacturers does not constitute approval or official endorsement of these products by the U.S. Government.

the high-voltage procedure will effect a separation in a much shorter period of time. Time can be of great importance in manipulating polypeptides, since these compounds are readily attacked by omnipresent bacteria and can be thermally inactivated relatively easily under conditions of electrophoresis.

The instrument selected for the procedure was manufactured by Savant Instruments Incorporated. The electrophoresis chamber contained 6 l of buffer in each of two compartments, overlaid with 50 l of high flash point paint thinner (Varsol B®) as a coolant. Supplemental cooling was accomplished by tap water circulating through coils immersed in the paint thinner. The buffer employed in the procedure consists of a 0.05 *M* solution of sodium tetraborate, adjusted to a pH of 9.4 with sodium hydroxide. The buffer was used for 10 to 12 electrophoretic runs before a change in pH was noted.

The starting material for the purification of vasopressin was a product manufactured by Parke-Davis, Pitressin®, an aqueous solution of the pressor and antidiuretic principle of the posterior pituitary gland. Forty milliliters of this solution was freeze-dried to reduce its volume for spotting on the filter paper. The resulting powder was taken up in 0.8 ml of distilled deionized water, a sufficient amount to just dissolve the solid material. The solution was applied as a streak with a disposable capillary pipet to the center of an 18 × 42 in. piece of filter paper. The width of the streak was kept to less than 0.5 cm, and was dried between applications with a forced-air drier. The paper was transferred to the support rack, carefully saturated with buffer, and immersed in the electrophoresis chamber. Electrophoresis was performed for 2 h at 2500 V. At the completion of the run, the rack was removed and set in front of a fan for air drying at room temperature. A strip was cut from each side of the paper, including about 0.5 cm of the streak. These strips were stained by dipping into ninhydrin solution and heated to 100°C for 5 min to develop the color. They were then returned to their original positions alongside of the unstained portion and fastened to it with tape. Several bands were noted on the stained portion (Fig. 2), corresponding to electrophoretically separable polypeptides. The band closest to the anodal side of the line of application migrated with an oxytocin standard. The compound of interest, vasopressin, corresponded to the band closest to the cathode. Fig. 3 shows an electrophoretogram of the purified product and synthetic arginine-8-vasopressin (furnished gratis by Sandoz Pharmaceuticals Inc.).

A narrow strip, corresponding to this latter band, was cut from the unstained portion of the sheet parallel to the line of application. This strip was placed in a glass chromatography column and eluted by slowly dripping deionized distilled water on the top of the strip. Twenty milliliters of eluate was collected over a period of about 15 min. This solution was freeze-dried, yielding 15 mg of a fluffy white powder, of which approximately 75 % was vasopressin and 25 % was sodium tetraborate from the buffer.

It was quite apparent that a considerable fraction of the starting material was not vasopressin. Some of it had the electrophoretic properties of oxytocin, while the remainder of the material migrating to the anode apparently consisted of lower molecular weight fragments.

Although the procedure described here is relatively simple and straight-forward, certain precautions should be observed for optimal results. It is important that Pitressin® solution be kept refrigerated and sterile prior to use in order to prevent

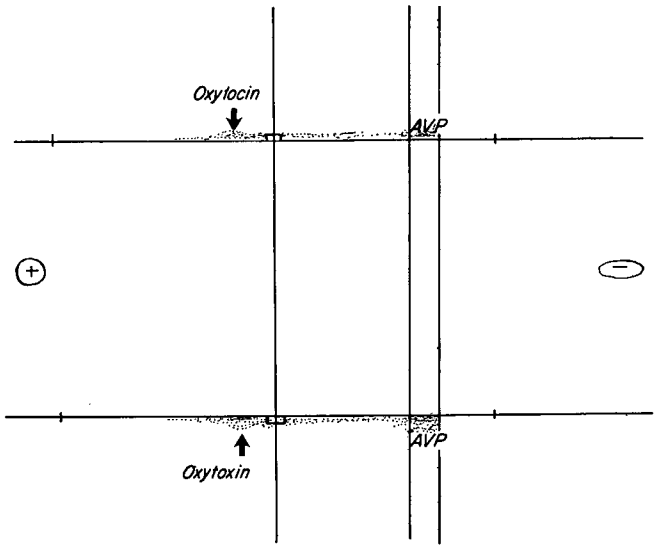


Fig. 2. Electrophoretogram of Pitressin®. The two outer edges are stained with ninhydrin, showing electrophoretic nonhomogeneity of the preparation. The parallel lines at the negative side of the center delineate the vasopressin fraction which is subsequently eluted. Arrows point to the fraction corresponding to oxytocin.

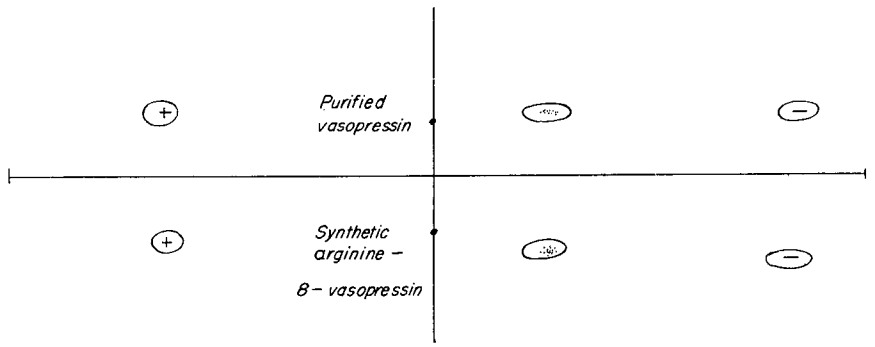


Fig. 3. Electrophoretogram of purified vasopressin and synthetic arginine-8-vasopressin, stained with ninhydrin. The mobilities of the two compounds are identical.

decomposition. Freeze-dried Pitressin®, in our experience, can be kept at freezer temperature without sterile precautions for several months. When it is reconstituted with water, it should be electrophoresed promptly. The temperature of the electrophoresis chamber should be kept at room temperature or only slightly above during the run. Electrophoresis current should be kept below 200 mA so that suspension of water in the coolant is minimal, thereby reducing markedly elution of the Pitressin® from the paper. Buffer should be applied to the paper very carefully; it should be allowed to wet the streak by osmotic action rather than by direct application, which causes the applied material to assume a larger area on the paper. The band containing vasopressin should be eluted as soon as possible after drying the paper, but storing the strip overnight in a freezer does not seem to be detrimental. The eluate should be

freeze-dried immediately after elution; the resulting powder can be kept at freezer temperature for several months.

Purified antidiuretic hormone (vasopressin) can thus easily be obtained from commercial posterior pituitary extract by the use of preparative high voltage electrophoresis. The resulting product appears to be chemically identical to the synthetic hormone.

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CHROM. 3353

Electrophoretic behaviour of copper(II) histidinate in the presence of the other copper(II) amino acid chelates

Recently[1] we studied the electrophoretic behaviour of copper(II) amino acid chelates at different pH values. We noticed that complexes of dicarboxylic amino acids and histidine behaved somewhat unusually. Splitting of the former into two distinctive Cu^{2+} positive spots may be due to the structure of these complexes, as suggested by PFEIFFER AND WERNER[2]. On the other hand at pH 11.98 copper(II) histidinate yields two spots, one of which moves to the anode and the second to the cathode. WIELAND AND FISCHER[3], in 1948, also observed the somewhat peculiar behaviour of copper(II) histidinate in the presence of copper(II) lysinate, *viz.* the formation of a third spot during the electrophoretic separation of the two complexes. They suggested that this third spot was the mixed complex copper(II) histidino–lysinate. Owing to the fact that coordination of histidine with Cu^{2+} ions is unusual (see for example references 4, 6, 8) and because the kinetic lability of copper(II) amino acid complexes in aqueous solution restricts the formation of mixed amino acid complexes, the authors have studied the electrophoretic behaviour of copper(II) histidinate in the presence of the other chelates more extensively.

Thus we found that the existence of the third spot described by WIELAND AND FISCHER is observable only in the case of copper complexes of basic amino acids (with the exception of α,β-diaminopropionic and α,γ-diaminobutyric acids owing

to their low mobilities). A similar effect with Cd^{2+}, Ni^{2+} and Zn^{2+} complexes was not observed. Nor did the third spot form when copper(II) complexes of basic amino acids were separated from each other.

Quite different results were obtained when the separation of a mixture of complexes and free amino acids was made. In the first case, when a mixture of copper(II) complexes of basic amino acid and L-histidine was separated, we detected on the electropherogram the copper complex of the amino acid and copper(II) histidinate only. On the other hand, mixtures of copper(II) histidinate and basic amino acid after separation yield a third spot as well as copper complexes of both histidine and the basic amino acid (see Table I).

TABLE I

Separation of pairs	*Mobilities ($cm^2/V \cdot cm$)*		
	Cu^{2+}-histidinate	*Third spot*	*Other part of pair*
Cu^{2+}-histidinate + Cu^{2+}-ornithinate	$0.51 \cdot 10^{-4}$	$1.11 \cdot 10^{-4}$	Cu^{2+}-ornithinate $1.66 \cdot 10^{-4}$
Cu^{2+}-histidinate + Cu^{2+}-lysinate	$0.51 \cdot 10^{-4}$	$1.06 \cdot 10^{-4}$	Cu^{2+}-lysinate $1.64 \cdot 10^{-4}$
Cu^{2+}-histidinate + Cu^{2+}-argininate	$0.50 \cdot 10^{-4}$	$0.98 \cdot 10^{-4}$	Cu^{2+}-argininate $1.44 \cdot 10^{-4}$
Cu^{2+}-histidinate + lysine	$0.51 \cdot 10^{-4}$	$1.09 \cdot 10^{-4}$	Cu^{2+}-lysinate $1.64 \cdot 10^{-4}$
Cu^{2+}-lysinate + histidine	$0.50 \cdot 10^{-4}$	—	Cu^{2+}-lysinate $1.62 \cdot 10^{-4}$

Further we found that the phenomenon described is observed only at pH 7.5 and depends on the composition of the buffer used. The concentration of the complex applied and changes in potential gradient have little effect.

In order to investigate the characteristics of the third spot, in all the cases where the formation of this third spot was observed, the latter was eluted from the paper and subjected to electrophoresis under the conditions described. On visualisation (both for Cu^{2+} ions and for the amino acids) the presence of copper(II) histidinate was always demonstrated. This result, in addition to the facts described above, *i.e.* the existence of the third spot only in the case of copper(II) complexes, its formation only in the course of separation of a mixture of copper(II) histidinate and free basic amino acids, but not when free histidine and copper complexes of basic amino acids were separated, and furthermore dependence on the pH value and composition of buffer, suggests that the third spot is more likely to be the result of interaction between copper(II) histidinate and complexes of basic amino acids, in the sense of coulombic attraction, than to be due to the formation of mixed amino acid complexes. Our conclusions are based on the fact that, in all cases, the third spot was only detected as copper(II) histidinate and further on the literature reports on histidine coordination. According to ALBERT[4], the structure of copper(II) histidinate in aqueous solution is unsymmetrical. One molecule of histidine is attached to copper normally, but the second is not attached to the Cu^{2+} through all three histidine sites[5]. The weakly coordinating donor centres (probably carboxylate oxygen, because all nitrogen donor atoms lie in the plane[6]) might be displaced by the water molecule[7]

and that may interact with the free ω-amino group of the copper(II) complex of basic amino acids. Formation of unsymmetrical copper complexes is due to the strong tendency of copper(II) ions to form square planar structures. Furthermore the dependence of the phenomenon observed on the pH value may be caused both by the composition of copper(II) histidinate in aqueous solution as such which changes with the pH value[8] giving rise to flexidentate chelation of histidine, and also by the fact that at pH 7.5 ω-amino groups of copper(II) complexes of basic amino acids have a positive charge.

The electrophoretic mobilities are given in Table I.

Experimental

Copper chelates were prepared from the L-isomers of amino acids according to the method of ABDERHALDEN AND SCHNITZLER[9].

10 μl of an 0.01 *M* solution of chelates were applied on Whatman No. 1. paper. Electrophoresis was carried out in a horizontal Tatrachema apparatus (Č.S.S.R.) under the following conditions: 0.1 *M* CH_3COONa buffer, pH 7.5, ionic strength 0.1; potential gradient 15 V/cm.

The electropherogram was sprayed with a solution of rubeanic acid and ninhydrin, respectively, to detect the spots.

All mobilities given in Table I were corrected for electroosmosis, glucose being used as zero marker.

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CHROM. 3365

Separation of arsenite and arsenate ions by paper chromatography

Study of a methanol–ammonia–water solvent system

The possibility of separating elements in different valency states is of importance in many fields of scientific research. A simple sensitive method for this purpose is paper chromatography. The possibility of separating arsenic in the two valency states is of importance in toxicological chemistry because of the different toxicity of arsenic-(III) and (V) compounds. There are not many publications available dealing with the chromatographic separation of the two valency states of arsenic.

The main difficulty in ascending paper chromatography of the two valency states of arsenic is the interference of some metals, especially Sb(III, V), Sn(II,IV), Fe, Cu and Zn. Also the R_F values for separating As(III) and As(V) were not sufficient. This fact was proved by a systematic study using mixtures of acetone, methyl ethyl ketone or methanol, and hydrochloric or nitric acid as solvents[1].

POLLARD, MCOMIE AND BANISTER[2] separated As(III) from As(V) with R_F 0.55 and 0.75, respectively, using ascending paper chromatography in the solvent mixture conc.HCl–water–ether–methanol (4:15:50:30, by vol.). Carefully standardized conditions were necessary in order to obtain completely reproducible R_F values. By the ascending method with butanol saturated with 1 N HCl, LEDERER[3] separated successfully As(III) and As(V) from Ge(IV) with R_F 0.84, 0.52 and 0.26, respectively. The mixtures with butanol as solvent required several hours for development. OVERBY, BOCCHIERI AND FREDRICKSON[4] described a paper chromatographic method for qualitative and quantitative estimation of arsenate-^{74}As, arsenite-^{74}As, arsenilic-^{74}As acid and related organic arsenicals. The two solvent systems: acetonitrile–HNO_3–water (78:2:20, by vol.; with an up-flow for 2 h or down-flow for 3 h) and isopropyl alcohol–water (7:3, by vol.; using an overnight or 8-h descending development) are described to give good separations of sodium arsenite from sodium arsenate.

The method depending on the use of an alkaline solvent mixture (ethanol–pyridine–ammonia (0.88)–water (60:20:4:16, by vol.)) was described by ELBEIH AND ABOU-ELNAGA[5] for the analysis of anions based on paper chromatography. The R_F values for the separation of arsenate from arsenite were not sufficient (R_F AsO_4^{3-} = 0.03 and AsO_3^{3-} = 0.12). YAMAGUCHI[6] recommended the solvent system methanol–1 N NH_4OH (3:1) for the effective separation of arsenite from arsenate (with R_F 0.54 and 0.00, respectively).

Since a speedy separation is desirable in toxicological analysis, attention has been focussed upon fast-moving solvents. Therefore we studied in detail the chromatographic behaviour and possibility of separating the two valency states of arsenic using the methanol–ammonia–water solvent system.

Experimental

Paper chromatography. Solutions of arsenites and arsenates were prepared from analytical grade reagents of arsenic trioxide or arsenic pentoxide or sodium hydrogen arsenate, respectively. Before being used the paper strips were thoroughly washed with 1 N HNO_3 and water (redistilled with a quartz apparatus) until the water eluant was not acidic. 20 μg (20 μl) of As were placed on a purified strip of Whatman No. 1

paper (23 × 15 cm) and developed by the ascending method in a solvent mixture of methanol–ammonia–water. Before being used, methanol was dried by means of metallic sodium and distilled in the usual way. The length of the run was 18 cm from the starting line. After development the chromatograms were removed and the solvent was allowed to evaporate in a current of air at room temperature.

Detection. An ammoniacal silver nitrate solution (2 N $AgNO_3$ in 10 % ammonia) was used to visualize the spots. Silver arsenite gives a visible yellow spot and silver arsenate a pale brown one. The detection was more sensitive when U.V. light was used. The U.V. light source was an Analytic Quartzlamp, Hanau, $\lambda = 360$ nm. The spots were then blue. They are distinctly visible for about 15 min, then the paper darkens. For the other metals tested a 0.1 % solution of 4-(2-thiazolylazo)resorcinol (TAR) in ethanol (96 %) was used. The paper strips were exposed to ammonia vapour after being sprayed with this reagent.

Results

Ionic mobility of sodium and ammonium arsenites as well as arsenates was studied as a function of the ammonia concentration at a constant methanol–water ratio of 8:2 by volume (see Fig. 1). The concentration of ammonia in methanol was in the range 2×10^{-4} N to 8×10^{-1} N NH_4OH. The R_F values were calculated to the second organic front. The maximum mobility of sodium arsenite, as well as arsenate, was found at a concentration of 4×10^{-4} N NH_4OH and was practically constant up to a concentration of 8×10^{-4} N. With a further increase in concentration of ammonium hydroxide, the R_F values of arsenite as well as arsenate decreased. On the other hand, the mobility of ammonium arsenate and arsenite under the same conditions was somewhat different. The constant R_F values of ammonium arsenite were observed from 2×10^{-4} N to 6×10^{-3} N NH_4OH and were slightly decreased with a further increase in concentration of ammonium hydroxide. The decrease of R_F values of ammonium arsenate was considerable, as can be seen in Fig. 1. From 2×10^{-1} N NH_4OH the R_F values of ammonium arsenite and arsenate were practically

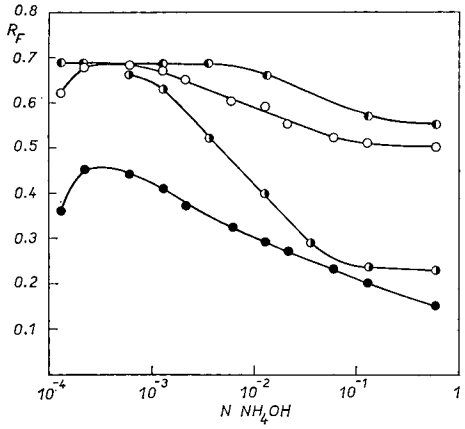


Fig. 1. Relationship between the R_F values of arsenite and arsenate ions and ammonium concentration in the methanol–ammonium hydroxide solvent system (8:2, v/v). (○) Na_3AsO_3; (◐) $(NH_4)_3AsO_3$; (●) Na_3AsO_4; (◑) $(NH_4)_3AsO_4$.

constant. We observed that the spots with the increasing concentration of ammonium hydroxide were more concentrated.

The effect of the methanol to water ratio on the mobility and separation of sodium arsenate and arsenite was studied at a constant concentration of 2×10^{-1} *N* NH_4OH (Fig. 2). The R_F values increased with the hydration of methanol and this effect was greater in the case of arsenate than in that of arsenite. At the methanol–water ratio of 1.2:1 the curves intersect at an R_F value of 0.65.

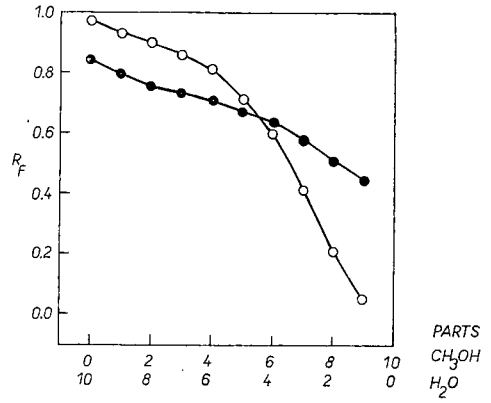


Fig. 2. Relationship between the R_F values of arsenite and arsenate ions in the methanol–ammonia–water solvent system and a different ratio of methanol–water at the constant concentration of 2×10^{-1} *N* NH_4OH. (○) Na_3AsO_4; (●) Na_3AsO_3.

The R_F values of Na_3AsO_3, $(NH_4)_3AsO_3$, Na_2HAsO_4, Na_3AsO_4 and $(NH_4)_3AsO_4$ after development in the solvent system methanol–1 *N* NH_4OH (8:2) are given in Table I. It was found that sodium nitrate tends to retard the movement of arsenate ions and this effect was greater at a lower ammonium hydroxide concentration of the solvent system. Addition of sodium nitrate (0.4 %) to the above-mentioned solvent system results in elongated spots and very low R_F values of arsenate ions (0.08), while the R_F values of arsenite ions did not change. The successful separation and identifi-

TABLE I

THE $R_F \times 100$ VALUES OF ARSENITE AND ARSENATE IN AMMONIACAL METHANOL, ETHANOL, AND ACETONE SYSTEMS

Compound	*Solvent systems*			
	MeOH–NH₃ 1 N (8:2)	*MeOH–NH₃ 1 N–0.4% NaNO₃* (8:2)	*(Me)₂CO–NH₃ 1 N* (8:2)	*EtOH–NH₃ 1 N* (8:2)
Na_3AsO_3	51	50	12	31
$(NH_4)_3AsO_3$	57			
Na_2HAsO_4	22	8	0	3
Na_3AsO_4	21	8	0	3
$(NH_4)_3AsO_4$	25	8	0	3
Time of run for 18 cm	140 min, 20°	140 min, 20°	140 min, 17.2°	345 min, 17.2°

cation of Na_3AsO_3 and Na_3AsO_4 after developing in the solvent system methanol–1 *N* NH_4OH (8:2) are shown on a photograph of a chromatogram. 10 μg of As (10 μl) of each compound was applied (Fig. 3). On separation of a mixture of arsenite and arsenate ions a decreased R_F value of arsenite was found.

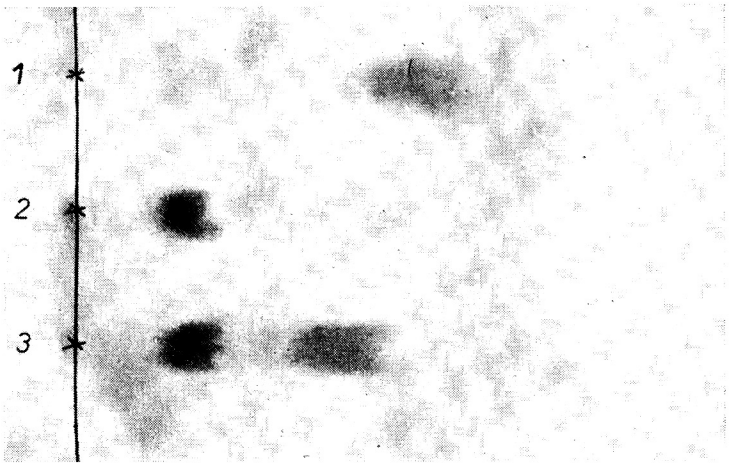


Fig. 3. Chromatogram of a separation of arsenite and arsenate ions. Solvent system: methanol–1 *N* NH_4OH (8:2, v/v), ascending. Length of run: 18 cm; 140 min at 20°. Detection: 2 *N* $AgNO_3$ in 10% ammonium hydroxide. As(III) gives a yellow spot, As(V) a brown spot; under U.V. light, blue absorbency. 1 = Na_3AsO_3; 2 = Na_3AsO_4; 3 = mixture of Na_3AsO_4 and Na_3AsO_3.

Sodium arsenate and arsenite were also developed in the ammoniacal ethanol and acetone solvent systems. These solvents were unsuitable for separating As(III) from As(V) because of very low R_F values of arsenite as well as of the arsenate remaining on the starting line (Table I); therefore many elements, especially iron, copper and antimony, interfere in the separation.

Conclusions

From our experiments it is evident that the best solvent system for the separation of arsenite and arsenate ions and for obtaining well developed spots of both is

TABLE II

THE R_F × 100 VALUES OF SOME METALS OBTAINED IN THE METHANOL–AMMONIUM HYDROXIDE SYSTEM (8:2, v/v)

Sequence of separation: (Ba or Cu)—(Cr(VI) or As(V) or Zn)—(Mo(VI) or W(VI)—As(III)—Ag.

Metal (20 μg)	R_F × 100	*Metal* (20 μg)	R_F × 100	*Metal* (20 μg)	R_F × 100
Ag	60	Al, Au, Bi	0	Ca, Cd, Mg, Sr, Tl(I), V(V)	strong tailing from the starting point
As(III)	52	Ce(III, IV)	0		
As(V)	20	Co(II)	0		
Ba	7	Cr(III)	0		
Cr(VI)	17	Fe(III)	0		
Cu	10	Hg, Mn, Pb	0		
Mo(VI)	30	Sb(III, V)	0	Ni	68 (the tailing spot)
W(VI)	30	Sn(II, IV)	0		
Zn	21	U(VI)	0		

methanol–1 N NH_4OH in the ratio 8:2 by volume. The R_F values of arsenite and arsenate ions were highly reproducible and were not greatly influenced by the basicity of the methanol but they were greatly affected by the methanol–water ratio.

This solvent system produced two fronts; the first one, the wet front, with R_F 0.65 and a second, organic, dry front. All R_F values were calculated to the second organic front. The first wet front did not change with the basicity of the solvent system (from 2×10^{-3} N – 2×10^{-1} N NH_4OH) and only slight changes were observed on increasing the aqueous part. With a methanol–water ratio of 5:5 the wet front had the R_F value 0.75. The wet front did not interfere with the R_F values of arsenic.

The advantage of the methanol–1 N NH_4OH (8:2) solvent system is the separation of arsenic from 13 elements which remain at the starting point. A further seven elements (barium, copper, chromium(VI), molybdenum, silver, tungsten and zinc) are separated from arsenic(III) according to their different R_F values. The R_F values found for some other metals are given in Table II. Calcium, cadmium, magnesium, nickel, strontium, thallium(I) and vanadium(V) interfere with their tailing spots. Chloride ions were detected in this solvent system with R_F 0.63 and nitrate ions with R_F 0.74. The time required for chromatographic analysis of arsenic does not exceed 2.5 h.

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CHROM. 3380

Separation of anionic chromium(III) thiocyanate complexes by displacement analysis*

The thiocyanate complexes of Cr(III) hold exceptional interest for studies of systematic differences among complex ions, *e.g.*, the effect of charge on adsorption. They are inert, the full set containing one through six ligands are known, and their stabilities have been measured[1]. The present work concerns preparation of solutions of the anionic complexes substantially free of other anions. KAUFMAN AND KEYES[2] have demonstrated separation of the anionic Cr(III) thiocyanate complexes using a cellulose anion exchanger with $HClO_4$ in aqueous acetone as eluant. We have modified their procedure to permit displacement analysis instead of elution analysis, thereby avoiding use of acetone and contamination by extraneous anions. Prior removal of SCN^- was found necessary from the starting mixtures of Cr complexes; Fe^{3+}-loaded cation exchanger proved useful for this purpose.

Experimental

Reagents. The salt $K_3Cr(SCN)_6$ was obtained from Alpha Inorganics, Inc. Our analyses indicated the presence of 2 % excess KSCN by weight. Mixtures of the chromium(III)-thiocyanate complexes were obtained by heating 0.5 *M* $K_3Cr(SCN)_6$ at 50° overnight to achieve equilibrium. From the results of BJERRUM[1], we estimate that the resulting solution contained 12 % $Cr(SCN)_6^{3-}$, 28 % $Cr(SCN)_5^{2-}$, 37 % $Cr(SCN)_4^-$, 19 % $Cr(SCN)_3$ and 4 % $Cr(SCN)_2^+$.

The cellulose anion exchanger, Cellex T, was obtained from Bio-Rad Laboratories, Richmond, Calif. This exchanger is a triethylaminoethyl cellulose with an exchange capacity of 0.53 mequiv./g.

Removal of free thiocyanate. Solutions prepared from commercial $K_3Cr(SCN)_6$ were used to displace the lower complexes as adjacent bands. To avoid presence of free SCN^- in the eluate, removal of the KSCN impurity from the $K_3Cr(SCN)_6$ was desired. In addition, better column behavior (*i.e.* flatter band fronts) was obtained when free SCN^- was removed from the mixture of complexes initially loaded on the anion-exchanger column. The mixture as originally prepared contained 1.7 moles free SCN^- per mole Cr(III).

A separation was developed making use of the tendency of SCN^- to invade Fe^{3+}-loaded cation exchanger. Beads of cation exchanger in the Fe^{3+}-form become red in dilute aqueous KSCN, providing visual evidence for SCN^- absorption. In contrast, anionic Cr(III) thiocyanate complexes are excluded from the Fe^{3+}-form cation exchanger.

For the present work, 80 ml of cation exchanger was prepared using 150 mesh resin (Dowex 50W X8). Ferric nitrate in 0.001 *M* HNO_3 was used for conversion to the Fe^{3+}-form. The converted resin was loaded into a 30 cm column with 2 cm of K^+-form cation exchanger at the bottom. Two ml of 0.5 *M* $K_3Cr(SCN)_6$ (or two ml of the mixture of Cr(III) complexes) was added to the top of the column and eluted with water. The deep blue Cr(III) complexes were, except as noted later, eluted as a

* This work was carried out under the auspices of the U.S. Atomic Energy Commission.

narrow band immediately after the column free volume (about 30 ml) was displaced. No free SCN^- could be detected in the product[3]. In the case of the mixture of complexes, a small fraction of the Cr was retained at the top of the column, presumably as a result of absorption of cationic complexes.

Preparation of aqueous $K[Cr(SCN)_4(H_2O)_2]$ *and* $K_2[Cr(SCN)_5(H_2O)]$. Columns were prepared by adding 3.2 g of anion exchange cellulose to 40 ml of 1 *M* KSCN, shaking vigorously to obtain a fine slurry and adding the slurry to the column while applying suction. Columns of 0.78 cm^2 cross-section were used; the resin bed occupied 17 cm of length and the head was adjusted to maintain a flow of 0.5 ml/min.

Two ml of the mixture of Cr complexes was treated to remove free SCN^- as described above, further diluted to 50 ml with water, and loaded onto the top of the column. Elution was carried out using 0.0033 *M* $K_3Cr(SCN)_6$. To avoid decomposition of $Cr(SCN)_6^{3-}$, the column and solutions were cooled to 10° with circulating chilled water, and the experiments were carried out in subdued light.

Results

The brightly colored mixed band was sharply defined on the white resin as the separation began. This band was displaced down the column as $Cr(SCN)_6^{3-}$ was added; the resin at the trailing edge of the mixture assumed the bright violet characteristic of adsorbed $Cr(SCN)_6^{3-}$. After displacement through about 5 cm, separate dark blue and bluish-violet bands formed from the mixed band.

Chemical analyses of the eluate fractions were performed with a Cary Model 14 MR spectrometer. Chromium was determined as chromate[4] and presence of free SCN^- was checked through formation of the ferric complex[3]. The number of bound SCN^- per Cr(III) was calculated using the known, fixed concentration of potassium ions in the eluate (0.01 *M*). The results are shown in Fig. 1.

Discussion

The results demonstrate efficient separation of the non-labile complexes $Cr(SCN)_4(H_2O)_2^-$ and $Cr(SCN)_5H_2O^{2-}$ by displacement along a column of anion exchange cellulose. A variety of displacing anions were tried including ClO_4^-, SO_4^{2-},

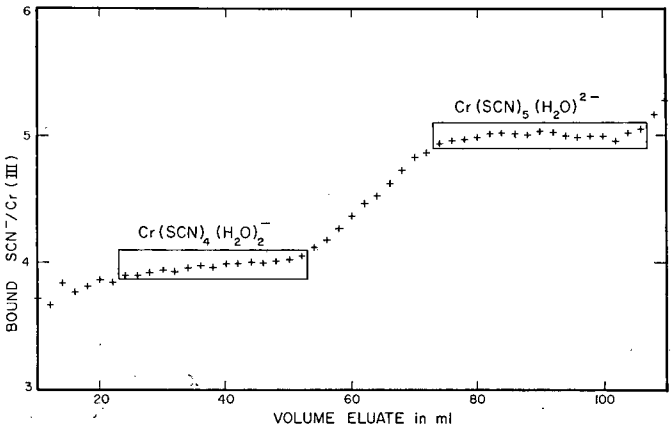


Fig. 1. Preparation of aqueous $K[Cr(SCN)_4(H_2O)_2]$ and $K_2[Cr(SCN)_5H_2O]$ by displacement analysis on anion exchange cellulose.

$P_4O_{12}^{4-}$, $Fe(CN)_6^{4-}$ and $Cr(SCN)_6^{3-}$. Only the last of these proved at all satisfactory.

The fairly substantial volume of eluate containing both the tetra- and pentathiocyanato complexes should not be taken to indicate that the adsorptivities of these ions are similar. Instead, it reflects primarily the tilting of the band fronts which was evident visually. Successful separations require band lengths which are great relative to the column lengths over which the tiled or fluted band fronts extend. SPEDDING AND POWELL[4] discuss this and related requirements in connection with their pioneering work on displacement analysis of rare earths.

As pointed out by KAUFMAN AND KEYES[2], cellulose ion exchangers appear promising for separating a variety of inorganic ions which are strongly absorbed on conventional ion exchange resins. The relatively low density of ionic sites offers a special convenience with displacement analysis; in the present experiments, for example, each milliequivalent of adsorbed ion occupied about 8 ml of cellulose exchange bed so that rather small samples still produced bands of adequate length. From our experience, we feel displacement analysis deserves wider application in preparative inorganic chemistry.

The procedure described for removal of free SCN^- involves a novel application of ion exclusion[5]. The SCN^- ions adsorbed by the Fe^{3+}-form cation exchanger must be accompanied by K^+ to maintain charge balance. Microscopic examination of sections cut from large resin beads confirms that we are not dealing with a surface effect; the red characteristic of ferric thiocyanate complexes is evident throughout the resin. The KSCN may be eluted with water, much as CH_3COOH is eluted during its separation from HCl by ion exclusion[6]. One of us (G.L.G.) has undertaken study of related systems for separation of the halides. A variety of other applications appear possible.

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CHROM. 3361

Chromatography on ion exchange papers

XXIII. Further results on ion exchange with organic solvents*

Our previous work with ion exchange papers and mixtures of aqueous acids and organic solvents[1] has been extended to cover a wider range of solvents. Previously only alcohols and acetone had been studied, and we wanted to investigate more polar solvents (acetic acid and ethyl acetate), a very non-polar solvent (benzene) and phenol (an aromatic polar solvent).

The ion exchangers examined were a cationic sulphonic resin (Amberlite SA-2 paper), an anionic quaternary ammonium resin (Amberlite SB-2 paper), and a cellulose anion exchanger (Macherey, Nagel quaternary ammonium cellulose paper); control chromatograms were also run on ordinary (Whatman No. 1) filter paper. The techniques employed were those described previously[1].

TABLE I

R_F VALUES OF INORGANIC IONS IN MIXTURES OF AQUEOUS HCl AND ACETIC ACID (FROM 0% TO 80%) (HCl = 2.4 *N* THROUGHOUT)

Papers: SA-2 = Amberlite SA-2 paper (sulphonic resin paper)
SB-2 = Amberlite SB-2 paper (strongly basic quarternary ammonium resin paper)
MN = Macherey Nagel strongly basic ion exchange cellulose paper with quarternary ammonium groups
W 1 = Whatman No. 1 pure cellulose paper

Metal ion	*Paper*	% *HAc*			
		0	*30*	*50*	*80*
Fe(III)	SA-2	0.57	0.51	0.36	0.10–0.53
	SB-2	0.57	0.04	0.05	0
	MN (basic)	0.87	0.72	0.55	0.75
	W 1	1	0.82	0.68	0.73
Co(II)	SA-2	0.46	0.39	0.33	0.04–0.55
	SB-2	1	1	0.15; 0.84	0
	MN	0.92	0.88	0.69	0.42
	W 1	1	0.85	0.72	0.46
Ni(II)	SA-2	0.46	0.39	0.33	0.32
	SB-2	1	1	0.78	0.41
	MN	0.92	0.88	0.74	0.33
	W 1	1	0.85	0.72	0.30
Cu(II)	SA-2	0.59	0.61	0.59	0.49
	SB-2	0.75	0.20	0.06	0
	MN	0.89	0.80	0.62	0.42
	W 1	1	0.84	0.72	0.43
Ba(II)	SA-2	0.04	0.06	0.05	0.04
	SB-2	1	0.92	0.88	0.81
	MN	1	0.81	0.89	0.68
	W 1	0.89	0.71	0.64	0.67

(*continued on p. 293*)

* Parts XX, XXI and XXII of this series are notes in *J. Chromatog.*, 21 (1966) 499; 22 (1966) 200; and 29 (1967) 306.

TABLE I (*continued*)

Metal ion	*Paper*	*% HAc*			
		0	*30*	*50*	*80*
Zn(II)	SA-2	0.04–0.47	0.04–0.51	0.04–0.44	0–0.31
	SB-2	0	0	0	0
	MN	0.55	0.42	0.43	0.42
	W 1	1	1	1	0.51
Sr(II)	SA-2	0.26	0.16	0.09	0.06
	SB-2	1	1	1	0.62
	MN	0.88	0.85	0.77	0.60
	W 1	0.90	0.82	0.69	0.57
Ce(III)	SA-2	0.10	0.03	0.01	0.01
	SB-2	1	1	1	0.70
	MN	1	1	0.84	0.49
	W 1	1	0.90	0.69	0.62
La(III)	SA-2	0.13	0.06	0	0.02
	SB-2	1	1	1	0.76
	MN	1	1	0.84	0.52
	W 1	1	0.86	0.76	0.60
Sm(III)	SA-2	0.12	0.06	0.01	0.02
	SB-2	1	1	1	0.70
	MN	1	1	0.82	0.45
	W 1	1	0.90	0.69	0.59
ReO_4^-	SA-2	0.50	0.64	0.66	0.56
	SB-2	0.03	0.08	0.12	0.17
	MN	0.57	0.61	0.65	0.72
	W 1	0.70	0.73	0.72	0.59
TcO_4^-	SA-2	0.19	0.39	0.45	0.17–0.50
	SB-2	0	0.01	0.03	0.03
	MN	0.60	0.60	0.60	0.64
	W 1	0.65	0.69	0.73	0.58
$AuCl_4^-$	SA-2	0.03	0.10	0.10	0.21
	SB-2	0	0	0	0.01
	MN	0.28	0.44	0.48	0.67
	W 1	0.47	0.64	0.67	0.58

Benzene holds so little HCl that the paper strips were moistened with aqueous HCl so as to obtain conditions similar to those in equilibrium experiments.

The results obtained are given in Tables I–IV. Acetic acid has the same effect as a polar alcohol, *i.e.* it fixes hydrated ions more strongly, increases the complexation of reversible chloro complexes, and desorbs anions soluble in organic solvents (*e.g.* ReO_4^-). Benzene does not extract any of the ions tested to any great extent. We were surprised that even ReO_4^- and $AuCl_4^-$ are preferentially held on the resin.

Phenol does not extract any of the ions examined, with the exception of Rb, Cs, ReO_4^- and TcO_4^-. Ethyl acetate also generally gave low R_F values even with ions otherwise readily extractable such as $AuCl_4^-$.

TABLE II

R_F VALUES OF SOME INORGANIC IONS ON A STRONG ANIONIC RESIN (SB-2) PAPER MOISTENED WITH 2.4 *N* HCl AND DEVELOPED WITH BENZENE

Metal ion	R_F
$AuCl_4^-$	0
ReO_4^-	0
Co	0.01
Cu	0.02
Ni	0.05
Sr	0.04
La	0.04
Fe	0
Ce	0.03

TABLE III

R_F VALUES OF SOME INORGANIC IONS WITH MIXTURES OF PHENOL AND AQUEOUS HCl OF VARIOUS CONCENTRATIONS (1:1)

Papers: SA-2 = Amberlite SA-2 paper (sulphonic resin paper)
SB-2 = Amberlite SB-2 paper (strongly basic quarternary ammonium resin paper)
MN = Macherey Nagel strongly basic ion exchange cellulose paper with quarternary ammonium groups
W 1 = Whatman No. 1 pure cellulose paper

Ion	*Paper*	*2N HCl*	*3N HCl*	*4N HCl*
Fe	SA-2	0	0	0
	SB-2	0.02T*	0.01	0
	MN	0T	0.02	0.01
	W 1	0.02	0.02	0.01
Co	SA-2	0	0	0.01
	SB-2	0.05T	0.02	0.03
	MN	0.03T	0.03	0.03
	W 1	0.02T	0.02	0.02
Ni	SA-2	0	0	0
	SB-2	0.04T	0.02	0.03
	MN	0.02T	0.03	0.01
	W 1	0.02	0.02	0.01
Cu	SA-2	0	0	0.01
	SB-2	0.04T	0.02	0
	MN	0.03T	0.02	0.01
	W 1	0.03	0.02	0.02
Sm	SA-2	0	0	0
	SB-2	0.05T	0	0.04
	MN	0.05T	0	0.01
	W 1	0.01	0	0.01
La	SA-2	0	0	0
	SB-2	0.05T	0	0.04
	MN	0.03T	0	0.01
	W 1	0.02	0	0

* T = Tail or elongated spot.

(*continued on p. 295*)

TABLE III (*continued*)

Ion	*Paper*	*2N HCl*	*3N HCl*	*4N HCl*
Ce	SA-2	0	0	0
	SB-2	0.05T	0	0.02
	MN	0.04T	0	0.02
	W 1	0.02	0	0–0.14
Sr	SA-2	0	0	0
	SB-2	0	0	0.04
	MN	0.03T	0	0.01
	W 1	0.01	0	0
Ba	SA-2	0	0	0
	SB-2	0	0	0.02
	MN	0	0	0
	W 1	0.03	0	0
Zn	SA-2	0	0.03	0
	SB-2	0	0.02	0
	MN	0	0.03	0
	W 1	0	0	0
K	SA-2	—	—	—
	SB-2	0.15	0.15	0.16
	MN	0.10	0.16	0.09
	W 1	0.08	0.10	0.06
Rb	SA-2	—	—	—
	SB-2	0.33	0.42	0.41
	MN	0.34	0.41	0.33
	W 1	0.23	0.24	0.22
Cs	SA-2	—	—	—
	SB-2	0.72	0.77	0.73
	MN	0.71	0.64	0.70
	W 1	0.44	0.48	0.33
ReO_4^-	SA-2	0.23	0.23	0.23T
	SB-2	0	0.01	0
	MN	0.26	0.28	0.24
	W 1	0.27	0.29	0.22
TcO_4^-	SA-2	0.07	0.07	0.06
	SB-2	0	0	0
	MN	0.26	0.20	0.19
	W 1	0.27	0.20	0.19
$AuCl_4^-$	SA-2	0–0.10	0–0.14	0–0.10
	SB-2	0	0	0
	MN	0.17	0.26	0.13
	W 1	0.30	0.30	0.23

TABLE IV

R_F VALUES OF SOME INORGANIC IONS WITH MIXTURES OF ETHYL ACETATE AND AQUEOUS HCl (1:1)

Papers: SA-2 = Amberlite SA-2 paper (sulphonic resin paper)
SB-2 = Amberlite SB-2 paper (strongly basic quarternary ammonium resin paper)
MN = Macherey Nagel strongly basic ion exchange cellulose paper with quarternary ammonium groups
W 1 = Whatman No. 1 cellulose paper

*Ion**	*Paper*	*2N HCl*	*3N HCl*
Fe	SA-2	0.05–0.32	0–0.30
	SB-2	0–0.11	0–0.06
	MN	0.07–0.35	0–0.22
	W 1	0.09–0.29	0–0.18
Cu	SA-2	0.02	0
	SB-2	0	0
	MN	0.01	0
	W 1	0.01	0
$AuCl_4^-$	SA-2	0.06T	0.07–0.23
	SB-2	0.02	0.03
	MN	0.03–0.15	0.06–0.25
	W 1	0.26–0.51	0.23–0.61
Zn	SA-2	0.05	0.10T
	SB-2	0	0
	MN	0.03	0.04
	W 1	0.06	0.11
TcO_4^-	SA-2	0.04	0.05
	SB-2	0.01	0
	MN	0.04	0.05
	W 1	0.06	0.06
ReO_4^-	SA-2	0.05	0.09
	SB-2	0.01	0
	MN	0.04	0.07
	W 1	0.06	0.08

* K, Rb, Cs, Co, Ni, Sm, Sr, La, Ce, Ba all have $R_F = 0$ on all papers and in all solvents.

The results presented here do not seem to have any analytical applications. However previously published data did find use in explaining the mechanisms involved in ion exchange with organic solvents[2] and the present results might also find use in this direction.

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News

7th International Symposium on Gas Chromatography and its Exploitation

The 7th International Symposium on Gas Chromatography and its Exploitation, organised by the Gas Chromatography Discussion Group of the Institute of Petroleum (in co-operation with the Danish Chemical Society), will be held at Falkoner Centret, Copenhagen, from June 25th–28th, inclusive.

PROGRAMME

Morning sessions

Mr. E. M. Bens and Mr. C. M. Drew, Dr. A. B. Littlewood, Prof. J. E. Lovelock and a Scandinavian scientist have been invited to present the Plenary Lectures at the commencement of each morning session. The following submitted papers have been accepted for presentation:

Tuesday, June 25th, 1968

Theory, new applications and techniques

Thick layer graphitized carbon black open tubular columns: performance and analytical applications, by G. Goretti, A. Liberti and G. Nota (Italy)

The influence of time dependent adsorption on dry support surface in qualitative and quantitative GLC, by M. Krejci and K. Hana (Czechoslovakia)

Analysis of olefin mixtures by combination of capillary–gas chromatograph and mass spectrometer, by G. Schomburg and D. Henneberg (Germany)

Retention indices of alkanes through C_{10} and alkenes through C_8 and relation between boiling points and retention data, by A. Matukuma (Japan)

The study of liquid–liquid mixtures using medium-pressure gas–liquid chromatography: solutions of fluorinated benzenes in *n*-octadecane, by A. J. B. Cruickshank, B. W. Gainey and C. L. Young (Great Britain)

Wednesday, June 26th, 1968

Novel applications of chromatography

Pyrolysis gas chromatography of biological macromolecules, by M. V. Stack (Great Britain)

Determination of the configuration of asymmetric compounds by gas chromatography of diastereoisomers, by B. Halpern and J. W. Westley (U.S.A.)

Cyclic boronate esters in the characterization of diols by gas chromatography–mass spectrometer, by C. J. W. Brooks and J. Watson (Great Britain)

Analysis of ultra traces of aluminium in uranium by gas chromatography, by C. Genty, C. Houin and R. Schott (France)

The determination of the permeability of plastic tubes, by I. L. Stansfield (Great Britain)

An apparatus for the detection of interfaces between products in pipelines, by E. R. Adlard, A. J. Davies and A. Evans (Great Britain)

GLC for on-stream octane rating of stabilized catalytic reformates, by G. I. Jenkins, N. G. McTaggart and B. L. H. Watkin (Great Britain)

Thursday, June 27th, 1968

Techniques associated with chromatography

The effect of temperature on the performance of liquid chromatographic columns, by R. J. Maggs and T. E. Young (Great Britain)

Microporous polymers as stationary phases in chromatography with high pressure gases or supercritical fluids, by S. T. SIE, J. P. A. BLEUMER AND G. W. A. RIJNDERS (The Netherlands)
On-line high resolution mass spectrometry of organic compounds eluted from a gas chromatograph, by J. R. CHAPMAN, M. BARBER AND W. A. WOLSTENHOLME (Great Britain)
System for the identification of gas chromatographic peaks by pyrolysis gas chromatography with high speed temperatures programming, by R. R. GOFORTH AND W. E. HARRIS (Canada)
An automatic laboratory chromatograph, by P. J. KIPPING AND C. A. SAVAGE (Great Britain)
Digital recorder display of radioactive and integrated chromatograms, by P. A. T. SWOBODA AND E. H. A. PRESCOTT (Great Britain)

Friday, June 28th, 1968

The exploitation of chromatographic performance
Automatic digital computing integrator for gas chromatography, by Y. VOLLERS, A. SCHURINGA AND C. C. HOMS (The Netherlands)
Gas chromatographic data handling with on-line data acquisition computers, by R. D. MCCULLOUGH (U.S.A.)
Digital data acquisition and computer identification of gas chromatographic eluates, by C. MERRITT, JR., J. WALSH AND D. H. ROBERTSON (U.S.A.)
Small on-line computer for GC data handling, by F. BAUMANN AND D. WALLACE (U.S.A.)
A gas chromatographic computer, by J. G. KARCHL (U.S.A.)
The application of a computer GC system to laboratory analysis, by E. J. LEVY, I. RADVANY, D. GOLAND, L. CHANT AND T. M. MALATESTA (U.S.A.)

Afternoon sessions

On the first afternoon session there will be discussion in about twenty informal panels. Each table will have a definite subject for discussion, prominently displayed. Among the subjects to be discussed will be Quantitative Analysis, Essential Oils and Flavours, Biomedical Applications, Pyrolysis, Apparatus, Liquid Chromatography and Auxiliary Techniques.

Reports from the panels will be presented on the third afternoon (Thursday) in related groups with general discussion following each group.

On the final afternoon conventional discussion sessions will be held and the topics include Detectors, Column Technology, and Process Chromatography. The meeting will close with a Brains Trust Session.

For further information on accommodation, registration etc., see the preliminary announcement in Vol. 32, pp. 201–203 of this journal.

NINTH CONGRESS OF THE INTERNATIONAL SOCIETY FOR FAT RESEARCH

The IXth I.S.F. Congress will be held in Rotterdam, The Netherlands, from September 16th–21st, 1968. The technical programme will consist of plenary lectures by invited speakers, and about 100 papers to be presented before five sections:

Lipids (biochemistry, nutrition)
Oils and Fats (analysis, structure, synthesis)
Oils and Fats (technology)
Soaps and Detergents (physical chemistry, analysis, technology)
Chemicals (fatty acid derivatives, detergent raw materials)

Sessions of these five sections will, in part, be held concurrently. The plenary lectures will be given by:

Dr. H. J. DUTTON: hydrogenation of fats

Prof. Dr. E. LEDERER: les applications de la spectrométrie de masse à la détermination de substances lipidiques complexes d'intérêt biologique

Prof. Dr. J. LYKLEMA: thin liquid films

Prof. Dr. F. LYNEN: chemical mechanisms and biological regulation of the biosynthesis of lipids

The closing date for final registration for the Congress is April 15th, 1968. The preliminary programme, giving the titles of papers to be presented, detailed information on technical excursions, social functions, lady's programme etc., can be obtained from: Secretariat IXth I.S.F. Congress, Unilever Research Laboratorium, P.O. Box 114, Vlaardingen, The Netherlands.

New apparatus

Phoenix Precision Instrument Co., Philadelphia, Pa. announces a new *automatic recording amino acid analyzer* which incorporates many new and improved features over previous models.

The new Phoenix Model K-8000-C Analyzer is a self-contained instrument for the automatic analysis of amino compounds in protein hydrolyzates, protein free plasma and other physiological fluids, hydroponic solutions, tissue extracts, culture media, foods and pharmaceuticals.

The newest feature includes a dual-speed multi-point strip chart recorder, which in connection with the accelerated analysis system permits eight complete protein or peptide hydrolyzate analyses per 24-hour day using less sample amounts and at increased sensitivity. Additional new features of the Model K-8000-C include precision bore high-pressure type chromatographic columns as standard equipment; a column selector manifold for directing of effluents from accessory and other optional columns into the analyzing system without changing capillary lines; a dual-temperature column circulating water bath; a solid state regulated d.c. power supply; a large fold-out work-bench for mounting of accessory equipment on either side of the cabinet and more.

A new accessory 6600 Variable Gradient System can be incorporated into the Model K-8000-C allowing for single column, variable gradient operation (after K. A. Piez and L. Morris of the National Institute of Health). The Phoenix Model K-8000-C Analyzer is 76 in. h × 48 in. w × 26 in. d. Height to workbench is 36 in. and height to pull-out desk is 30 in.

For further information apply to the publisher under reference No. Chrom. N-94.

Manufacturers' literature

The *Camag Bibliography Service*, No. 11, November 1967 on thin-film chromatography has just been received. This issue also contains descriptions of (i) the Camag self-filling micropipette, (ii) the Camag Chromatocharger and (iii) aluminium oxides for column chromatography.

Carlo Erba, Scientific Instruments Division, Milan has recently sent to us their *Short Notes*, No. 2–67, which contain descriptions of the apparatus which they are currently manufacturing.

Facts and Methods, December, 1967, Vol. 8, No. 6 from Hewlett/Packard has been received, which contains information on:

(i) T.C. detector limits for tracer gases,
(ii) capillary column analysis of aromatic solvents, and
(iii) analysis of automobile exhaust gases.

E. Merck AG, Darmstadt has published the following *catalogues*: (i) Amino acids and derivatives. (ii) Enzymes. (iii) Steroids and steroidal glycosides. (iv) Synthetic chemicals. (v) Nucleotides and derivatives.

Smaller catalogues are included containing descriptions of: (a) 2,2-biquinoline (Cuproin) as a reagent for the determination of copper, and (b) chloranilic acid (3,6-dichloro-2,5-dihydroxy-*p*-benzoquinone) used as a reagent for calcium, sulphate and chloride.

Chromatographic thin-film handbooks Nos. I–VI were also received at the same time. The methods described in detail cover a variety of common products and samples.

For further information apply to the publisher under reference No. Chrom. N-102.

Just issued by the Phoenix Precision Instrument Co., Philadelphia, Pa. is a data sheet on the new *Amino Acid Micro-Analyzer*, model M6800, an instrument which has the capability of providing fully-automatic high-speed liquid chromatography and which is designed primarily for the small laboratory.

The data sheet MA-865 reflects the many new improvements to the Micro-Analyzer including restyled cabinetry for convenience of operation and easier accessibility to columns from the front.

For further information apply to the publisher under reference No. Chrom. N-83.

CHROM. 3373

THE OBSTRUCTIVE COEFFICIENT AND STATIONARY PHASE DISTRIBUTION OVER SOLID SUPPORT SURFACES IN GAS CHROMATOGRAPHY

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(Received January 4th, 1968)

SUMMARY

A relationship has been suggested for the explicit expression of the obstructive coefficient dependent on the basic characteristics of the supporting material and stationary liquid. In addition, the influence of support pore volume and the distribution of the support pores filled with various volumes of the stationary liquid on the value of the obstructive coefficient was investigated. The difference between the penetrability of material whose surface had been modified with trimethylchlorosilane and of that with an unmodified surface has been verified.

The distribution of the liquid on a solid support not only influences the mass transfer from the gaseous (mobile) to the liquid (stationary) phase and diffusion processes in the liquid phase, but also diffusion processes in the gaseous phase, due to the internal pore structure of the supporting material. The influence of the porous medium on such processes has been characterized by the obstructive coefficient, γ[1–3],

$$\gamma = CT^{-2} \tag{1}$$

where the influence of the constriction C and tortuosity T of the support channels on the longitudinal diffusion of sorbate in the gaseous phase are described. Attention has been given to various geometrical patterns of column packing[2], as well as to the influence of such an arrangement on the shape of channels in the packing material. A qualitative description of the role of the internal structure of material has also been made[4]. There is no doubt that the internal pore structure enhances the penetrability of loosely packed material[5] and, consequently, also the obstructive coefficient. It can be assumed[4] that the resulting obstructive coefficient corresponds to the sum of the obstructive coefficients representing the resistance against diffusional flow in interstitial space, γ_M, and that in the internal support pores, γ_V.

$$\gamma = \gamma_M + \gamma_V \tag{2}$$

The coefficient γ_M is unambiguously characterized by the shape and arrangement of the column packing. Thus, if the volume of coating liquid V_L does not exceed

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the internal pore volume V_ε, the value of γ_M will not change with various volumes of the coating liquid.

We will therefore restrict ourselves to the dependence of γ_V on the volume of coating liquid.

THEORETICAL

Let us suppose that a major part of the solid support surface is constituted of its internal pores. This assumption holds for materials with surface areas of about 1 $m^2 \cdot g^{-1}$, *i.e.* for the supports most widely used—diatomaceous supports (Chromosorb W) and firebrick supports (Sterchamol, Chromosorb P). Under these conditions, most of the coating liquid will collect in support pores[6,7].

Let us describe the distribution of pore radii, r, with respect to pore volume by the equation

$$dV = \alpha(r)dr \tag{3}$$

Since the relation[3] below holds true

$$S^+\bar{r}/2 = V_\varepsilon \tag{4}$$

where S^+ is the dry surface area and $\bar{r}$ the mean value of pore radius relative to pore volume, we can write for the relative area[7]:

$$\rho = \frac{S}{S^+} = \frac{1}{V_\varepsilon}\int_{r_{min}}^{r_{max}} \alpha(r)dr \tag{5}$$

where S is the area of supporting material coated with liquid and r_{min} and r_{max} are the minimum and maximum radii of pores filled with liquid. The relative area does not depend on the absolute dry area of the support[8] and is a function of the distribution of support pore radii as well as of the volume of coating liquid. It can be derived[7] that

$$\rho = \frac{V_\varepsilon - V_L}{V_\varepsilon} \frac{\bar{r}}{\bar{r} + r(V_L)/\beta} \tag{6}$$

where β is a constant.

Let us further consider a single particle of the supporting material. The diffusional flow through this particle is proportional to the overall cross-section communicating from one side of the particle to the opposite one. Assuming that all of the pores are equal in length, then

$$\frac{d\gamma_V}{dV} = \frac{d\gamma_V}{dr}\frac{dr}{dV} = 0 \tag{7}$$

A change in the radius (cross-section) of pores r_i will be proportional to the change in the obstructive coefficient; thus

$$d\gamma_V = k\alpha(r)dr \tag{8}$$

where k is a constant. In respect to the number of pores in a particle of a particular radius

$$\gamma_V = \sum_1^n \gamma_{Vi} \tag{9}$$

For the sake of simplification, the total resistance of the system against diffusional flow can be represented by analogy to a system of parallel wire conductors of the same length and radius. The overall conductivity C of the system is given by the sum of reciprocal values of the resistors. If we select the system of resistors so that the distribution of their radii will correspond to the distribution function (3) (the radius of a wire resistor is denoted r'), we may write, in analogy to eqn. (9)

$$C = k_r \sum_1^n \frac{1}{r'_i} \tag{10}$$

where k_r is a constant.

With regard to eqns. (2), (7) and (8), the intraparticle obstructive coefficient can be written as

$$\gamma_V = k \int_{V_\varepsilon}^{V_\varepsilon - V_L} \mathrm{d}V = k \int_{r_{\min}}^{r_{\max}} \alpha(r)\mathrm{d}r \tag{11}$$

Combining eqns. (11) and (5) we obtain an explicit relation for the intraparticle obstructive coefficient as a function of the relative area and volume of the internal pores,

$$\gamma_V = kV_\varepsilon\rho \tag{12}$$

If the entire diffusion flow is confined to the internal structure of the pores of a single particle, then $k = 1$ and in accordance with presupposition (7) $\mathrm{d}\gamma_V/\mathrm{d}V = 0$ also.

The proportionality constant is $0 < k \leqslant 1$, and it indicates the part of the total diffusion flow of sorbate between two points in the column which will be confined to the diffusion through the particle and around the particle, *i.e.* through the interstitial space V_M.

Let us again illustrate the influence of the interstitial column space by analogy to electrical wiring (Fig. 1). The internal obstructive coefficient γ_V is analogous to the output voltage E_2 from the divider of input voltage E_1 (analogous to ρ). The resistors in the diagram represent the interstitial volume, V_M', and internal volume V_ε'. In analogy with eqn. (12) we may write

$$E_2 = \frac{V_\varepsilon'}{V_\varepsilon' + V_M'} E_1 \tag{13}$$

With regard to eqn. (13), eqn. (12) can be rewritten as

$$k = 1/(V_\varepsilon + V_M) \tag{14}$$

and in a final form

$$\gamma_V = \frac{V_\varepsilon}{V_\varepsilon + V_M} \rho \tag{15}$$

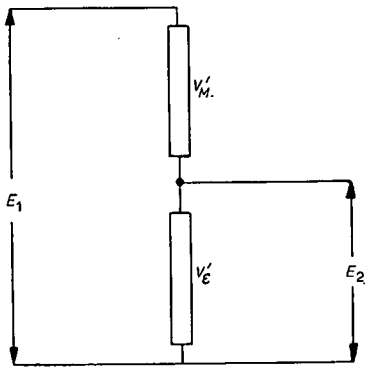


Fig. 1. Equivalent electrical circuit.

The limiting conditions are:

for $V_L = 0$, $\gamma_V = \dfrac{V_\varepsilon}{V_M + V_\varepsilon}$

for $V_L = V_\varepsilon$, $\gamma_V = 0$

for $V_M \to 0$, $\gamma_V \to \rho$

for $V_M \gg V_\varepsilon$, $\gamma_V \to 0$

With regard to eqn. (6), the internal obstructive coefficient can be expressed as a function of the volume of the internal pores, of the coating liquid, and of the distribution of support pore radii.

$$\gamma_V = \frac{1 - V_L/V_\varepsilon}{1 + V_M/V_\varepsilon} \cdot \frac{\bar{r}}{\bar{r} + r(V_L)/\beta} \tag{16}$$

By silanizing the support surface we obtained changes in the chemical character of the surface[8,10] and in the structure of internal support pores[11] which are reflected by a change of the dependence of the relative area on the volume of coating liquid[7] ($\rho = f(V_L)$). Structural (geometrical) changes of the surface indicate a closer (less penetrable) structure of silanized uncoated supports. For silanized supports, therefore, $(V_\varepsilon)_{sil} < (V_\varepsilon)$ and, consequently for V_M = constant and according to eqn. (16) also $(\gamma_V)_{sil} < (\gamma_V)$.

The first addition of liquid onto a silanized support surface does not cause closure of the smallest pores and, consequently, γ_V will not decrease with the increasing volume of the liquid, in the same way as is the case with an unsilanized surface. Silanization of the surface will therefore decrease the $B = 2\gamma D_g$ term in the diffusion equation for small volumes applied, whereas with larger volumes of liquid, the term B will grow as compared with the same term for unsilanized materials.

EXPERIMENTAL

Materials

Supports. Chromosorb P (Carlo Erba, Milan, Italy) particle size 0.25 mm–0.50

mm; Chromosorb P modified with trimethylchlorosilane, particle size 0.25–0.50 mm and 0.15–0.25 mm.

Liquid phase. Squalane (Carlo Erba, Milan, Italy).

Solvent. Ether.

Gases. Electrolytic hydrogen, nitrogen (Technoplyn, N. E., Ostrava), air, liquid nitrogen (Technoplyn, N. E., Komárov).

Methods

The diffusion coefficient and the obstructive coefficient were measured by the elution method[2]. Diffusion was measured in the system methane–hydrogen. At the temperature chosen (50°), methane was not sorbed on the supports used.

The surface areas of both activated and coated supports was measured by the dynamic desorption method[12]. A mixture of 20 % nitrogen and 80 % hydrogen (v/v) was used for the measurements.

Liquid nitrogen was used as coolant.

Apparatus

The obstructive coefficients were measured in the thermostat of an Argon-Pye instrument using a Vibron electrometer, model 33C (England), and a flame ionization detector constructed by the authors.

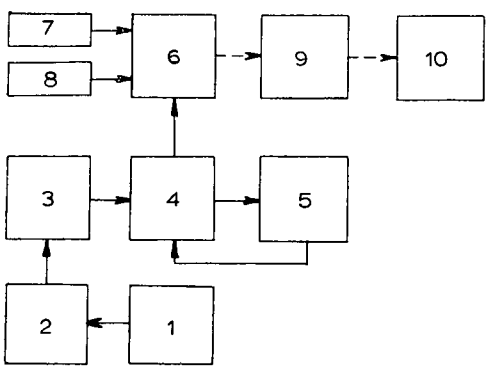


Fig. 2. Schematic representation of the apparatus.

Electrolytic hydrogen from a pressure cylinder (1) (Fig. 2) was passed *via* a pressure reducer (2), pressure gauge (3) and shut-off valve (4) into the column (5) and flame ionization detector (6). The sample was introduced directly into the column. A glass column was used (I.D. 3.6 mm, 740 mm long). In addition, a mixture of air (7) and hydrogen (8) was fed to the detector. The detector signal was transmitted by the electrometer (9) to a KBT recorder (10) (K. Marx Werke, Magdeburg, D.D.R.).

The specific area of the supports was measured with an instrument for dynamic desorption[13] measurements. The instrument was connected to a Pye integrator and a Honneywell-Brown recorder.

RESULTS AND DISCUSSION

The obstructive coefficients were calculated using the equation[2]

$$\frac{d\sigma_t^2}{dt} = 2\gamma D_g/u^2$$

where σ_t^2 is the standard deviation of the band of the diffusing material and u is linear velocity of the carrier gas. The diffusion coefficient D_g for the system hydrogen–methane was measured in an empty column (D_g = 0.765 $cm^2 \cdot sec^{-1}$) at 50° and 1.08 atm and correlated with a tabulated value[14] corrected for the given temperature and pressure (D_g = 0.760 $cm^2 \cdot sec^{-1}$). The value 0.76 $cm^2 \cdot sec^{-1}$ was used for calculating the obstructive coefficient. The error of the measurement calculated from six measurements was $\vartheta = \pm 2.8$ %. The error involved in the determination of γ is higher because it is dependent on several geometrical factors (arrangements of packing material in column, accuracy of support sizing, abrasion of support during application of stationary phase and column packing).

The intraparticle obstructive coefficients were calculated from eqn. (16) for various volumes of coating liquid applied on to Chromosorb P and trimethylchlorsilane modified Chromosorb P (particle size 0.15–0.25 mm and 0.25–0.50 mm). The distribution function for pore radii with respect to pore volume and the mean pore radii were taken from ref. 11. The structural constant β was calculated from the previously derived relation[7]. The internal pore volume was determined using eqn. (4) where S^+ has been determined by experiment. We have calculated the mean square deviation for the volume of internal pores calculated in the above manner, measured by experiment, and quoted in ref. 11, as $\pm$ 6.35 %. The magnitude of the interstitial space V_M was calculated as four tenths of the total volume of column reduced to 1 g column packing. The calculated intraparticle obstructive coefficients are listed in Table I.

TABLE I

CORRELATION OF CALCULATED AND MEASURED OBSTRUCTIVE COEFFICIENTS

Support	*Particle size (mm)*	*% squalane*	V_L $(ml \cdot g^{-1})$	ρ	$r(V_L) \cdot 10^4$ *(cm)*	γ_V *by eqn. 16*	$\gamma_{V\,exp.}$	$\gamma_V/\gamma_{V\,exp.}$
Chromosorb P	0.25–0.50	0	0	1.000	0	0.528	0.533	0.991
		1	0.0125	0.520	0.10	0.294	0.303	0.970
		5	0.0625	0.405	0.18	0.205	0.221	0.928
		10	0.125	0.314	0.26	0.152	0.155	0.981
		20	0.250	0.168	0.33	0.105	0.096	1.094
Chromosorb P + TMCHS	0.25–0.50	0	0	1.000	0	0.483	0.484	0.998
		1	0.0125	0.785	0.09	0.380	0.397	0.957
		5	0.0625	0.570	0.16	0.276	0.273	1.011
		10	0.125	0.373	0.25	0.181	0.196	0.924
		20	0.250	0.280	0.32	0.134	0.116	1.155
Chromosorb P + TMCHS	0.15–0.25	0	0	1.000	0	0.510	0.495	1.030
		1	0.0125	0.785	0.09	0.401	0.417	0.962
		5	0.625	0.570	0.16	0.292	0.305	0.957
		10	0.125	0.373	0.25	0.191	0.185	1.035
		20	0.250	0.280	0.32	0.143	0.140	1.021

TABLE II

EXPERIMENTAL AND CALCULATED CONSTANTS K (EQN. 17)

Support	*Particle size (mm)*	$K_{exp.}$	K *by eqn. 17*	K_{exp}/K
Chromosorb P	0.25–0.50	0.543	0.528	1.028
Chromosorb P + TMCHS	0.25–0.50	0.466	0.483	0.965
Chromosorb P + TMCHS	0.15–0.25	0.517	0.511	1.012

A change of the obstructive coefficient with respect to the volume of coating liquid will be manifested mainly by a change in the intraparticle obstructive coefficient. The dependence of γ on the relative area ρ is a straight line with a slope (Fig. 3A)

$$K = V_\varepsilon/(V_\varepsilon + V_M) \tag{17}$$

We compared the slope of the straight line relationship of γ/ρ obtained experimentally with the slope calculated from eqn. (17) (Table II). In the expression for the obstructive coefficient γ, the interstitial obstructive coefficient γ_M is represented by the intercept on the axis of ordinates. Since it is not only the intraparticle obstructive coefficient (*viz.* 16) but mainly the interparticle obstructive coefficient which is dependent[2] on the interstitial space V_M, a change in the interstitial space brings about a change in the slope K as well as in the intercept on the axis of ordinates, γ_M.

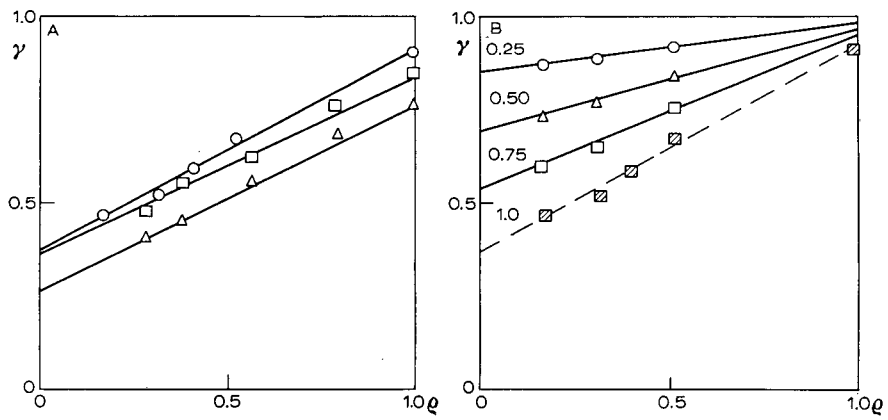


Fig. 3. (A) The dependence of the obstructive coefficient, γ, on the relative area, ρ, for: (○) Chromosorb P (0.25–0.50 mm); (□) Chromosorb P + TMCHS (0.25–0.50 mm); (△) Chromosorb P +TMCHS (0.15–0.25 mm). (B) The dependence of the obstructive coefficient, γ, on the relative area, ρ, for various times of diffusion in the porous material (Chromosorb P).

We have verified by experiment the influence of the interstitial space on the constant K_{exp} (eqn. 17). We placed in the column half of the previous amount of packing so that one half of the column remained empty. Thus, different densities of column packing were replaced by different times of diffusion of the gas in the porous and empty column spaces. We have in this way replaced columns varying in their interstitial spaces by a single column on which the obstructive coefficients were measured. (Fig. 3B).

From the slopes obtained by experiment (Fig. 3B) we have calculated k by eqn. (14). We also have determined k from the relation

$$k = \frac{1}{V_e + \left(\frac{t_e}{t} V_{Me} + \frac{t_f}{t} V_{Mf}\right)} \tag{18}$$

where t is the total time of diffusion of the substance in the column, t_e is the time of diffusion of the solute in the empty column space, t_f is the time of diffusion of the solute in the packed part of column, V_{Me} is the dead-volume of the empty column and V_{Mf} is the interstitial space between the particles of the packed column part. Correlation of k values calculated by eqns. (14) and (18) is shown in Table III.

TABLE III

EXPERIMENTAL AND CALCULATED CONSTANTS k FOR CHROMOSORB P (EQN. 18)

t_f/t	V_M ($ml \cdot g^{-1}$)	$k_{exp.}$ by eqn. 14	k by eqn. 18	$k_{exp.}/k$
1.00	1.033	0.467	0.457	1.022
0.75	1.894	0.350	0.327	1.070
0.50	3.617	0.225	0.210	1.071
0.25	8.783	0.108	0.101	1.069

The effect of surface silanization on the structure of internal support pores is obvious from the plot of relative support area against the volume of coating liquid (Fig. 4) and from the dependence of the obstructive coefficient on the volume of coating liquid for Chromosorb P and Chromosorb P modified with trimethylchlorosilane (Fig. 5).

The specific area of material modified with trimethylchlorosilane (3.97 $m^2\ g^{-1}$) and the intraparticle obstructive coefficient of the latter (0.484) are smaller than the specific area (5.78 $m^2\ g^{-1}$) and the intraparticle obstructive coefficient (0.533) of the

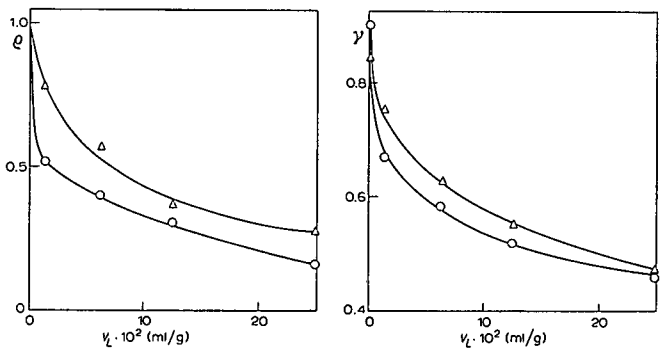


Fig. 4. The dependence of relative area, ρ, on the applied volume of liquid, V_L, for: (○) Chromosorb P; (△) Chromosorb P + TMCS.

Fig. 5. The dependence of the obstructive coefficient, γ, on the applied volume of liquid, V_L, for: (○) Chromosorb P; (△) Chromosorb P + TMCS.

uncoated unmodified support. This agrees with the assumption as to a lesser penetrability of the modified supports in contrast to the unmodified ones. On application of the first portions of the liquid, the intraparticle obstructive coefficient for an unmodified support decreases at a greater rate than does that for a modified support. The difference between the intraparticle obstructive coefficients of a modified and an unmodified support increases to a particular volume of liquid applied (approximately 0.01 ml g^{-1}) (Fig. 6). The course of the plot agrees with the assumption that initial additions of liquid do not close the smallest pores in the modified support surface and the structure which had been less penetrable is becoming more penetrable than that of unmodified supports. With successive applications of liquid the difference becomes less and the obstructive coefficients for modified and unmodified surfaces approach each other.

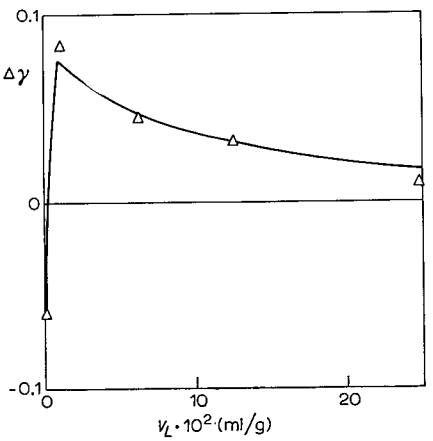


Fig. 6. The plot of the differences in the obstructive coefficients *versus* the volume of coating liquid.

The intraparticle obstructive coefficient represents about 50 % or more of the total obstructive coefficient. Thus filling up the intraparticle pore space with a stationarly liquid also pronouncedly influences the value of the total obstructive coefficient.

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A STUDY OF THE EVAPORATION OF THE STATIONARY PHASE FROM GAS-LIQUID CHROMATOGRAPHIC COLUMNS

ESTERS OF PHTHALIC ACID AND SOME OF THEIR ISOMERS

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SUMMARY

The present work is an attempt at more complete investigation of the evaporation of liquid phases from gas–liquid chromatographic columns. The validity of a general expression, found by the authors, is shown for 23 stationary phases, *viz.* phthalates and their isomers.

Some constants for the phases under investigation which might be useful in gas chromatography are tabulated.

The successful use of the gas–liquid chromatography for analytical and non-analytical purposes is only possible when a suitable stationary phase has been found. Besides a high selectivity, a fundamental requirement for such a phase is its low volatility under operating conditions.

Until recently, no good objective method for characterization of the phases was suggested from this view-point. The data published in the literature have been found empirically.

In our previous paper[1], in which di-*n*-propyl phthalate was used as a model stationary phase, it was shown that the quantity of phase evaporated from the column could be calculated from the equation:

$$\ln m = \frac{B}{T} + \ln V + C \qquad (1)$$

where m is the quantity of the stationary phase evaporated from the column in g, V is the volume of the gas passed through it in ml, T is the temperature in °K and B and C are constants.

It was shown also that the evaporation of the stationary phase does not depend on the gas flow rate and on the nature and particle size diameter of the inert support.

Eqn. (1) may be used for several purposes.

In the first place it is possible to calculate the liquid phase losses from the

column and to make corrections in the values of the retention volumes if the phase has a high volatility.

From the quantity of the evaporated phase, the concentration of its vapour in the gas flow at a certain temperature may be found, or the temperature at which a certain concentration is reached. Such data are very important when it is necessary to estimate in advance whether the liquid phase volatility at a given temperature will permit the use of a more sensitive detector.

At the same time, from the value of the constant B, the molar latent heat of vaporization, ΔH_v, of the phase under investigation may be calculated from:

$$\Delta H_v = -RB + RT \tag{2}$$

Eqn. (1) permits the calculations mentioned above, if in addition to the experimental parameters T and V, the values of B and C are known.

The present study was undertaken as an attempt to demonstrate the validity and the application of eqn. (1). Some phthalic esters and their isomers were chosen as phases for investigation.

EXPERIMENTAL

Apparatus

All measurements were carried out with an apparatus which had good reproducibility for the experimental conditions in the gas chromatographic column (Fig. 1).

The inlet pressure of the inert gas was reduced by means of coarse (1) and fine (2) reducing valves before entering the column (4).

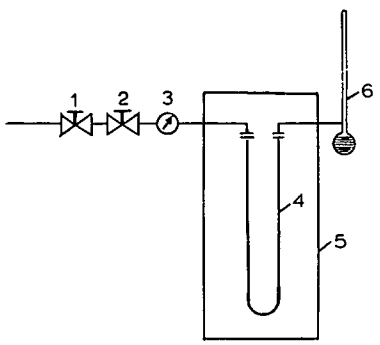


Fig. 1. Apparatus for liquid phase evaporation. 1 = Coarse reducing valve; 2 = fine reducing valve; 3 = manometer, 4 = column, 5 = air thermostat, 6 = soap flow meter.

The column was a 50 cm glass U-shaped tube with a 4 mm internal diameter. The inlet part of the column was always filled with glass spheres (1 mm diameter). In this way, the inlet gas was preheated to the column temperature before entering the chromatographic packing. The chromatographic column was enclosed in an air thermostat (5) with temperature control to within $\pm$ 1°.

Pure dry nitrogen was used as inert gas. The outlet flow rate was measured with a soap flow meter (6) at the end of the column. The measurements were carried out

with gas flow rates of 60 to 120 ml/min. These rates were not corrected by the compressibility factor, because the column resistance was very low.

Ground unglazed tile, with a particle size diameter 0.2–0.4 mm was used as solid support. The weight of the liquid phase was 10 % of the weight of the chromatographic packing.

With exception of the dinonyl phthalate (BDH Ltd.), all of the stationary phases were synthesized by the authors, as described in other papers[2,3].

Calculations

The evaporation of the stationary liquid phase was checked by weighing the column.

TABLE I

CONCENTRATION OF STATIONARY PHASE VAPOUR IN THE CARRIER GAS AT DIFFERENT TEMPERATURES

No.	*Phase*	*$A \cdot 10^6$ g/ml at the following temperatures (°C)*								
		80	*100*	*120*	*140*	*160*	*180*	*200*	*220*	*240*
1	Di-*n*-propyl phthalate	0.9	2.8	7.0	16.5	—	—	—	—	—
2	Di-*n*-butyl phthalate	0.3	1.5	4.5	10.8	—	—	—	—	—
3	Di-*n*-hexyl phthalate	—	—	0.8	2.5	7.8	16.6	—	—	—
4	Di-*n*-octyl phthalate	—	—	—	0.6	2.1	6.4	18.0	—	—
5	Di-*n*-decyl phthalate	—	—	—	—	0.3	1.1	4.2	9.3	—
6	Diphenyl phthalate	—	—	—	0.6	2.7	5.3	10.0	—	—
7	Dibenzyl phthalate	—	—	—	0.8	1.8	3.6	8.4	—	—
8	Di-β-phenylethyl phthalate	—	—	—	—	0.9	2.2	4.6	8.4	—
9	Di-γ-phenylpropyl phthalate	—	—	—	—	—	1.7	4.3	7.7	25.5
10	Diisopropyl phthalate	1.4	4.7	17.3	46.3	—	—	—	—	—
11	Diallyl phthalate	0.4	1.8	8.4	30.2	—	—	—	—	—
12	Di-*sec.*-butyl phthalate	0.6	1.8	5.2	14.0	—	—	—	—	—
13	Diisobutyl phthalate	0.5	2.0	5.1	15.8	—	—	—	—	—
14	Dinonyl phthalate	—	—	—	0.8	2.2	7.9	19.8	—	—
15	Di-*n*-hexyl isophthalate	—	—	0.9	2.8	6.4	14.5	—	—	—
16	Di-*n*-hexyl terephthalate	—	—	0.5	1.2	4.1	10.0	—	—	—
17	Dicyclohexyl phthalate	—	—	0.2	0.9	3.3	11.0	—	—	—
18	Dibornyl phthalate	—	—	—	0.9	1.6	3.4	8.8	—	—
19	Catechol dibutyrate	0.9	3.3	9.9	26.8	—	—	—	—	—
20	Catechol dibenzoate	—	—	—	0.7	2.5	6.0	16.8	—	—
21	Di-*n*-hexyl 4-nitrophthalate	—	—	—	0.4	1.3	3.6	10.7	—	—
22	Di-*n*-butyl tetrachloro-phthalate	—	—	0.4	1.5	5.3	17.5	—	—	—
23	Di-*n*-butyl phthalyl-bis-glycolate	—	—	—	0.6	2.4	7.7	28.2	—	—

The concentration of the liquid phase vapour in the carrier gas at different temperatures was calculated from the liquid phase losses at a certain gas velocity and for a constant time.

The concentration of the liquid phase in the carrier gas depending on the temperature is shown in Table I.

Fig. 2. shows the relationship between concentration of the liquid phase in the carrier gas at different temperatures and is represented by the function $\ln A = f(1/T)$, where A is the concentration of the liquid phase, di-*n*-hexyl phthalate, in the carrier gas.

From the function $\ln A = f(1/T)$ it is easy to calculate, or to find graphically the values of B and C, as well as the temperature at which a concentration limit will be reached.

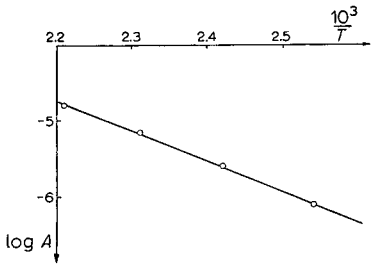


Fig. 2. Plot of the logarithm of the phase vapour concentration in the carrier gas *vs.* the inverse of the temperature. Liquid phase: di-*n*-hexyl phthalate.

RESULTS AND DISCUSSION

According to the data in the literature[4], detectors with a low sensitivity tolerate a concentration of liquid phase vapour in the carrier gas up to 10^{-6} g/ml, while those with a higher sensitivity have a concentration limit of 10^{-8} g/ml.

TABLE II

MAXIMAL OPERATING TEMPERATURES AND VALUES OF B, C AND ΔH_v FOR THE PHASES UNDER INVESTIGATION

No.	*Phase*	*Max. operating temperature (°C)*		$B \cdot 10^{-3}$	C	ΔH_v *(kcal/mole)*
		10^{-6} *g/ml*	10^{-8} *g/ml*			
1	Di-*n*-propyl phthalate	86	21	−2.989	2.427	14.4
2	Di-*n*-butyl phthalate	95	33	−3.642	3.868	17.4
3	Di-*n*-hexyl phthalate	130	60	−4.092	4.277	19.6
4	Di-*n*-octyl phthalate	148	87	−4.980	5.815	23.8
5	Di-*n*-decyl phthalate	178	120	−6.238	7.832	29.4
6	Diphenyl phthalate	135	49	−3.062	1.493	14.9
7	Dibenzyl phthalate	146	65	−3.363	2.026	16.3
8	Di-β-phenylethyl phthalate	163	87	−3.899	3.997	18.7
9	Di-γ-phenylpropyl phthalate	169	99	−4.550	4.274	22.8
10	Diisopropyl phthalate	79	18	−3.699	4.666	17.6
11	Diallyl phthalate	92	42	−4.688	6.841	22.2
12	Di-*sec*-butyl phthalate	89	28	−3.279	3.062	15.7
13	Diisobutyl phthalate	90	28	−3.455	3.514	16.5
14	Dinonyl phthalate	145	80	−4.767	5.400	22.8
15	Di-*n*-hexyl isophthalate	118	43	−3.627	3.119	17.5
16	Di-*n*-hexyl terephthalate	135	69	−4.242	4.393	20.3
17	Dicyclohexyl phthalate	141	82	−5.073	6.246	24.1
18	Dibornyl phthalate	151	75	−3.849	3.081	18.5
19	Catechol dibutyrate	82	24	−3.541	3.997	16.9
20	Catechol dibenzoate	146	94	−3.723	2.994	18.0
21	Di-*n*-hexyl 4-nitrophthalate	157	92	−4.832	5.259	23.0
22	Di-*n*-butyl tetrachloro-phthalate	134	76.5	−4.933	6.129	26.4
23	Di-*n*-butyl phthalyl-bis-glycolate	148	94	−5.592	7.282	26.5

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In the following text "maximal operating temperatures" will be the name given to the temperatures at which the concentration of the liquid phase vapour in the carrier gas is 10^{-6} or 10^{-8} g/ml. The data for the maximal operating temperatures of the liquid phases under investigation are shown in Table II.

The determination of the maximal operating temperatures was carried out graphically, while the values of the constants B and C and the molar latent heats of vaporization ΔH were calculated.

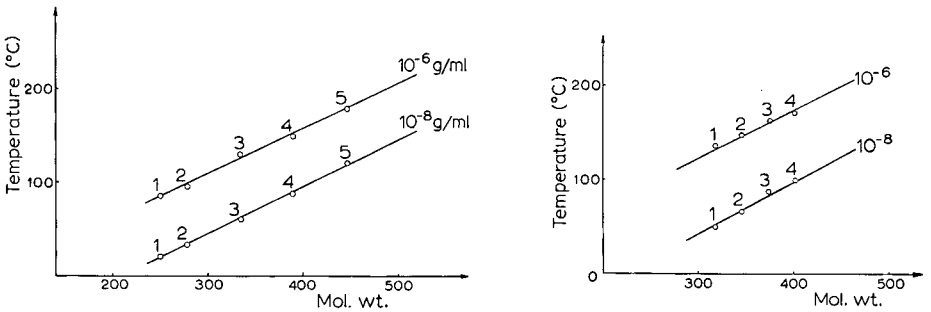


Fig. 3. Plot of the maximal operating temperatures *vs.* the molecular weight of the phases. 1 = Di-*n*-propyl phthalate; 2 = di-*n*-butyl phthalate; 3 = di-*n*-hexyl phthalate; 4 = di-*n*-octyl phthalate; 5 = di-*n*-decyl phthalate.

Fig. 4. Plot of the maximal operating temperatures *vs.* the molecular weight of the phases. 1 = Diphenyl phthalate; 2 = dibenzyl phthalate; 3 = di-β-phenylethyl phthalate, 4 = di-γ-phenylpropyl phthalate.

It is necessary to stress that the maximal operating temperatures at the concentration of 10^{-8} g/ml were found by extrapolation.

The data in Table II are in good agreement with those published for some of the phases investigated. For instance, NABIVACH AND DAL[5] give the maximal operating temperatures (10^{-6} g/ml) as follows for: di-*n*-butyl phthalate = 80–100°; di-*n*-octyl phthalate = 140°; dinonyl phthalate = 130°; di-*n*-decyl phthalate = 175°; diphenyl phthalate = 130°; and di-*n*-butyl tetrachlorophthalate = 125°C.

The phases whose structure changes regularly show a regular change in the corresponding maximal temperatures.

For example in Figs. 3 and 4 the dependence between the molecular weight and the maximal operating temperature for phthalic esters with general formula

$$o\text{-}C_6H_4\begin{matrix}\nearrow COO(CH_2)_nX\\ \searrow COO(CH_2)_nX\end{matrix}$$

is shown graphically in Fig. 3 for X = CH_3 and in Fig. 4 for X = C_6H_5. It is evident that the values of the maximal operating temperatures are a linear function of the molecular weight.

In accordance with the known data for the boiling points of organic compounds with normal and iso structure, the esters of the alcohols with iso chains have lower maximal operating temperatures than those of alcohols with normal chains.

The maximal operating temperature of the unsaturated diallyl phthalate shows a considerable rise in comparison with the maximal operating temperature of di-*n*-

propyl phthalate. Comparing the three isomeric di-*n*-hexyl esters of phthalic acid, di-*n*-hexyl isophthalate has the lowest volatility and di-*n*-hexyl terephthalate the highest.

The phases di-*n*-propyl phthalate and its isomer catechol dibutyrate have practically identical temperatures of operation. However, there is a difference between diphenyl phthalate and its isomer catechol dibenzoate. The values of the maximal operating temperatures of the diphenyl phthalate are lower than those of the catechol dibenzoate.

If one compares the maximal operating temperatures of di-*n*-butyl tetrachlorophthalate (mol. wt. 416) and di-*n*-nonyl phthalate* (mol. wt. 418), it can be seen that the second phase has higher maximal operating temperatures. This fact shows that a better decrease in volatility might be achieved if the ester molecule is made heavier by esterifying with a higher alcohol than by chlorination of the benzene ring.

The introduction of a nitro group into the benzene ring of di-*n*-hexyl 4-nitrophthalate (mol. wt. 379) in comparison with the di-*n*-octyl phthalate (mol. wt. 390) has a more sensitive influence than the chlorination.

The accumulation of the ester groups in the molecule of the ester, as is the case with di-*n*-butyl phthalyl-bis-glycolate (ml. wt. 394) does not give any advantage from the view-point of the liquid phase evaporation, if one compares its maximal operating temperatures with those of di-*n*-octyl phthalate.

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* The maximal operating temperature is found from Fig. 3. The commercial dinonyl phthalate used in this work is usually di-3,5,5-trimethylhexyl phthalate.

CHROM. 3388

EVALUATING DISPERSION IN GEL PERMEATION CHROMATOGRAPHY

I. THEORETICAL ANALYSIS*

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SUMMARY

Dispersion (peak spreading, zone broadening) in gel permeation chromatography (GPC) results from a variety of mechanisms, largely independent and additive in their effects. Among the more important of these, in addition to instrumental contributions, is expected to be eddy diffusion within the chromatographic columns. An experimental approach to the study of dispersion in GPC is proposed, based on existing theories and concepts from gas chromatography and liquid–liquid dispersion in packed beds.

INTRODUCTION

Ever since the early realization that virtually all polymer systems contain a distribution of molecular weights, the study of the details of such a distribution has been a problem of major concern. It was not until the development of gel permeation chromatography (GPC) by MOORE[1] that a practical solution to this problem evolved.

With reference to the usual definition of chromatography[2], GPC may be defined as a chromatographic method in which a liquid phase (polymer solution) percolates through a stationary bed (porous gel), the separation depending on the ability of the solute molecules to penetrate into the gel. The amount of internal pore volume accessible to a given molecular species depends on its size, which in simple cases correlates with its molecular weight. Since higher-molecular-size species can permeate less of the internal pore structure of the gel, they are eluted first. (With MOORE[3], we prefer the terminology of gel permeation to gel filtration[4] or molecular-sieve chromatography[5].)

For a given set of experimental conditions, the volume at which a single solute species is eluted from the column depends on the extent to which it permeates the gel, each molecular size having a characteristic elution volume or (at constant flow rate) elution time. The relations between elution volume, molecular size, and polymer chain

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structure are now rather well understood[6], and we will not discuss this aspect of the subject.

For a variety of reasons, however, not all molecules of a single species are eluted at the same time, resulting in a spread of elution or retention times about a mean. This zone broadening or peak spreading is, in our opinion, the major factor imposing an ultimate limit on the accuracy with which molecular-size distributions can be determined by GPC. Much attention has been given to the empirical correction of chromatograms for dispersion[7–10] but to date no comprehensive theory for this recently-developed technique has appeared. In addition, very little work has been published on liquid-system dispersion in packed beds at low Reynolds numbers. It is the purpose of this paper to develop a method for studying dispersion in GPC columns, with the eventual hope of arriving at a satisfactory theory to explain both the dispersion and the permeation processes.

THEORY

The separation process in a chromatographic column can be considered to occur in a series of hypothetical steps, within each of which equilibrium is achieved between solute concentrations in the mobile and stationary phases. In analogy with distillation theory, each step is termed a plate and is considered to correspond to a specified height of the column. Although the actual separation does not occur in this manner, the concept of height equivalent to a theoretical plate (HETP) is very useful for characterizing column efficiency[11]. The number N of theoretical plates is related to the average retention time T_r and its standard deviation σ by $N = (T_r/\sigma)^2$, and the HETP is simply L/N, where L is the column height. For a Gaussian peak, the width of the chromatogram measured at one-half its height is $2.36\,\sigma$.

We assume that gel permeation chromatography is based on a two-phase model, in which the stationary phase consists of the liquid within the pores of the gel, and the mobile phase is the liquid contained in the interstitial volume, *i.e.*, everywhere outside the gel. We now write a diffusion equation describing each phase.

The diffusion equation for the solute concentration in the mobile phase may be written:

$$\partial C_l/\partial t + U(\partial C_l/\partial x) = D(\partial^2 C_l/\partial x^2) + (k/F_l)\,(C_s - C_l/K) \qquad (1)$$

where C is concentration, t is time, F is volume fraction, and x is distance along the column. The subscripts l and s denote the mobile and stationary phases, U is the interstitial velocity (flow rate), D the effective longitudinal-dispersion coefficient, k the mass-transfer coefficient, and K the distribution coefficient.

Excluding the last term on the right-hand side, this equation is conventional and describes all aspects of the process except for permeation. Its use requires the assumption of plug flow.

The contribution of permeation, described by the last term on the right in eqn. (1), is given by the product of a mass-transfer coefficient k and a driving force equal to the difference in solute concentration across the boundary for permeation. To evaluate this concentration difference, we assume that the solute in the stationary phase (concentration C_s) is in equilibrium with that in the mobile phase at the entrance to the pore. The solute in the mobile phase at this point is assumed to have a

concentration differing from its bulk concentration in the mobile phase, C_l, by a factor $1/K$, where K is the distribution coefficient for permeation. This implies a linear isotherm for the permeation process.

Similarly, the diffusion equation for the solute concentration in the stationary phase may be written:

$$\partial C_s/\partial t = (k/F_s)\,(C_l/K - C_s) \tag{2}$$

By making these equations dimensionless, solving them simultaneously by Laplace-transform techniques, and introducing the boundary conditions ($C(0,t) = C_0(t)$, a unit injection impulse at $t = 0$, and $C(\infty,t) = 0$, it is possible to obtain VAN DEEMTER's equation[11]:

$$\text{HETP} = 2D/U + 2F_sK'U/(1 + K')^2k \tag{3}$$

where $K' = F_s/KF_l$. This approach does not restrict VAN DEEMTER's equation to Gaussian distributions, since no mention was made of the form of the resulting chromatogram. Neither is there any restriction on the nature of the injection impulse, provided that its variance is subtracted from that of the final chromatogram.

Taking the first moment of the distribution of retention times gives the average retention time T_r, while the second moment gives its variance. T_r is related to the distribution coefficient by the equation $T_r = 1 + K'$. This relation is in a dimensionless form such that with no permeation, $T_r = 1$.

Experimentally, care must the taken that the retention time and variance of the sample-injection step are negligible or are subtracted from those observed, to obtain the contribution of the column alone.

The longitudinal-dispersion coefficient is assumed[12] to be the sum of contributions from each of the dispersion mechanisms operating in the column:

$$D = \phi D_l + \lambda U d_p + hR^2U^2/D_r \tag{4}$$

where ϕ is a tortuosity factor, D_l the mobile-phase diffusivity, λ an eddy-diffusion factor, d_p the effective particle diameter, h a velocity-profile constant, R the column radius, and D_r is a radial-diffusivity coefficient. Following DORWEILER[13], we assume[14] that D_r varies in the same way as does the velocity profile across the column, and can be approximated by the sum $\phi D_l + \lambda U d_p$.

On the right-hand side of eqn. (4), the first term represents the tortuosity-corrected liquid diffusivity, or "molecular diffusivity". The second term is the "eddy diffusivity", taking into account the particle diameter and the characteristics of the column packing. The third term accounts for velocity-profile effects and radial dispersion. If, as is usual in liquid systems, the molecular diffusivity is negligibly small compared to the eddy diffusivity, the dispersion coefficient becomes a linear function of flow rate. This was confirmed by LEVENSPIEL AND BISCHOFF[15], who showed that for axial dispersion in liquid systems with fixed beds, D/Ud_p was approximately constant for Reynolds numbers from less than 1.0 to 100. VAN DEEMTER did not include a velocity-profile term in his equation for D.

By utilizing high-molecular-size solutes that do not permeate the gel, it is possible to characterize dispersion in the interstitial regions of the column. Since there is no mass transfer, eqn. (3) reduces to HETP $= 2D/U$, and D is readily studied as a function of operating and column-packing variables. Knowing D, F_s and F_l, it is

possible to evaluate the distribution and mass-transfer coefficients for homologous low-molecular-size solutes which permeate the gel. We believe that studies of this sort offer the possibility of obtaining insight into the mechanisms of the GPC separation.

DISCUSSION

Alternate approaches

In a recent theory for zone broadening in GPC based on well-known concepts in gas chromatography, GIDDINGS AND MALLIK[16] assumed that the individual dispersion mechanisms act independently and contribute additively to the total dispersion observed. An eddy-diffusivity term was coupled with one describing the mobile-phase resistance to mass transfer. LEPAGE *et al.*[17] recently applied this theory to the case of permeating solutes with porous silica beads.

Another experimental approach was taken by HENDRICKSON[18], who postulated that peak spreading obeys an equation of the form

$$\overline{W}_b^2 = \overline{W}_m^2 + \overline{W}_a^2 + \overline{W}_i^2 + \overline{W}_d^2 + \overline{W}_s^2 \qquad (5)$$

where $\overline{W}_b$ is the observed width (at the base) of the chromatogram, and the other terms represent, in order, the contributions to that width from the molecular-size distribution of the test sample, the apparatus, spreading in the interstitial volume within the column, diffusional spreading due to holdup of molecules within the pores of the gel, and sorption. HENDRICKSON attempted to evaluate the last four contributions, and thus obtained $\overline{W}_m$ by difference. TUNG *et al.*[8] utilized a reverse-flow scheme to isolate the contribution of longitudinal mixing to peak broadening. This approach required the assumption that the chromatogram was Gaussian.

It has been suggested[19] that a capacitive effect, produced by stagnant-flow areas in a packed column, may occur in liquid systems. This case was analysed by TURNER[20], who pictured the fluid as occupying a channel with distributed pockets of stagnant liquid. The transport of solute into or out of the "dead volume" was assumed to be controlled only by molecular diffusion. This approach was extended[21] by dividing a packed bed into a turbulent region with complete mixing and a stagnant volume with incomplete mixing controlled by molecular diffusion. It is conceivable that such stagnant volumes (exclusive of the pores in the gel) could exist in GPC columns in the form of regions where the local fluid flow rate was very small compared to the bulk flow rate. They would be less important in the GPC of macromolecules as compared to small molecules because of their widely-different diffusivities. While it would be difficult to prove the presence or absence of such regions, it is important to recognize that they could exist.

Advantages of the present approach

The residence-time distribution for any packed-bed system is closely related to chromatographic efficiency. For example, GIDDINGS AND MALLIK[16] draw an analogy between the causes of peak broadening in GPC and in gas chromatography. One might expect, however, that because the retention mechanisms differ, neither GIDDINGS' nor VAN DEEMTER's equations would describe GPC adequately. The real value in these approaches may lie in studying why they do *not* describe the GPC process accurately.

Perhaps more directly applicable are the models proposed to describe the spread of residence times caused by dispersion in packed beds: the diffusion model and the cascade-of-mixers model[22]. Our approach is based on a diffusion model embodying plug flow and expressing results in terms of an effective dispersion coefficient which includes velocity-profile effects as well as diffusivity.

We feel that the method we have outlined can provide a fruitful approach to a better understanding of GPC. The success of this approach depends on one's ability to obtain accurate dispersion data, and this in turn rests on the need to minimize and correct for extra-column contributions from the apparatus and from the finite width of the sample-injection pulse. We have made a detailed study of these factors, the results of which, together with preliminary dispersion data, will be presented in Part II of this series[23]. We and others[24] have observed significant non-Gaussian behavior (tailing) in these extra-column contributions, which can seriously limit the accuracy and column-efficiency of the GPC apparatus.

While HETP is widely used to describe zone broadening in packed-bed processes, its use has a disadvantage in that the HETP of a given column may change significantly with the nature of the solute and with other operating variables, such as flow rate. Thus, SMITH AND KOLLMANSBERGER[25] showed that HETP decreased linearly with decreasing flow rate for permeating solutes, except for a downward concavity at very low flow rates, as predicted by GIDDINGS' proposal[16]. The theory presented here similarly predicts the downswing, followed by a sharp increase at extremely-low flow rates where molecular diffusion becomes important.

In addition to the extra-column contributions mentioned, a thorough study of dispersion in GPC will have to deal with such complexities as velocity-profile effects, molecular diffusion, eddy diffusion, stagnant-flow areas, non-Fickian diffusion, non-linear permeation isotherms, viscosity effects with macromolecular solutes, sorption, particle-size effects, and column-packing variables, to mention only a few. Each of these will have to be evaluated in an as nearly independent fashion as possible prior to the development of a detailed understanding of the mechanism of the GPC process.

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CHROM. 3389

EVALUATING DISPERSION IN GEL PERMEATION CHROMATOGRAPHY

II. THE INJECTION–DETECTION SYSTEM*,**

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SUMMARY

The standard injection–detection system in the Waters Gel Permeation Chromatograph can lead to significant contributions to peak broadening and retention time when single columns or short columns are used in the gel permeation chromatography of macromolecular solutes. Use of a micro refractometer cell and elimination of all excess tubing, including the heat exchanger, led to significant reductions in contributions to peak width at half-height (0.065 to 0.019 in. at standard recorder-chart speed and a flow rate of 1 cc/min) and to retention time (0.166 to 0.040 in.) from the injection–detection system. Anomalies in the chromatograms such as spurious peaks and severe tailing were also eliminated. HETP data as a function of flow rate agreed with theory (Part I of this series); HETP for a nonpermeating macromolecular solute was always greater than that for a permeating low-molecular-weight solute (1.4×10^{-3} *vs.* 0.8×10^{-3} ft. at 1 cc/min). Values of D/Ud_p were approximately constant near 5.0 for Reynolds' numbers between 0.03 and 0.3.

INTRODUCTION

Gel permeation chromatography (GPC), as introduced by MOORE[2], has become in a very short time one of the most powerful analytical tools available to polymer chemists. A wide variety of polymer types covering a molecular-weight range from that of monomers to several million has been successfully analyzed. GPC offers a convenient method for on-stream analysis in commercial production units. The introduction of a preparative-scale unit[3] should make available gram-size quantities of narrow-distribution polymer fractions for research purposes. Detailed information on the molecular-weight distribution of a sample, which previously would have taken weeks to obtain, can usually be obtained by GPC in a matter of hours. Not since the introduction of the light-scattering method of DEBYE[4] has a new technique had such an impact on polymer science in the first few years of its existence.

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** For Part I of this series, see ref. 1.

The GPC process is basically a liquid–solid chromatographic method in which the separation is carried out by passing a dilute polymer solution through a column containing a rigid, but highly porous, crosslinked polystyrene gel packing. The solute molecules permeate the gel according to their volume in solution[5–7]. Since the higher-molecular-size species have less pore volume to permeate, they are eluted first. As is true with all chromatographic processes, dispersion during flow through the packed column and associated tubing and detector causes a spread in retention times about the mean. This peak spreading is a major factor limiting the accuracy of GPC analyses of polydisperse polymers.

One great advantage of GPC is its speed compared to that of other characterization methods. As GPC becomes adapted for on-stream analysis in commercial production units, speed of analysis will become increasingly important. For shorter column lengths, corrections for extra-column broadening can also become important. To evaluate accurately dispersion occurring within the column, and to apply the theory presented in Part I of this series[1], it is necessary to apply corrections for the finite width of the injection–detection pulse and for broadening arising from extra-column contributions. In this paper, the nature and importance of these contributions is examined. The injection and detection systems of the Waters Gel Permeation Chromatograph were studied without a column and with both standard and micro refractometer cells. After proper correction for extra-column effects, data were obtained and are presented for the dispersion of both permeating and nonpermeating samples, determined as a function of flow rate using a single column.

EXPERIMENTAL

A Waters Associates (Framingham, Mass.) Model 100 Gel Permeation Chromatograph was used with tetrahydrofuran as the solvent at room temperature. Recorder chart speeds up to 60 in./h were used to allow accurate measurement of peak widths at half-height. The instrument was used without a column to study the characteristics of the injection–detection system; both a standard refractometer cell (volume 70 μl) and a micro cell (volume 10 μl) were used, with a 1/16-in. null glass. Dispersion was studied with a column designated by Waters as 250 Å. The minimum molecular weight of polystyrene eluted at the interstitial volume of this column (21.94 cc) should be approximately 10,000. As determined with *o*-dichlorobenzene (ODCB) at a flow rate of 1 cc/min, this column had approximately 1200 plates/ft. In the dispersion studies, ODCB was used as a permeating solute and a narrow-distribution polystyrene (PS) (Pressure Chemicals Co., Pittsburgh, Pa.; $\overline{M}_w$ = 160,000; $\overline{M}_w/\overline{M}_n$ = 1.06) as a nonpermeating macromolecular solute.

Modifications made to the Chromatograph during study of the injection–detection system are described in the next section.

RESULTS

The injection–detection system

To evaluate the extra-column contributions to peak broadening, samples were injected through the sample loop directly into the refractometer cell, with no column in the system. The sample loop furnished with the instrument, consisting of 81.18 in.

of 1/16-in. O.D. hypodermic tubing, contains approximately 2 cc. It is common practice to inject from half to all of this material (injection times of 1–2 min at a flow rate of 1 cc/min) when using multiple-column assemblies for analytical purposes. Solute concentrations normally range from 0.25–1 wt. %.

When operating under these conditions with a 0.5 % PS solution, we observed considerable tailing (asymmetry) in the injection–detection pattern with no column in the system. As the flow rate was lowered, a double peak was produced. Injection–detection patterns for ODCB showed considerably less tailing. Similar effects have been noted by others[8–10].

To minimize these effects, we made several changes in the chromatograph flow system, *viz.*

(1) The size of the injection loop was reduced from 81.18 in. to 5.18 in. of 1/16-in. O.D. hypodermic tubing, corresponding to a volume of 0.13 cc.

(2) All excess tubing (5–6 ft.) between the sample-injection loop and the refractometer was eliminated.

(3) The sample and reference inlets to the refractometer were interchanged because we observed that this improved the pattern.

These changes reduced peak broadening in the injection–detection system considerably but improved the tailing only slightly. As shown in Fig. 1, the peaks appeared to approach symmetrical (Gaussian) shape at high flow rates, but showed considerable tailing at low flow rates and severe anomalies in an intermediate region

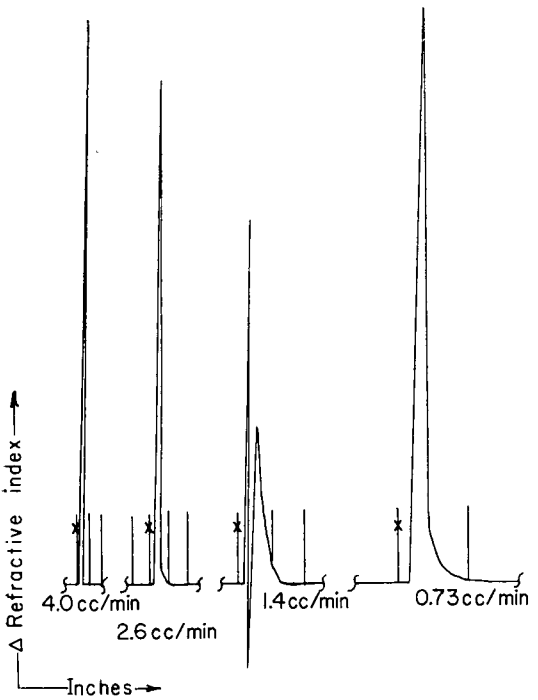


Fig. 1. Injection–detection patterns with standard refractometer cell, at the indicated flow rates. Flow system modified as described in the text; 0.1 % PS in THF at room temperature; standard recorder-chart speed: 6 in./h.

(1.1–1.6 cc/min). With ODCB, however, peaks were nearly symmetrical at all flow rates (Fig. 2).

To insure that these phenomena were not peculiar characteristics of our own instrument, we repeated the experiments on another chromatograph belonging to Waters Associates, with essentially the same results. No appreciable tailing was observed with ODCB as solute at any flow rate, whereas solutions of PS showed both tailing and other anomalies, including a sharp negative deflection just before the peak, a characteristic also seen by OSTERHOUDT AND RAY[8].

It seems probable, particularly in view of the difference in behavior between the two sides of the refractometer cell, that these anomalies result from the flow pattern and mixing characteristics in the cell. We believe that at high flow rates, sufficient mixing occurs in the cell to prevent severe distortion of the peak. At lower flow rates with the more viscous solutions of polymeric solutes, poor mixing causes the tailing and other anomalies. With ODCB as the solute, these are not observed because of its lower viscosity and higher diffusivity.

Since the results of these flow disturbances can still be seen with single columns in the instrument, it is essential to minimize them and correct for them if dispersion in the column is to be studied accurately. (It should be noted that these flow disturbances are usually insignificant when the Waters Instrument is used with more than two columns.) Following a suggestion of MOORE AND HENDRICKSON[11], we explored the use of a micro refractometer cell as a means of reducing both the breadth and the tailing of the injection–detection peak.

The Waters micro refractometer cell has an internal volume of 10 μl compared to 70 μl for the standard cell, and a flow pattern more nearly duplicating that in capillary tubing. In addition to installing such a cell, we removed several feet of capillary tubing included in a heat-exchanger block located between the columns and the refractometer. While this heat exchanger is required to achieve temperature

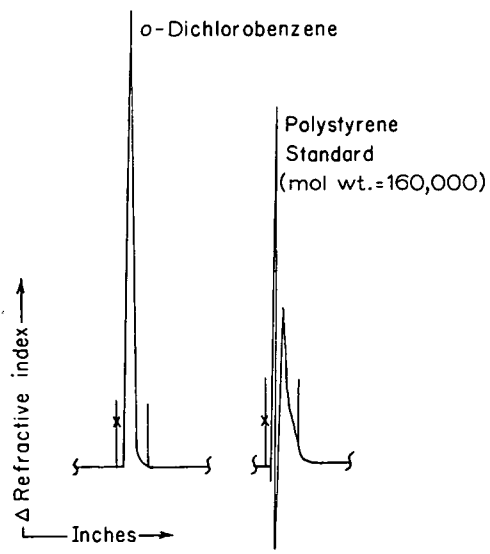


Fig. 2. Injection–detection patterns with standard refractometer cell. Experimental conditions as in Fig. 1; samples: 0.5 % ODCB and 0.1 % PS; flow rate: 1.45 cc/min.

equilibration in the sample and reference streams when operating at elevated temperature, it has proved unnecessary in our work at room temperature. We passed the liquid stream into the micro refractometer cell from either the column or our shortened injection loop with the shortest possible length of capillary tubing. Where necessary, the location of the injection valve and loop was changed to keep tubing lines no more than a few inches long.

Injection patterns obtained with the chromatograph modified in this way are shown in Fig. 3. It can be seen that tailing was greatly reduced, and other anomalies eliminated, over the entire range of flow rates used in this work. In other experiments, we found that the micro cell gave approximately three times the sensitivity and one half the peak width obtained with the standard cell.

Column end-fittings

The effect of column end-fittings on peak broadening was assessed separate from that of the remainder of the system by preparing a "column" by joining two such fittings together with a minimum of space between them. The use of these end-fittings increased peak broadening slightly. More experiments are planned in this area since we have not yet determined whether the flow pattern is identical with that at the ends of a packed column.

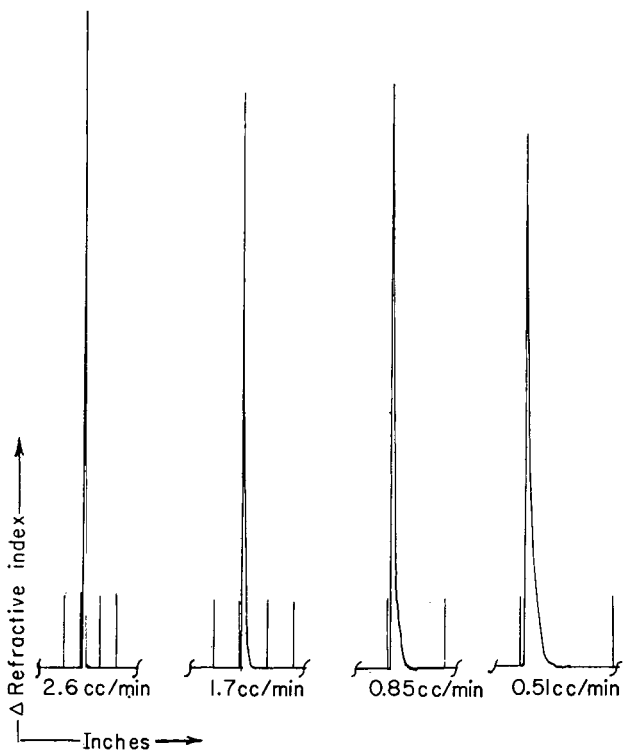


Fig. 3. Injection–detection patterns with micro refractometer cell, at the indicated flow rates. Flow system modified as described in the text; 0.1 % PS in THF at room temperature; standard recorder-chart speed: 6 in./h.

Corrections for extra-column effects

Fig. 4 shows the variation with reciprocal flow rate of the width at half-height of the injection–detection pulse. The relationships are linear within experimental error, with the broadening greater for ODCB than for PS, and the end-fittings increasing the broadening slightly.

The average residence time of the pattern was also found to increase linearly with reciprocal flow rate, as shown in Fig. 5. The end-fittings and associated tubing almost doubled the residence time at a given flow rate for PS. We do not yet understand why slightly longer residence times appear to be observed for ODCB.

From the curves of Figs. 4 and 5, accurate corrections to any chromatogram can be made for residence time and peak broadening associated with the injection–detection system.

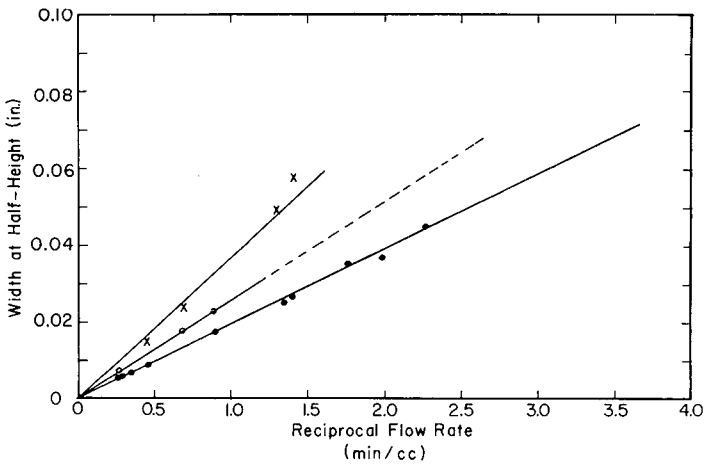


Fig. 4. Extra-column contributions to peak broadening. (●) 0.1% PS, end-fittings not included; (○) 0.1% PS, end-fittings included; (×) 0.5% ODCB, end-fittings included. Conditions same as in Fig. 3.

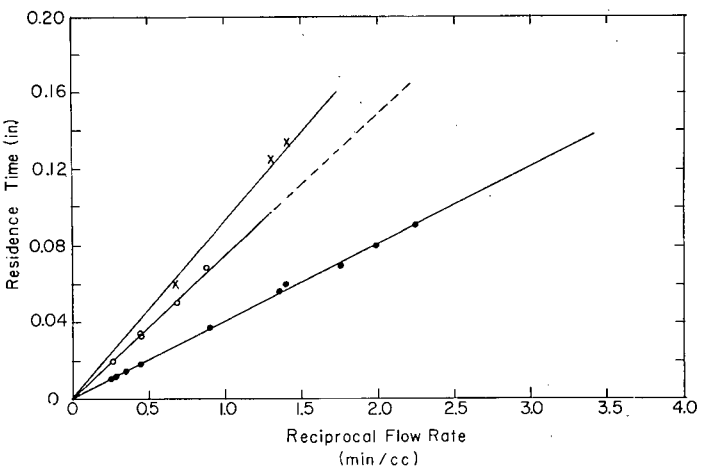


Fig. 5. Extra-column contributions to residence time. Same identifications as for Fig. 4; other conditions same as in Fig. 3.

Dispersion data

Dispersion data were obtained with a single 250 Å column, using ODCB as a permeating solute and PS as a nonpermeating macromolecular solute. At each flow rate, retention time and peak width at half-height were corrected for extra-column effects using the data of Figs. 4 and 5. Values of height equivalent to a theoretical plate (HETP) were calculated from the average residence time (location of peak), peak breadth and column length as described in Part I[1]. These results are given in Fig. 6.

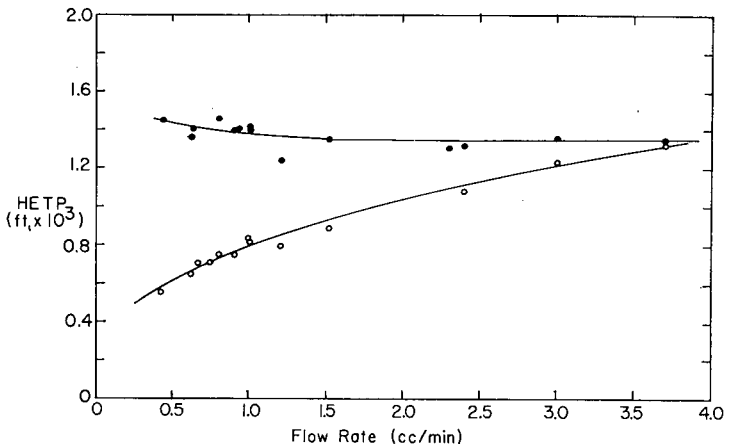


Fig. 6. Effect of flow rate on HETP for permeating (○, 0.5% ODCB) and nonpermeating (●, 0.1% PS) solutes. Single 250 Å column; other conditions same as in Fig. 3.

For the nonpermeating solute PS, the effective longitudinal-dispersion coefficient was calculated from the relation (Part I) HETP $= 2D/U$ where U is the flow rate. Numerical values leading to this calculation are given in Table I. It can be seen from Table I that, with the modified flow system, corrections for extra-column peak broadening were indeed negligible.

The dispersion characteristics of the high-efficiency column used in these experiments can be illustrated by plotting D/Ud_p (the inverse of the Peclet number), where d_p is the particle diameter of the column-packing gel, against the Reynolds number. Such a plot is shown in Fig. 7; the value of d_p was taken as 40 μ, which is in the range of the actual particle size. The region designated as typical for liquid axial dispersion was taken from LEVENSPIEL's work[12] on the axial dispersion of solutes in packed beds of uniform-size spherical particles.

DISCUSSION

Our results show that accurate dispersion data can not be obtained from experiments with single GPC columns using a standard refractometer cell in the Waters Chromatograph and retaining the large amount of hypodermic tubing incorporated in the normal flow system. Flow anomalies associated with this injection–detection system were, however, virtually eliminated in our instrument by changing to a micro refractometer cell, minimizing the amount of tubing in the system (including elimination of the heat exchanger), using short injection times (5–10 sec at a

TABLE I

DISPERSION CHARACTERISTICS OF A MACROMOLECULAR SOLUTE (PS) WITH A 250 Å COLUMN[a]

Flow rate (*cc/min*)	*Residence time*[b] (*in.*)	*Variance*[b] (*in.*$^2 \times 10^4$)	*HETP* (*ft.* $\times 10^3$)		*U* (*ft./min*)	*Reynolds number*[c]	D[d] ($ft.^2/min \times 10^4$)
			Uncorrected	*Corrected*			
0.43	5.015	91.186	1.46	1.45	0.080	0.029	0.580
0.62	3.476	41.300	1.38	1.37	0.115	0.041	0.788
0.63	3.425	41.413	1.42	1.41	0.116	0.042	0.818
0.79	2.714	26.937	1.47	1.46	0.147	0.053	1.073
0.90	2.439	20.856	1.41	1.40	0.164	0.059	1.148
0.92	2.320	19.002	1.42	1.41	0.172	0.062	1.212
0.98	2.204	17.235	1.43	1.42	0.181	0.065	1.285
1.00	2.156	16.175	1.40	1.39	0.186	0.067	1.292
1.20	1.771	9.640	1.24	1.23	0.226	0.081	1.390
1.52	1.418	6.820	1.36	1.35	0.282	0.101	1.903
2.29	0.922	2.758	1.30	1.30	0.434	0.155	2.821
2.39	0.886	2.617	1.34	1.33	0.451	0.161	2.999
2.99	0.700	1.662	1.35	1.35	0.571	0.204	3.854
3.68	0.565	1.076	1.35	1.34	0.708	0.253	4.743

[a] 0.1% PS in THF at room temperature; micro refractometer cell and modified flow system as described in the text.
[b] Data corrected for extra-column effects and converted to standard recorder-chart speed.
[c] Calculated assuming $d_p = 40\ \mu$, solution density 0.89, solution viscosity 0.50 cP.
[d] Calculated assuming $d_p = 40\ \mu$.

flow rate of 1 cc/min), and reducing solute concentration. For example, the injection–detection system gave rise to a residence time of 0.166 in. and a peak width at half-height of 0.065 in. for 0.1 % PS at 1 cc/min, using the standard refractometer cell and normal flow system; these figures were reduced to 0.040 in. and 0.019 in., respectively, with the micro cell and other modifications to the system. The effect of column end-fittings will increase both sets of figures somewhat, but the comparison will not change.

Both the correction factor for width at half-height and that for residence time, plotted as functions of flow rate in Figs. 4 and 5, respectively, are greater for ODCB

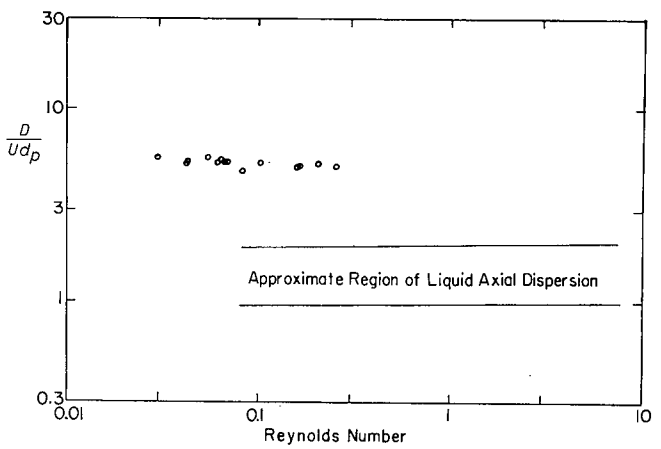


Fig. 7. D/Ud_p *vs.* Reynolds' number for nonpermeating solute (0.1 % PS); conditions as in Fig. 6.

than for PS. We believe this reflects the larger diffusivity of ODCB as compared to the macromolecular solute, giving rise to better radial mixing with ODCB. Most of the effect of column end-fittings, also shown in these figures, lies in the larger residence time associated with the fittings and additional tubing required; they contribute little to the peak broadening.

Dispersion data corrected for the injection–detection system characteristics, Fig. 6, show that HETP varies little with flow rate for a nonpermeating solute, as predicted by the theory presented in Part I[1]. For a permeating solute, ODCB, HETP drops with flow rate decrease in a concave-downward curve observed by others[7,9] using multiple-column assemblies and consistent with our approach[1] and that of GIDDINGS[13].

We believe it important to note that HETP for the nonpermeating solute is substantially higher than for the permeating solute at corresponding flow rates. This may result in part from the larger volume (interstitial plus pore volume) available to a permeating solute, and its correspondingly longer retention time. Even if the two values of HETP could be compared at equal elution volumes, however, we feel that a second effect, arising from the higher viscosity and lower diffusivity of a macromolecular solute, would be important.

The form of the relation for HETP,

$$\text{HETP} = (2D/U) + CU$$

where C describes the permeation effect, would suggest that HETP should be higher for a permeating solute if the two species had similar values of D. But, as indicated in Part I, D is made up of a molecular-diffusivity term (usually quite negligible in liquid systems), an eddy-diffusivity term, and a velocity-profile term. The velocity-profile term is inversely proportional to a radial-dispersion number, D_r. This number should be relatively large for low-molecular-weight solutes, and thus much larger for ODCB than for PS. In other words, velocity-profile effects are expected to be much more important for high polymers than for low-molecular-weight solutes in flow through packed beds.

Since D_r is large for ODCB, the second term in, and thus the value of, D is smaller for ODCB than for PS, leading to the observed lower values of HETP for this solute. This analysis assumes that the eddy-diffusivity term does not outweigh the velocity-profile term for macromolecular solutes. As yet we have no evidence on this point; its elucidation is one objective of our continuing studies.

From Fig. 7 it appears that values of D/Ud_p for a nonpermeating macromolecular solute are much higher than those predicted from classical dispersion studies in packed beds[12]. We believe this effect is also associated with the high values of D for macromolecular solutes, reflecting the importance of velocity-profile effects. Segregation effects may also be significant since the column-packing gels used in GPC contain a range of particle sizes. It seems likely that with more uniform column packings impermeable to nonviscous solutes such as ODCB, values of D/Ud_p would be more nearly within the region of liquid axial dispersion.

Clearly, the key to any successful treatment of gel permeation chromatography lies in separating and examining individually each process contributing to retention time and peak broadening. Any single column-efficiency parameter, such as HETP, of

necessity combines these individual contributions, and obscures an assessment of their relative importance.

ACKNOWLEDGEMENTS

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CHROM. 3360

FURTHER STUDIES ON THE CHROMATOGRAPHIC SEPARATION OF THE COLLAGEN MOLECULAR SUBUNITS

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SUMMARY

A study has been made of three chromatographic methods used for the separation of collagen molecular components. Two of the methods use a salt gradient, while the third uses a salt and pH gradient to elute the protein from the column. Optical absorption at 220, 230 and 278 mμ was used for analysis of the column effluent in one of the methods, in order to follow protein concentration and the distribution of the aromatic amino acids. These methods yield minor components which could correspond to acidic and basic telopeptides which are essential to the structure of collagen.

INTRODUCTION

The chromatographic separation of the collagen molecular subunits has been based mainly on the simple linear gradient technique of PIEZ and co-workers[1,2], which uses CM-cellulose and acetate buffers. In general, the column effluent from these separations has been analysed spectrophotometrically at 230 mμ using flow cells of relatively large volume (± 1 ml), or by chemical means using bulk fractions (5 to 10 ml). While the method of PIEZ may resolve all the components present in a monomeric extract, it has been shown by several authors (see for example TRISTRAM, WORRALL AND STEER[3], COOPER AND DAVIDSON[4], and DAVIDSON AND COOPER[5]) that this simple gradient does not give complete resolution of all components present in a polymeric extract. Therefore some authors report using strong alkali and even 6 *M* urea to recover all the protein (see for example VEIS AND ANESEY[6]), while the present authors have developed a combined pH and salt gradient using phosphate buffers[4]. It is therefore of interest to study the effect of pH on the stability and solubility of soluble collagen.

In addition, the chromatographic resolution of soluble collagen has been improved with the aid of the Beckman Spectrochrom automatic chromatography analyser, which employs a micro cuvette for optical analysis of the column effluent. This micro cuvette has volumes of about 0.05 and 0.01 ml in its dual path length compartment of 10 and 2.5 mm respectively, compared with about 1 ml in most flow cells of 10 mm path length. Further the Spectrochrom is capable of continuously analysing the column effluent at any three fixed wavelengths. The distribution of the important aromatic amino acids in relation to the collagen subunits has therefore

been studied by optical absorption at 278 mμ. These aromatic amino acids play an important role in both intra- and intermolecular bonding[7].

EXPERIMENTAL

Preparation of collagen

The preparation and purification of the neutral-salt-soluble and acid-soluble collagen has been described previously[8].

Column chromatography

The separation of heat-denatured soluble collagen was carried out using the following three methods:

(1) *Modified* PIEZ *method.* In this method a column kept at 40° was packed with CM-cellulose (Bio Rad Laboratories, Lot No. 4146) to give a bed 2.5 × 34 cm. The stock acetate buffer of PIEZ *et al.* (pH 4.8 and I = 0.5) was diluted to give their starting buffer, I = 0.06, and limiting buffer, I = 0.16. The linear gradient was prepared from 500 ml of each of these two buffers in separate flasks[1]. This was pumped onto the column (Beckman Accuflo pump) at 100 ml/h. After 900 ml of this gradient had been used, acetate buffer of a higher salt content (I = 0.26), prepared from the starting buffer by the appropriate addition of sodium chloride, was pumped through the column. The column effluent passed through the flow cell (1 cm path length, 1 ml volume) of a recording spectrophotometer (Beckman Model DB) set at 230 mμ, and was collected in 10 ml aliquots in a refrigerated fraction collector (Beckman Model 132). The collagen was dissolved in the starting buffer, to give solutions containing about 50 mg/25 ml, heat-denatured at 45° for 30 min, and run into the column with nitrogen at 10 p.s.i.

(2) *Spectrochrom method.* This method used the same column, column temperature, flow rate, gradient and sample preparation as above. The Spectrochrom was set to analyse the column effluent at 220, 230 and 278 mμ. At the same time continuous recordings of the conductivity and pH of the column effluent were obtained, which acted as a control on the gradient. The column effluent was collected in 10 ml aliquots by the drop counting method.

(3) *The pH and salt gradient method.* This method has been described in detail elsewhere[4].

Two dimensional high voltage electrophoresis and chromatography

A Shandon water-cooled HVE apparatus and Whatman No. 4 paper was used[9]. The electrophoresis was carried out in a formic and acetic acid buffer (pH 1.6) at 6,000 V for 25 min. The chromatography was done with equal volumes of 2:6-lutidine, collidine and water for 24 h at 20°. The method was standardised with a known mixture of pure amino acids. The amino acids were detected by spraying the dried paper with ninhydrin.

Optical rotation and mutarotation

These methods have been described previously[8,10]. A Perkin Elmer Model 141 digital-readout polarimeter was used at wavelengths of 589 and 365 mμ.

Ultracentrifugation

The same procedure was used as described previously[8].

Amino acid analysis

Amino acid composition was determined with a Beckman Unichrom amino acid analyser after hydrolysis with 6 *N* HCl under vacuum in sealed tubes at 100° for 24 h.

RESULTS AND DISCUSSION

Chromatographic separation of collagen components by the pH gradient method

A typical separation of acid-soluble collagen by the pH gradient method into α- and β-subunits plus the additional components a, b and c is shown in Fig. 1. From this it will be seen that the α- and β-subunits elute at about pH 5.0, while component a elutes at pH 8.0, and components b and c at pH 11.0. The fact that these components elute at such relatively high pH values leads to interesting speculation as to their nature and relation to the α- and β-subunits and tropocollagen. It was therefore considered important to follow the action of pH and temperature changes on the stability and solubility of acid-soluble collagen.

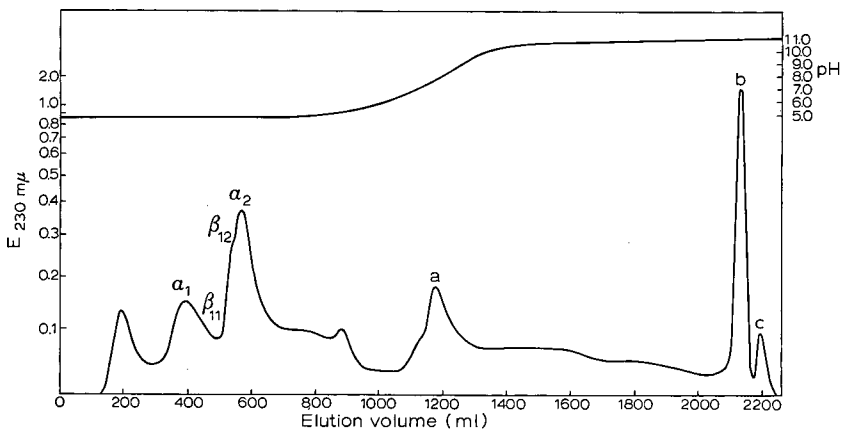


Fig. 1. Chromatography of 98 mg of acid-soluble collagen on CM-cellulose at 40° using a phosphate buffer gradient (see text). The upper curve shows the pH gradient.

In a series of experiments samples of acid-soluble collagen, as prepared and characterised in previous publications[5,8] were added to 0.15 *M* potassium acetate buffer (pH 4.8), which is a good solvent for mutarotation studies, to 0.1 *M* potassium phosphate buffer (pH 8.0), and to an alkaline phosphate buffer (pH 11.0). The latter corresponds to the solution used to elute components b and c from CM-cellulose[4]. The acid-soluble collagen was not readily soluble in any of these solvents at 4°, but on heating at 45° for 15 min the protein dissolved readily in all three instances. It is of interest to note that with the phosphate solution of pH 8 heating at 40° for 2 h did not result in the protein dissolving, but on raising the temperature to 45° the collagen dissolved readily. The melting point (T_m) of this acid-soluble collagen in 0.15 *M* acetic acid, as measured by optical rotation[5], was 38.1°.

After heating at 45° for 15 min to dissolve the collagen, these solutions were stored at 4° for one week. Samples of the solutions of known concentration were then heated at 45° for 15 min and immediately placed in a polarimeter tube thermostated at 15°. The mutarotation of these solutions was then followed for 48 h at 15° and 589, 578, 546, 436 and 365 mμ. The results as measured at 365 mμ are shown in Fig. 2. In Table I the specific rotation values are given for the original collagen solutions and for the solutions after heating and keeping at 15° for 48 h. From these results it is evident that at pH values of 4.8 and 8.0, and presumably at pH values in between these, the mutarotation and final specific rotation are largely unaffected, whereas at pH 11 the specific rotation is significantly reduced. The specific rotation at this pH ($[\alpha]_D^{15} = -198°$ and $[\alpha]_{365}^{15} = -649°$) is, however, still greater than the value for the rotation of α-helix-forming proteins and collagen in the denatured form ($[\alpha]_D^{15} = -90°$ to $-120°$, $[\alpha]_{365}^{15} = -450°$; HARRINGTON AND VON HIPPEL[11]), showing that even after heating at 45° and pH 11, acid-soluble collagen on cooling still retains a degree of helical content. At this pH the mutarotation after 48 h at 15° gave a 90 % recovery (Table I).

Ultracentrifugation at 35° of acid-soluble collagen after heating at 45° in the phosphate solution of pH 11 showed the presence of low molecular weight degradation products (Fig. 3), but a large proportion of material with a relatively high sedimen-

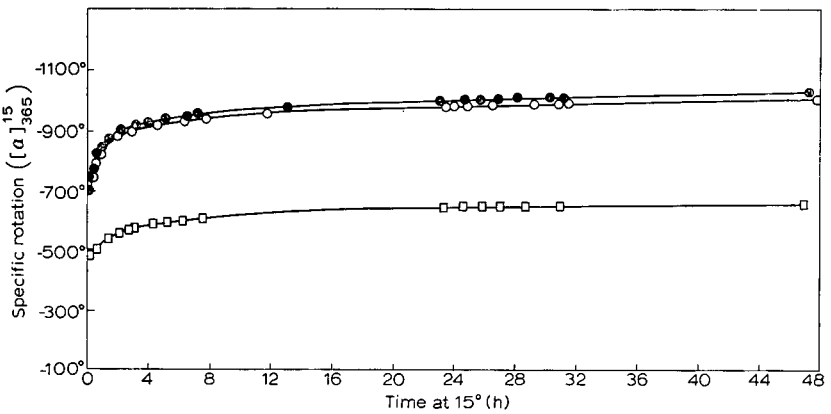


Fig. 2. Specific rotation ($[\alpha]_{365}^{15}$) as a function of time for solutions of acid-soluble collagen (1.40 mg/ml) at various pH values, heat-denatured at 45° for 15 min and then kept at 15°. (●) pH 4.8; (○) pH 8.0; (□) pH 11.0.

TABLE I

MUTAROTATION OF HEAT-DENATURED ACID-SOLUBLE COLLAGEN AT 15° AND VARIOUS pH VALUES

pH	*Specific rotation*			
	Initial		*After 48 h at 15°*	
	$[\alpha]_D^{15}$	$[\alpha]_{365}^{15}$	$[\alpha]_D^{15}$	$[\alpha]_{365}^{15}$
4.8	−318°	−1025°	−318°	−1025°
8.0	−292°	− 998°	−292°	− 998°
11.0	−206°	− 721°	−198°	− 649°

tation constant ($S_{20,w} = 2.2$) was still present. This may be compared (Fig. 3) with the sedimentation at 35° of acid-soluble collagen in formate buffer (pH 3.75) after denaturing at 45°. Here the α- and β-subunits are clearly shown ($S_{20,w} = 2.6$ and 3.4, respectively). It is evident from Fig. 3 that the alkaline treatment decomposes the β-subunit. DOTY AND NISHIHARA[12] and ASTRUP *et al.*[13] report a similar decrease in the β-subunit at pH values above 9.

While the above results show the effect of high pH on acid-soluble collagen as a whole, in the actual chromatography procedure used (Method 3) the pH gradient only reached high values after the bulk of the acid-soluble collagen had been eluted from the column. Therefore component b was isolated by this chromatography procedure

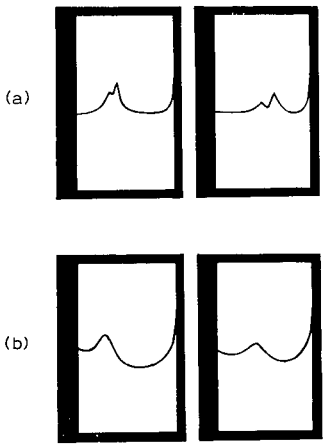


Fig. 3. Sedimentation patterns of (a) heat-denatured acid-soluble collagen in sodium formate buffer, pH 3.75, and (b) heat-denatured acid-soluble collagen in phosphate solution, pH 11.0. The photographs were taken after 80 and 120 min at 56100 rev/min and 35°. The solutions contained 0.4% of protein. Sedimentation is from left to right.

(Method 3), dialysed against several changes of distilled water and freeze-dried. This was then dissolved in potassium acetate buffer (pH 4.8) and its optical rotation measured. The specific rotation of component b ($[\alpha]_D^{15} = -159°$, $[\alpha]_{365}^{15} = -527°$) is only slightly greater than that of collagen in the denatured form ($[\alpha]_D^{15} = -90°$ to $-120°$, $[\alpha]_{365}^{15} = -450°$), showing that component b under the conditions of its preparation has a low helical content in comparison with tropocollagen.

Chromatographic separation of collagen components by the Spectrochrom method

A typical separation obtained by the method of PIEZ *et al.*[1] is shown in Fig. 4, where the optical absorption was measured at 230 mμ. The improved resolution using the micro cuvette of the Spectrochrom method is illustrated in Fig. 5. This shows the optical absorption at 220 mμ, due to peptide bonds, and at 278 mμ from the presence of aromatic amino acids. These results confirm previous findings[4,5] that the method of PIEZ *et al.*[1] does not give a complete picture of the chromatographic components. The components obtained using the Spectrochrom method (Fig. 5) are in many respects similar to those found by the more complicated and longer method of step-wise gradients[5], and by the pH-gradient method (Fig. 1). The distribution of the components, particularly the α- and β-subunits, was studied in detail for the separation

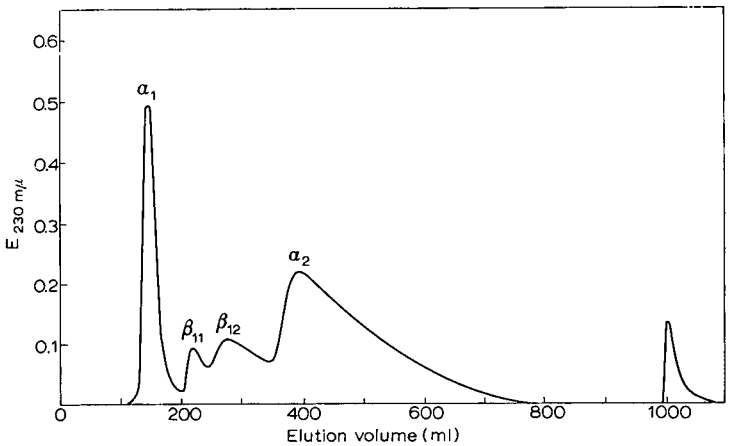


Fig. 4. Chromatography of 50 mg of acid-soluble collagen on CM-cellulose at 40° using a modified PIEZ gradient (see text).

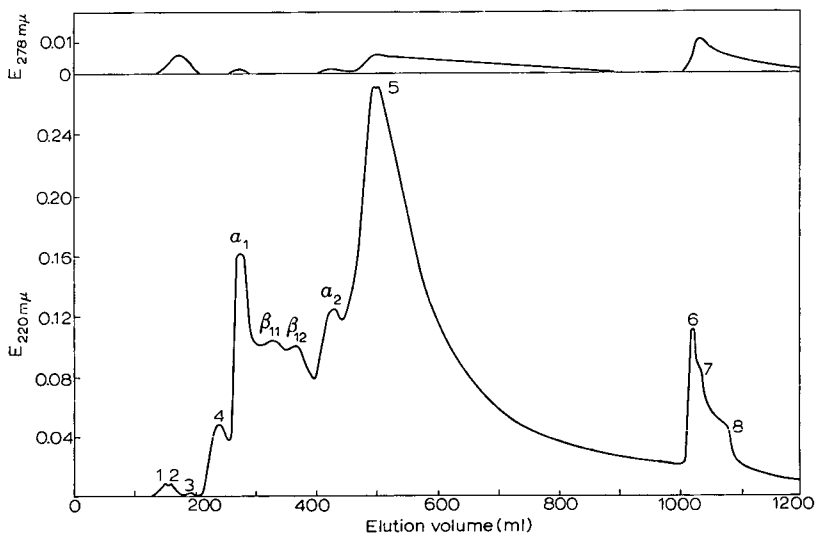


Fig. 5. Chromatography of 50 mg of acid-soluble collagen on CM-cellulose at 40° using the Spectrochrom method (see text). The lower curve represents the optical absorption at 220 mμ and the upper curve at 278 mμ.

obtained by the method of step-wise gradients[5], and was used here to confirm the allocation of the α- and β-subunits in the separation by the Spectrochrom method. Sedimentation studies have shown the absence of γ-components[5,8] in the collagen preparations used here. It is suggested that component 4 may correspond to the α_3-subunit[14]. Both methods were reproducible and gave similar results for several preparations of neutral salt-soluble and acid-soluble collagen, before and after lyophilisation.

The proportions of the various components are given in Table II. One of the most important features of these data is the relative proportions of components with optical absorption at 230 mμ, which is due to peptide bonds and is therefore a measure

of protein concentration, and the corresponding absorption at 278 mμ due to the presence of aromatic amino acids. Thus the minor components 1, 2, 3, 6, 7 and 8 which constitute only 6 % of the whole preparation contain about 43 % of the aromatic amino acids, while the α- and β-subunits comprise 29 % of the preparation and only contain 8 % of the aromatic amino acids. The major component 5 comprises 63 % of the preparation yet contains only 49 % of the aromatic amino acids. The presence of components similar to 1 to 8 in acid-soluble collagen was found by a completely different chromatography procedure on several preparations of soluble collagen[5].

TABLE II

COMPOSITION OF CHROMATOGRAPHIC COMPONENTS

Component	*%Composition*		
	PIEZ *method at 230 mμ*	*Spectrochrom method*	
		At 220 mμ	*At 278 mμ*
1, 2, 3	—	0.5	10.8
4	—	2.4	0
α_1	21	8.2	3.3
β_{11}	18	6.5	5.0
β_{12}		5.9	
α_2	61	8.3	
5		62.9	49.3
6, 7, 8	—	5.3	31.6

Components 1, 2 and 3 eluted close to the void volume of the column, and therefore have little affinity for the CM-cellulose, which is a cation exchanger. They are therefore acidic in nature. On the other hand, components 6, 7 and 8 required a high ionic strength or pH to elute them. These are therefore either polymeric forms[3,5], or are strongly basic in character. Components 1, 2 and 3 were separated by chromatography from the rest of the collagen, evaporated to dryness under vacuum, and then hydrolysed for 24 h at 100° in a sealed tube with 6 *N* HCl. These components were not dialysed against water to remove excess salt before hydrolysis, since there was some doubt as to the size of these polypeptides and therefore they could possibly have been lost on dialysis. Components 6, 7 and 8 were, however, dialysed against several changes of distilled water before being freeze-dried, and hydrolysed for amino acid analysis.

Both preparations were then analysed by high voltage electrophoresis followed by chromatography. Repeat runs showed that components 1, 2 and 3 contained no amino acids detectable by the method used, but that ninhydrin-positive material remained at the origin after electrophoresis and migrated in a long streak after chromatography. The inability to detect amino acids in this preparation was due to the low concentration, arising from the fact that only a few milligrams of this preparation were available (see below). Components 6, 7 and 8 were shown to contain lysine (+ + +), arginine (+ +), histidine (+ + +), alanine (+ + +), glycine (+ +), serine (+), valine (+), leucine (+), glutamic acid (+ +), and aspartic acid (+ +), where the plus signs indicate the intensity of the spots as assessed visually. In addition

TABLE III

AMINO ACID COMPOSITION (RESIDUES/1000 RESIDUES)

Amino acid	*Components 1, 2, 3*	*Components 6, 7, 8*	*Component 9 —corresponding to components 6, 7, 8 corrected for collagen*
Ala	44.0	103.2	64.8
Arg	Trace	47.8	66.1
Asp	122.1	47.6	51.8
Glu	191.7	95.4	136.7
Gly	263.7	307.7	237.0
His	Trace	10.8	25.1
Hyl	—	4.9	4.1
Hyp	—	62.5	0.0
Ile	—	13.0	17.4
Leu	—	24.1	26.5
Lys	Trace	34.5	52.9
Met	—	0.6	0.0
Orn	Trace	16.9	46.6
Phe	72.0	12.1	14.3
Pro	—	99.0	49.3
Ser	175.8	77.9	157.1
Thr	—	22.5	35.3
Tyr	130.6	5.6	10.5
Val	—	13.4	4.7

similar ninhydrin-positive material as that found in components 1, 2 and 3, but not corresponding to amino acids, was also present.

These two preparations were then analysed quantitatively for their amino acid content. The results are presented in Table III. In agreement with the findings from chromatography components 1, 2 and 3 contain relatively large amounts of aspartic and glutamic acids (313.8 residues/1000 residues) and small amounts of the basic amino acids, thus making these components acidic in nature. The amount of material available made it possible only to detect traces of the basic amino acids. It was also found in the analysis that relatively large amounts of ammonia were present which indicated that the acidic amino acids were present to some extent as amides. Therefore components 1, 2 and 3 could correspond to an acidic telopeptide. Also of significance are the relatively large amounts of glycine, phenylalanine and tyrosine, the latter two accounting for the optical absorption at 278 mμ found in the chromatography.

The analysis for components 6, 7 and 8 shows that these contain hydroxyproline, and therefore collagen. If all the hydroxyproline is assumed to be contained in the collagen, and at the elution position of components 6, 7 and 8 this would correspond to polymeric forms of collagen[5], then the amino acid analysis of components 6, 7 and 8 minus that of acid-soluble collagen corresponds to that shown in Table III. For the sake of clarity this is referred to as component 9. This analysis shows that component 9 contains 194.8 residues/1000 residues of basic amino acids and 188.5 residues/1000 residues of acidic amino acids. The analysis also showed the presence of relatively large amounts of ammonia (1.04 %, w/w amide nitrogen) which would indicate that the acidic amino acids are largely present as amides, thus making component 9 basic in nature. This confirms the deduction made from the chromato-

graphy data. Component 9 contains lower amounts of phenylalanine and tyrosine than components 1, 2 and 3, which is also confirmed by the chromatographic data at 278 mμ (Table II). Therefore components 6, 7 and 8 appear to contain collagen and polypeptides which could correspond to basic telopeptides.

The presence of large amounts of material other than amino acids in component 9, as found by high voltage electrophoresis followed by chromatography, was confirmed by the fact that the amino acid analysis only gave a recovery of 35.2 % of amino acids. The presence of ornithine in these components probably arises from the decomposition of arginine[15], although the hydrolysis conditions used had not previously produced ornithine in studies on collagen and the non-collagenous proteins of skin[8]. This would also account for the relatively large amount of ammonia found in these amino acid analyses.

In the amino acid analysis two unidentified peaks were obtained for both components 1, 2, 3 and 6, 7, 8. These eluted in the positions of glycerophosphoethanolamine and phosphoethanolamine, but still have to be identified. These could account for a portion of the large amount of non-amino acid material shown to be present.

In some respects, notably in the high contents of acidic amino acids, glycine, tyrosine and phenylalanine, the peptide in components 1, 2, 3 resembles the telopeptides obtained from calfskin tropocollagen by proteolytic enzymes[7] and the two cyanogen bromide peptides obtained from ratskin collagen[16]. The latter authors found that the NH_2-terminal region of ratskin collagen is unusual in being more acidic than collagen, in lacking hydroxyproline, and in not containing glycine in every third position. By present concepts this region cannot assume the helical configuration characteristic of collagen.

These acidic and basic peptides were found in repeat runs on four different preparations of acid-soluble collagen, and also in the re-chromatography of collagen molecular subunits[5]. As these peptides were present in very small quantities, the possibility that they are impurities in the collagen must be considered. The acid-soluble collagen preparations used in these studies, however, were extracted from skin after the neutral-salt-soluble and non-collagenous impurities had been removed, and the acid-soluble collagen was re-purified by different methods[5,8].

The first component obtained by the phosphate gradient chromatography method (Fig. 1) would appear to correspond to components 1, 2, 3 from the Spectrochrom method (Fig. 5), while components b and c from the phosphate method could correspond to components 6, 7, 8 of the Spectrochrom method. In the latter case the basic nature attributed to these components would agree with this conclusion. Optical rotation studies show that component b still retains some helical nature, which is in accord with the fact that amino acid analysis showed the presence of collagen in components 6, 7, 8.

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GEL FILTRATION CHROMATOGRAPHY OF FLUORESCENT PHENOLIC AND HETEROCYCLIC COMPOUNDS*

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SUMMARY

A systematic study has been made of the chromatographic behavior of individual phenolic and heterocyclic compounds on Sephadex G-15. The partition coefficients of these compounds with distilled water or an acid–salt solution as eluants were determined.

Compounds with a carboxylic acid substituent on the ring structure were found to be excluded from the gel beads with distilled water as the eluant. However, these compounds were adsorbed on columns developed with the acid–salt solution.

Phenolic and indolylic compounds with an amine group in the aliphatic side chain were bound to the gel beads by the cationic properties of the gel matrix. Development of the column with the acid–salt eluant afforded the fractionation of the phenolic and indolylic compounds.

Hydroxy, methoxy and dihydroxy substituents on the ring structure resulted in increased adsorption when the columns were developed under conditions in which ion-exclusion effects were not operative or suppressed.

The degree of adsorption by the gel matrix was found to be affected by the functional groups in the aliphatic side chain of the compounds. For an acid–salt column, the order of elution for either phenolic or indolylic compounds was in the following sequence: amine, glycol, and finally carboxylic acid derivatives.

A method is presented for the separation of carboxylic acid derivatives from amino acid or glycol derivatives with the distilled water eluant, and subsequently, for the fractionation of phenolic and indolylic compounds with the acid–salt eluant.

INTRODUCTION

Gel filtration chromatography affords a method for the fractionation and quantitative recovery of small and large molecules[1,2]. The primary applications of

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cross-linked dextran gel have been the separation of large molecules by virtue of the molecular dimensions or the removal of small molecules from larger ones. GELOTTE's report demonstrated the applicability of dextran Sephadex for the fractionation of small molecules[2]. Since then, systematic studies of the chromatographic behavior of small molecules on the gel material have been relatively few[3–5]. The recent review of adsorption phenomena on Sephadex by JANSEN has attempted to explain the interactions and anomalous behavior of small molecules on cross-linked dextran[6].

In the course of attempting to fractionate certain fluorescent aromatic and heterocyclic compounds on dextran gel Sephadex G-15, it became apparent that further studies were necessary to properly utilize the ion-exclusion, ion-exchange, and adsorption properties of the gel matrix. Our findings agree with other investigators on the chromatographic behavior of aromatic and heterocyclic compounds. However, examination of analogues with different functional groups has provided several new facets on the interactions of solutes with the gel matrix.

This paper deals with the chromatographic migration of aromatic and heterocyclic hormones, their metabolites, and certain structurally related analogues on columns of Sephadex G-15. In addition, a novel method is presented that permits the separation of phenolic or indolylic acids from analogous glycols, and the fractionation of phenolic and indolylic amines on a single chromatographic column.

METHODS AND MATERIALS

Column preparation and operation

The cross-linked Dextran, Sephadex G-15 (Pharmacia AB), used in the study had a particle size of 40–120 microns and a water regain of 1.5 g/g. The dry powder was mixed with glass distilled deionized water, and the fines were removed by repetitive mixing, sedimentation and decantation.

Glass columns with O-ring joints (Kontes Glass Co.) and a stopcock with a Luer joint delivery tip were employed. The column dimensions were 1.2 cm in diameter and 50 cm in length. A coarse frittered glass disc at the bottom of the column supported the gel column.

The chromatography columns were prepared by addition of the slurry of gel beads to a height of 42 cm of packed gel. With the aid of an extension tube clamped on to the top of the column, the packed gel was uniformly raised to an approximate height of 40 cm by applying pressure with a column of water from an elevated reservoir. With the stopcock closed, the gel beads were allowed to settle. Firm tapping of the column promoted further settling of the gel beads. Final compression of the gel column was accomplished by percolating distilled water from an elevated reservoir through the column for several hours. This procedure provided uniformly packed columns that exhibited no layering or banding of the gel when viewed with a strong light. A flow rate of 35 ± 4 ml/h was used for all the chromatographic runs.

All compounds were obtained from commercial sources and were used without further purification. Each compound was dissolved in 0.05 *M* phosphate buffer pH 6.1, and the amount applied to the gel column was varied according to the relative fluorescence of the compound.

A 0.2 ml volume of each compound was applied directly onto the gel surface and allowed to percolate into the gel beads. Three additions of 0.1 ml of eluant were

made to insure the complete introduction of the sample into the gel surface. The space above the surface was filled with 10 ml of the eluant, and the column was connected to the reservoir with a plastic tube.

The two eluants used for development of the gel columns were (a) deionized distilled water, and (b) 50 millimolar sodium chloride solution adjusted to pH 4.0 with dilute hydrochloric acid. The latter eluant will be referred to as the acid–salt solution in the text. The chromatographic behavior of the compounds was first studied with the distilled water equilibrated columns and subsequently with the acid–salt equilibrated columns.

Repetitive use of gel columns resulted in a compaction of the gel surface and a resultant decrease in flow rate of the eluant. This effect was counteracted by periodically stirring the top 2 cm of the gel beads and then allowing them to settle.

Fluorometric monitoring of columns

A fluorometer (Turner—Model 111) with a flow-cell attachment was used to continuously monitor the fluorescent content of the column effluent solution. A potentiometric recorder (Bausch & Lomb—VOM-5), set at a speed of 3 inches/hour, provided a tracing of the elution of each compound. The fluorometer lamp (Westinghouse F4T5/UV 31) had a continuous emission from 270 to 340 nanometers (nm) and a peak emission at 306 nm. The light filter system consisted of a primary filter (Baird-Atomics Interference Type) with peak transmission at 280 nm, and a secondary filter (Corning CS No. 7-60) with peak transmission at 360 nm. The aperture openings used were at the 1 × or 3 × setting. All of the compounds, with the exception of xanthurenic acid, gave a fluorescence response with the described lamp and light filter combination. The latter compound gave an absorption response.

RESULTS

Presentation of data

The elution volume (V_e) for each compound represents the volume of eluant required for elution from a 40 cm column of Sephadex G-15 with a total bed volume (V_t) of 50 cc. The V_e measurement was made from the time of introduction of the sample onto the gel surface until the point of maximum fluorescence on the recorder tracing. The fluorescence elution tracings obtained for most of the compounds were essentially quasi-symmetrical peaks.

The partition coefficient (K_{av}) for each compound was calculated from the equation,

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

where V_0 is the void volume of the column and V_t is the total volume of the gel beads. The V_G, determined with Blue Dextran 2000 (Pharmacia AB), was found to be 20 ml.

Reproducibility of elution volumes

The consistency of column operation was evaluated by repetitive chromatography of individual compounds using distilled water as the eluant. A mixture of three compounds was used to test the reproducibility of elution volumes on an acid–

TABLE I

REPRODUCIBILITY OF ELUTION VOLUMES (V_e) ON SEPHADEX G-15

	V_e (ml) for each run			
	No. 1	*No. 2*	*No. 3*	*No. 4*
Individual Compounds: Water Eluant				
5-Hydroxy-indoleacetic acid	29	30	32	—
3-Methoxy-4-hydroxy-phenethylglycol	71	70	—	—
Mixture: Acid–Salt Eluant				
Metanephrine	51	52	53	53
Tryptamine	113	117	121	123
Serotonin	144	150	157	162
	Ratios			
Separation Factors				
Tryptamine–Metanephrine	2.24	2.24	2.31	2.34
Serotonin–Metanephrine	2.86	2.86	2.90	3.09
Serotonin–Tryptamine	1.28	1.28	1.29	1.32

salt column. The results are shown in Table I. Although there were shifts in the elution volume of individual compounds, calculation of separation ratios showed that the relative fractionation of the compounds remained fairly consistent.

Distilled water elution study

Carboxylic acid, amino acid, or glycol derivatives of heterocyclic or phenolic compounds were found to be readily eluted by distilled water (Table II). Those compounds with a carboxy group were eluted with relatively small volumes of eluant, regardless of the structure of the parent compound. The small elution volumes for compounds with a prominent carboxy substituent is due to the ion-exclusion properties of the gel matrix.

TABLE II

WATER ELUTION OF AROMATIC AND HETEROCYCLIC ACIDS AND GLYCOLS

Compound	*Quantity* (μg)	V_e(ml)	K_{av}
Kynurenic acid	5	29	0.30
5-Hydroxy-indoleacetic acid	10	29	0.30
4-Hydroxy-phenyllactic acid	65	30	0.33
3-Methoxy-4-hydroxy-mandelic acid	65	31	0.37
3-Methoxy-4-hydroxy-phenylacetic acid	65	31	0.37
3,4-Dihydroxy-phenylacetic acid	65	31	0.37
Anthranilic acid	1	32	0.40
Indole-3-acetic acid	10	32	0.40
Xanthurenic acid	20	32	0.40
Vanillic acid	10	35	0.50
Tyrosine	100	51	1.03
3,4-Dihydroxyphenylalanine	100	58	1.27
3-Methoxy-4-hydroxy-phenethylglycol	100	71	1.70
3,4-Dihydroxy-phenethylglycol	100	91	2.37

The two phenolic amino acids that were tested showed slightly larger elution volumes. This effect could be attributed to an interaction of the alpha amino group with the carboxylic group. Interestingly, the alpha hydroxy group or the ortho amino group on some of the carboxylic acid compounds had no effect on the exclusion of the acidic analogues.

Both derivatives of phenethylglycol also required large volumes of water for elution. These results suggest that the phenolic amino acid and glycol derivatives are adsorbed by the gel matrix.

The effects of ring substitutions, such as methoxy, hydroxy or dihydroxy groups, on the relative elution volumes were apparent only for those compounds that were adsorbed. Furthermore, the two dihydroxy derivatives were adsorbed to a greater degree than tyrosine or 3-methoxy-4-hydroxy-phenethylglycol.

Acid–salt elution study

Compounds with an aliphatic amine group, applied to distilled water equilibrated columns, could not be eluted with distilled water. Elution of amines could be effected only after incorporation of small amounts of electrolytes or dilute acid into the eluant. This binding of amines by the gel is due to weak cationic properties of the gel. The elution volumes and K_{av} values for a series of phenolic and indolylic amines obtained with the acid–salt eluant are shown in Table III.

The most prominent feature in Table III is the larger volumes of eluant required for the elution of the indolylic amines when compared with the phenolic amines. Apparently, the indoles are adsorbed to a greater degree by the gel matrix than the phenolic amines.

Although the acid–salt eluant tends to neutralize the ion-exchange properties of the gel, there is still some evidence of a residual ionic interaction with some of the derivatives. Evidence for this effect is shown by smaller elution volumes for mono- and dimethyl- derivatives of the primary amines. The following pairs of compounds

TABLE III

ACID–SALT ELUTION OF AROMATIC AND HETEROCYCLIC AMINES

Compound	*Quantity* (μg)	$V_e(ml)$	K_{av}
Tyramine	100	55	1.17
DL-Metanephrine	100	55	1.17
DL-Octopamine	100	58	1.27
3-Methoxy-4-hydroxy-phenethylamine	100	60	1.33
DL-Normetanephrine	100	62	1.40
L-Epinephrine	100	62	1.40
DL-Norepinephrine	100	69	1.67
3,4-Dihydroxy-phenethylamine	100	72	1.73
N,N-Dimethyl-tryptamine	40	87	2.23
N,N-Dimethyl-5-hydroxy-tryptamine	4	113	3.10
Tryptamine	35	115	3.17
N-Acetyl-5-methoxy-tryptamine	20	130	3.67
5-Methoxy-tryptamine	40	148	4.27
Serotonin (5-hydroxy-tryptamine)	40	157	4.57
N-Acetyl-5-hydroxy-tryptamine	10	249	7.63
Indole	100	328	10.27

illustrating this effect are: metanephrine–normetanephrine; epinephrine–norepinephrine; N,N-dimethyltryptamine–tryptamine and N,N-dimethyl-5-hydroxy-tryptamine–serotonin.

From the results for methylated derivatives, a similar reduction in elution volume with the two N-acetylated compounds was anticipated. However, both an increase and a decrease in elution volumes was noted. The N-acetyl derivative of 5-methoxy-tryptamine showed a decrease in elution volume of 18 ml, whereas the volume required for the elution of N-acetyl-5-hydroxy-tryptamine was 92 ml greater than for the parent compound.

The addition of a functional group to an aromatic or heterocyclic "nucleus" had a definite effect on the elution volumes with the acid–salt column. Changing from a 4-hydroxy to a 3-methoxy-4-hydroxy or to a 3,4-dihydroxy derivative resulted in a progressively larger K_{av} value. This effect was observed for both the phenethylamine and phenethanolamine type compounds. The elution volumes for tryptamine, 3-methoxy-tryptamine and serotonin showed a similar relationship for the indolylic amines.

Since ion-exchange effects are suppressed by electrolyte-containing eluants, the chromatographic behavior of some of the indolylic acids, phenolic acids, and glycols was reexamined with the acid–salt elution system. Table IV shows the elution volumes of these compounds in each of the elution systems. The findings from this comparative study are:

(a) The two compounds, dihydroxyphenylalanine and 3-methoxy-4-hydroxy-phenethylglycol that were adsorbed on the distilled water equilibrated column, behaved identically on the acid–salt column.

(b) The four compounds that were excluded by distilled water were adsorbed by the gel with the acid–salt eluant.

(c) The phenolic acids tended to be well separated from the indolylic acids.

Furthermore, it was noted that the acidic analogues of certain compounds were adsorbed to a greater degree than the corresponding amines on the acid–salt column. The elution volumes shown in Table V for four pairs of acid and amine analogues clearly illustrate this point.

Two stage elution chromatograms

The results obtained from the study of single compounds with the two eluant systems were then applied to effect the separation of a selected mixture of compounds. The mixture was applied to a distilled water equilibrated column and distilled water

TABLE IV

COMPARISON OF ELUTION VOLUMES OF COMPOUNDS ON DISTILLED WATER OR ACID–SALT COLUMNS

Compound	*Water (ml)*	*Acid–salt (ml)*
5-Hydroxy-indoleacetic acid	29	250 (T)*
3-Methoxy-4-hydroxy-mandelic acid	31	65
3-Methoxy-4-hydroxy-phenylacetic acid	31	88 (T)
Indole-3-acetic acid	32	203 (T)
3,4-Dihydroxyphenylalanine	58	59
3-Methoxy-4-hydroxy-phenethylglycol	71	68

* T = Tailing of peaks.

was percolated through the column for 3 h. The eluant was then changed to the acid–salt solution and allowed to run for 5 h. The fractionation of the mixture of phenolic and indolylic compounds using this two stage elution system is shown in Fig. 1.

In the fractionation of the mixture of compounds, the three properties of the gel matrix, ion-exclusion, ion-exchange and adsorption were utilized.

TABLE V

ELUTION VOLUMES OF AMINE AND ACID ANALOGUES WITH THE ACID–SALT ELUANT

Compound	V_e
Metanephrine	55
3-Methoxy-4-hydroxy-mandelic acid	65
3-Methoxy-4-hydroxy-phenethylamine	60
3-Methoxy-4-hydroxy-phenethylacetic acid	88
Tryptamine	115
Indole-3-acetic acid	203
Serotonin	157
5-Hydroxy-indoleacetic acid	250

DISCUSSION AND CONCLUSIONS

Our investigation of the chromatographic behavior of aromatic and heterocyclic compounds on Sephadex has uncovered new relationships on the fractionation of these classes of compounds by the gel material. The adsorption of aromatic and heterocyclic compounds noted by FLODIN[1] and the ion-exclusion and ion-exchange properties of the gel reported by GELOTTE[2] were also observed in the present study. However, the systematic examination of phenolic and indolylic acids, amines, amino acids, glycols, ring structure and aliphatic substitutions of this study now provides a greater insight for the application of Sephadex for the fractionation of complex mixtures of small molecules.

The exclusion and adsorption effects that are operative on distilled water

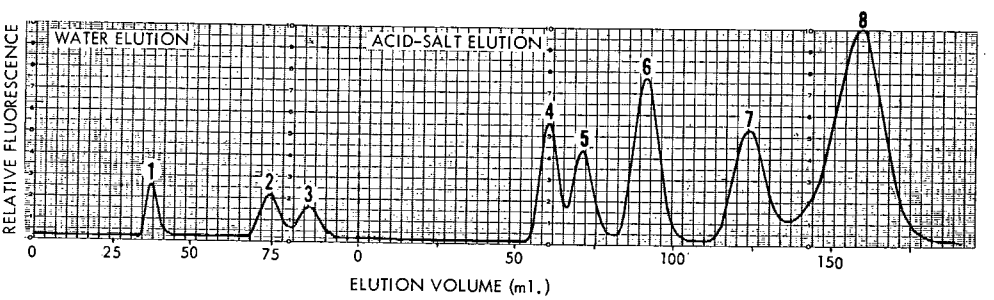


Fig. 1. Fractionation of phenolic and indolylic compounds on Sephadex G-15. Distilled water was used for the first phase and 0.05 *M* sodium chloride, pH 4.0 solution for the second phase of column development. Column size: 1.2 × 40 cm. 1 = Vanillic acid (1 μg); 2 = 3-methoxy-4-hydroxy-phenethylglycol (100 μg); 3 = 3,4-dihydroxy-phenethylglycol (100 μg); 4 = normetanephrine (200 μg); 5 = 3,4-dihydroxy-phenethylamine (200 μg); 6 = N,N-dimethyltryptamine (40 μg); 7 = tryptamine (10 μg); 8 = serotonin (10 μg).

columns afford a method for the separation of compounds with carboxy groups from the amino acid and glycol derivatives. From the data in Table II it is apparent that the nature of the ring structure or the nuclear substitutions have but a minimal effect on the chromatographic behavior of the monocarboxylic acids. However, the introduction of an alpha amino group tends to suppress the exclusion effect as was noted with the two phenolic amino acids. The exclusion of monocarboxylic acids, nucleotides and benzoic acid derivatives has been reported by GELOTTE[2] and WOOF AND PIERCE[5].

The phenolic amino acids and glycols that were adsorbed on the distilled water columns also showed the rather marked effects of nuclear substitutions. In both cases, the dihydroxy compounds had larger elution volumes than the corresponding 4-hydroxy or 3-methoxy-4-hydroxy derivatives. Similar results for a large number of phenols have been reported[5].

The ion-exchange properties, due to the negatively charged groups in the gel matrix, result in the binding of basic compounds on the distilled water column. Neutralization of the electrostatic interactions of solute and gel matrix by the incorporation of an electrolyte or acid into the eluant results in the elution of the basic compounds. The chromatographic behavior of basic compounds on an electrolyte-equilibrated column depends primarily on the degree of adsorption, and secondarily on the residual electrostatic charges on the gel beads. Both effects have been observed with the acid–salt column. Our conclusion for the existence of residual electrostatic charges (weak ion-exchange effects) is based on the larger partition coefficients obtained with the primary amines in comparison to the corresponding methylated or acetylated derivatives.

The planarity of a molecule markedly influences the degree of adsorption by the gel. Biphenyl and naphthyl derivatives have been reported to have larger elution volumes than simple phenols[5]. Our study with phenolic and indolylic compounds has shown a greater adsorption by the gel for the latter class of compounds.

Substitutions on the aromatic nucleus that extend the planarity of the molecule may result in either ion-exclusion or an increase in adsorption. WOOF AND PIERCE have shown that carboxy and nitro groups decrease adsorption, whereas methoxy and hydroxy groups increase the adsorption of compounds by the gel matrix[5]. These effects are attributed to the electron-donating properties of the substituent groups. Similar effects of methoxy and hydroxy groups on phenolic and indolylic compounds have been noted in our study.

The effect of the chemical nature of the aliphatic portion of an aromatic or heterocyclic compound on the chromatographic behavior has not been previously reported. Our finding of the stronger adsorption of carboxy derivatives in comparison to amine analogues on the acid–salt column led to an examination of the sequence of elution of compounds with different functional groups. The sequence found was in the following order: amines, alcohols and carboxylic acids. The examples illustrative of this elution sequence are 3-methoxy-4-hydroxy-phenethylamine, glycol and acetic acid derivatives with elution volumes of 60, 68 and 88 ml, respectively. This sequence of elution has been found to be primarily dependent on the pH of the eluant[7].

Finally, the two stage elution system used for the separation of a mixture of phenolic and indolylic compounds demonstrates the applicability of gel filtration chromatography for the facile separation of compounds. By using a distilled water

column, the exclusion of the strongly acidic compounds and the separation of amino acid and alcoholic compounds can be accomplished. A subsequent change to an electrolyte-containing eluant permits the elution and separation of phenolic and indolylic amines. Although the acidic compounds are not fractionated on the distilled water column, it is possible to collect this fraction and effect a separation of these types of compounds by rechromatography on an acid–salt column.

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CHROM. 3379

DÜNNSCHICHT-CHROMATOGRAPHISCHE AUSWERTUNG BEI DER BESTIMMUNG KLEINER DAMPFDRUCKE

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(Eingegangen den 29. Dezember 1967)

SUMMARY

The use of thin-layer chromatography in the determination of low vapour pressures

A procedure for the determination of vapour pressures using thin-layer chromatography is described, based on the saturation method. Vapour pressures in the order of 10^{-5} to 10^{-6} mm Hg can easily be determined. The separation of the compounds by thin-layer chromatography increases the selectivity of the method.

EINLEITUNG

Ein einfaches Verfahren für die Bestimmung von kleinen Dampfdrucken in der Grössenordnung von 10^{-2} mm Hg und darunter bietet die Überführungs- oder Gassättigungs-Methode. Ein trockenes Inertgas wird derart über die zu untersuchende Substanz geleitet, dass ein gesättigter, mit seinem Bodenkörper im Gleichgewicht befindlicher Dampf entsteht. Der Sättigungsdruck der zu prüfenden Substanz stellt sich daher als Partialdruck ein.

Die aus einer Sättigungs-Säule austretende Substanz kann entweder in fester oder flüssiger Form kondensiert und gewogen, oder in einem Lösungsmittel absorbiert werden[1]. Es ist aber auch möglich, die flüchtige Substanz auf eine gas-chromatographische Säule zu kondensieren und anschliessend gas-chromatographisch aufzutrennen[2]. Die letztgenannte Technik hat den Vorteil einer grossen Selektivität, da Begleitsubstanzen mit geringerem oder höherem Dampfdruck das Resultat nicht verfälschen, was besonders bei der Bestimmung von niederen Dampfdrucken der Fall sein kann. Die gas-chromatographische Methode hat jedoch den Nachteil, dass bei thermisch instabilen Verbindungen eine Zersetzung bei der Gas-Chromatographie auftritt. Wir suchten deshalb nach einer Methode, welche einerseits die Vorteile der gas-chromatographischen Dampfdruck-Bestimmung besitzt und anderseits die überführten Dämpfe selektiv bestimmen lässt.

In der nachfolgend beschriebenen Methode wird nun der aus der Sättigungs-Säule austretende Dampf auf einer Dünnschicht-Platte adsorbiert, dünnschicht-chromatographisch entwickelt und ausgewertet.

PRINZIP

Ein vollkommen getrockneter und gereinigter Inertgasstrom wird durch eine thermostatisierte Sättigungs-Säule geleitet, welche die auf einem inerten Träger aufgezogene Substanz enthält. Der aus der Sättigungs-Säule austretende Dampf wird direkt auf eine Dünnschicht-Platte (z.B. Kieselgel) adsorbiert. Für Substanzen, die einen Dampfdruck im Bereich von 10^{-4} bis 10^{-5} mm Hg besitzen, beträgt die überführte Menge bei einem Molekulargewicht von etwa 250 grössenordnungsmässig 10^{-6} g/l Inertgas.

Aus der Kenntnis der total durchgeflossenen Gasmenge, der kondensierten Dampfmenge, des Molekulargewichtes und der verwendeten Temperatur errechnet sich der Sättigungsdruck nach der Zustandsgleichung idealer Gase:

$$p \cdot V = n \cdot R \cdot T$$

Durch Umformen erhält man:

$$p_s = R \cdot \frac{m}{M} \cdot \frac{T}{V}$$

wobei

p_s = Sättigungsdruck, in mm Hg

$R = \frac{22.415 \cdot 760}{273} = 62.36 \frac{\text{Liter} \cdot \text{mm Hg}}{°\text{K}}$

m = Menge der überführten Substanz, in Gramm

M = Molekulargewicht

V = Volumen des Inertgases, gemessen am Ausgang der Sättigungssäule, in Liter

T = Temperatur der Sättigungssäule, in °K.

EXPERIMENTELLES

Apparatur (Fig. 1)

Als Inertgas wird für die Messung Stickstoff 99.99 % verwendet. Dieser gelangt nach doppelter Druckregelung durch eine Gasuhr in ein mit Magnesiumperchlorat gefüllten Trockenturm und von dort zur Entfernung letzter Feuchtigkeitsspuren durch ein mit Trockeneis/Äthanol gekühltes und mit Linde Molekularsieb 5A gefülltes Trockenrohr. Das trockene Gas gelangt nun in die Sättigungs-Säule, welche aus einem 1.5 m langen Pyrex-Glasrohr mit einem inneren Durchmesser von 4 mm besteht. Dieses Rohr wird mit einem mechanischen Gemisch von einem Teil der zu untersuchenden Substanz und 2–6 Teilen Kieselgur (20–30 mesh) gefüllt. Experimentelle Untersuchungen ergaben die gleichen Dampfdrucke, gleichgültig ob die Säule mit einem mechanischen Gemisch Substanz-Kieselgur gefüllt, oder ob die Substanz vorher mittels eines Lösungsmittels auf den Träger aufgezogen wurde. Durch Zugabe des Kieselgurs erreicht man eine Verbesserung der Permeabilität und eine Vergrösserung der Oberfläche. Die Sättigungs-Säule wird mit einem Thermostaten auf der

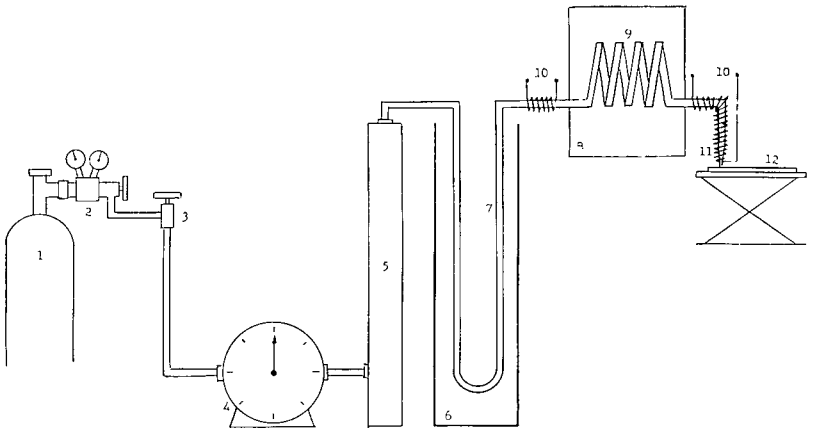


Fig. 1. Schema der Dampfdruck-Apparatur. (1) Stickstoff-Bombe, (2) Druckventil, (3) Feindruckregler, (4) Gasuhr, (5) Trockenturm, (6) Dewar-Gefäss, (7) Trockensäule (Molekularsieb 5A), (8) Temperaturbad, (9) Sättigungs-Säule, (10) Heizung für Säuleneingang und -ausgang, (11) Kapillarrohr, (12) Dünnschicht-Platte.

gewünschten Temperatur gehalten (Genauigkeit $\pm$ 0.3 °C). Als Temperaturbad eignet sich ein gut isolierter Chromatographie-Tank, in welchen die Sättigungs-Säule eingesetzt wird. Am Ende dieser Säule befindet sich ein kurzes gebogenes Zwischenstück, das zu einer Kapillare ausgezogen ist. Der innere Durchmesser der Kapillarspitze beträgt *ca.* 1 mm. Eingang und Ausgang der Säule, sowie das Kapillarrohr werden elektrisch beheizt, um Kondensation der mitgeführten Substanz zu vermeiden. Über einen Regeltransformator wird die Heizleistung so eingestellt, dass der Säulenausgang die gleiche Temperatur aufweist wie das Heizbad.

Die Glasverbindungen bestehen aus ätherdichten Schliffen. Am Ende des gebogenen Kapillarrohres befindet sich in einem Abstand von *ca.* 0.5 mm die 0.25 mm dick beschichtete Dünnschicht-Platte. Die Herstellung dieser Platte erfolgt nach den üblichen Vorschriften[3].

Der Durchfluss des Inertgases kann mit einem Feindruckregler konstant gehalten werden. Die Durchflussmenge wird mit einem Seifenblasen-Strömungsmesser, den man für kurze Zeit an den Säulenausgang anschliesst, einreguliert und gemessen.

Arbeitsbedingungen

Konditionierung der Sättigungs-Säule. Die Sättigungs-Säule kann vor Beginn der eigentlichen Messung konditioniert werden, um einen Teil der leichter flüchtigen Komponenten zu entfernen. Die Konditionierungsbedingungen hängen von der Reinheit und der Flüchtigkeit der zu untersuchenden Substanz ab.

Wahl der Durchflussgeschwindigkeit. Durch Änderung der Strömungsgeschwindigkeit des indifferenten Gases ist zu prüfen, ob die Sättigung des Gasstromes mit dem Dampf der Untersuchungssubstanz vollständig ist. Es ist bekannt, dass bei zu grosser Strömungsgeschwindigkeit wegen ungenügender Sättigung zu kleine Mengen übergeführt werden. Bei den vorgenommenen Prüfungen wurde der Durchfluss auf 1.2 l/Std. eingestellt. Eine doppelte Strömungsgeschwindigkeit ergab noch keine Abnahme der Überführung.

Ausführung der Bestimmung. Während einer bestimmten Zeit lässt man die Substanz auf der Dünnschicht-Platte adsorbieren. Dauer der Adsorption und Menge des Transportgases richten sich nach der approximativen Grösse des Dampfdruckes bei der vorgegebenen Temperatur. Je nach Identifizierungsmöglichkeiten (Eigenfarbe, Reagens, Fluoreszenzlöschung, etc.) genügen Mengen zwischen 0.1 und 50 μg. Anschliessend wird eine Eichreihe von der zu untersuchenden Substanz auf dieselbe Platte aufgetragen und mit einem passenden Laufmittel mitchromatographiert.

Verunreinigungen und Zersetzungsprodukte können in vielen Fällen aufgrund ihres verschiedenartigen Verhaltens im Dünnschicht-Chromatogramm von der Hauptkomponente getrennt werden. Die Bestimmung der überführten Substanzmenge kann mit Hilfe einer Eichreihe bei einiger Übung mit genügender Genauigkeit visuell erfolgen.

BEISPIELE UND DISKUSSION

Aus der Clausius–Clapeyron'schen Gleichung[1,2,4]

$$\frac{\mathrm{d} \ln p}{\mathrm{d}(1/T)} = \frac{-\Delta H}{R}$$

wobei

T = absolute Temperatur

ΔH = molare Verdampfungswärme

R = Gaskonstante

erhält man die bekannte Abhängigkeit für den Dampfdruck:

$$\log p_s = a - \frac{b}{T}$$

(a und b = Dampfdruckkonstante[1]).

Der Dampfdruck von *p*-Aminoazobenzol (F = 126 °C) wurde im Bereich von 50–70 °C bestimmt (vgl. Tabelle I). Die Regressionsgerade kann mit Hilfe der Methode der kleinsten Fehlerquadrate berechnet werden. Für die Dampfdruckkonstanten a und b wurden aus neun Messungen mit 95 % statistischer Sicherheit folgende Werte ermittelt:

$a = 15.276 \pm 3.245$

$b = 6388 \pm 1080$

Die Methode lässt sich sehr einfach mittels Spektralphotometrie überprüfen. Zu diesem Zweck wird das Inertgas nach dem Verlassen der Sättigungs-Säule durch 2–3 Absorptionsgefässe geleitet und anschliessend die überführte Substanzmenge spektralphotometrisch bestimmt.

Die Dampfdruckmessung mittels Dünnschicht-Chromatographie ergänzt die bisherigen gebräuchlichen Methoden, da sie gestattet, die Dampfdrucke bei niedrigeren Temperaturen zu erfassen. Bedingt durch die visuelle Auswertung der Eichreihe auf der Dünnschicht-Platte ist sie weniger genau als die Effusions- und die spektral-

TABELLE I

VERGLEICH DER EINZELNEN BESTIMMUNGSMETHODEN*

Temp. (°C)	*Dampfdruck (mm Hg)*			
	Überführungsmethode			
	(a) dünnschicht-chromatographisch	*(b) spektral-photometrisch*	*(c) gas-chromatographisch*	*(d) Knudsen Effusions-methode*[4]
50	$2.80 \cdot 10^{-5}$			
60	$1.62 \cdot 10^{-4}$			
70	$4.00 \cdot 10^{-4}$	$4.5 \cdot 10^{-4}$		
80		$1.1 \cdot 10^{-3}$	$1.29 \cdot 10^{-3}$	
90		$2.9 \cdot 10^{-3}$	$3.17 \cdot 10^{-3}$	
100			$1.16 \cdot 10^{-2}$	$7.15 \cdot 10^{-3}$
110			$3.15 \cdot 10^{-2}$	$1.82 \cdot 10^{-2}$
120				$4.40 \cdot 10^{-2}$

* (a) und (b) eigene Messungen, Mittelwerte aus je drei Bestimmungen;
(c) private Mitteilung von K. FRIEDRICH, Mittelwerte aus je drei Bestimmungen;
(d) Messwerte entnommen aus Lit. 4.

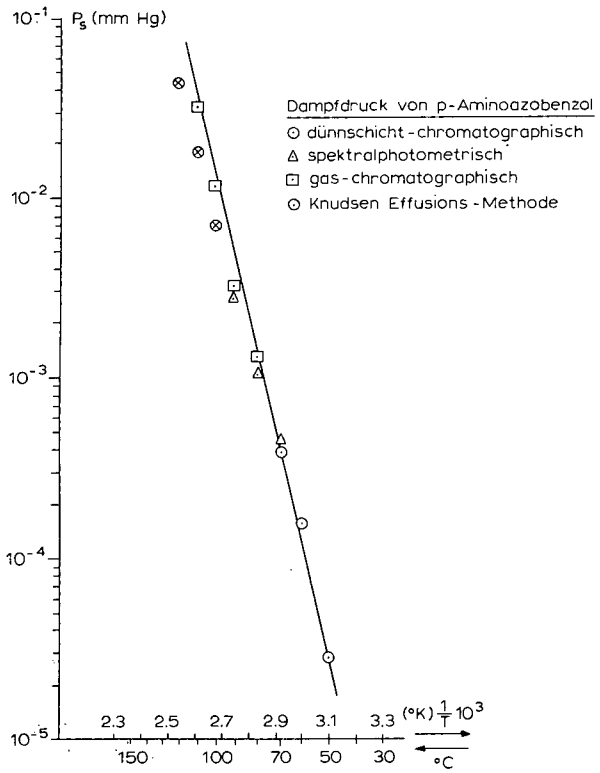


Fig. 2. Dampfdruckkurve von *p*-Aminoazobenzol, berechnet und extrapoliert aus der dünnschicht-chromatographischen Methode.

photometrische Methode. Sie besitzt aber den Vorteil, dass mit einem geringen apparativen Aufwand die überführten Dämpfe selektiv bestimmt werden können.

Die Dampfdrucke verschiedener Triazin-Herbizide, Fungizide, Insektizide und Farbstoffe wurden nach der dünnschicht-chromatographischen Methode gemessen (Fig. 2). Die Resultate wurden mit sehr guter Übereinstimmung durch die spektralphotometrische- und gaschromatographische Überführungsmethode sowie mit den bereits publizierten Literaturwerten[5–7] überprüft.

ZUSAMMENFASSUNG

Es wird ein einfaches Verfahren für die Bestimmung von Dampfdrucken bis zu 10^{-6} mm Hg mit Hilfe der Gassättigungsmethode beschrieben. Die überführte Substanzmenge wird auf einer Dünnschicht-Platte adsorbiert, entwickelt und ausgewertet.

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CHROM. 3370

AUFTRAGSGERÄT FÜR DIE ANALYTISCHE DÜNNSCHICHT-CHROMATOGRAPHIE

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(Eingegangen den 27. Dezember 1967)

SUMMARY

Apparatus for the loading of samples on to thin layer chromatography plates

An apparatus is described for the loading of samples on to thin layer chromatography plates. The dissolved substances are sprayed as a fine homogeneous strip (0.5 mm or more thick) without damaging the surface of the plate. The apparatus permits work on an analytical (from 3–4 μl) or on a preparative scale, and has great advantages for the loading of plates for quantitative densitometry.

Die Dünnschichtchromatographie mit ihren bekannten Vorteilen hat sich in den letzten Jahren auch bei der quantitativen Mikrobestimmung von Substanzen bewährt. Für die quantitative Auswertung von Dünnschichtchromatogrammen werden in der Hauptsache zwei Methoden angewendet:

(1) Die getrennten Verbindungen werden zusammen mit dem Sorptionsmittel aus der Schicht entfernt und nach Elution mit Hilfe einer herkömmlichen, meist spektroskopischen Methode bestimmt.

(2) Die quantitative Auswertung erfolgt direkt auf der Platte durch Messen der Fleckenflächen oder durch eine densitometrische Methode.

Die wichtigste Voraussetzung für beide Methoden ist die Ausbildung von scharf getrennten Substanzzonen. Diese Forderung lässt sich nur durch das gleichmässige Auftragen der zu trennenden Substanzgemische in Strichform ohne Verletzung der Trennschicht erfüllen. Es fehlt daher nicht an Versuchen, derartige Geräte zu konstruieren[1–5]. Die Substanzlösung wird gewöhnlich durch eine Kanüle in Tropfenform oder als Strahl auf die Platte appliziert. Nach unseren Erfahrungen sind jedoch die meisten beschriebenen Geräte mehr für den präparativen Einsatz als für den Routinebetrieb im analytischen Massstab geeignet. Für diesen Zweck hat sich bei uns das Prinzip bewährt, die Substanzlösung durch einen Gasstrom auf die Platte zu sprühen. Das dazu konstruierte Gerät mit einem Spezialauftragstisch erlaubt ein strichförmiges und absolut homogenes Auftragen in quantitativ-analytischem Massstab.

AUFBAU DES GERÄTES

(a) *Auftragsgerät*

Für unsere Zwecke haben wir eine im Handel befindliche Mikrospritzpistole

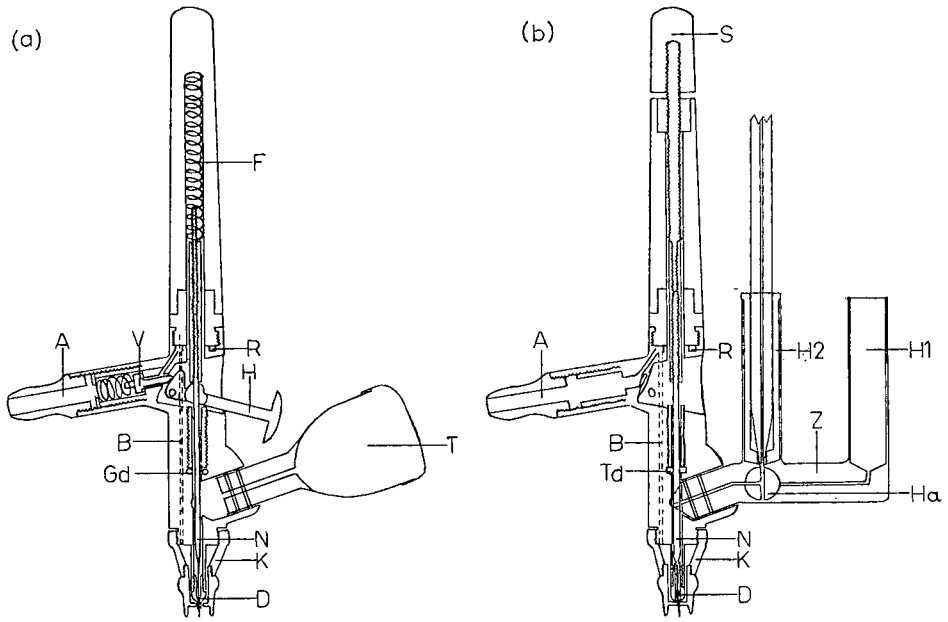


Fig. 1. (a) Sprühpistole der Fa. Desaga. Beschreibung siehe Text. (b) Modifizierte Sprühpistole für die analytische Dünnschichtchromatographie. Beschreibung siehe Text.

(Fa. Desaga) in mehreren Punkten modifiziert. Fig. 1a zeigt eine schematische Darstellung der ursprünglichen Spritzpistole. Diese arbeitet nach dem Bernoulli'schen Prinzip. Das Druckgas wird unter einem Betriebsdruck von 0.5–1.5 atm durch den mit dem Ventil V verschliessbaren Ansatz A in den Verteilerring R geleitet. Von da aus gelangt es durch zwei seitliche Bohrungen B in den Konus K und tritt durch den kreisförmigen Spalt zwischen Konus und Düse D aus. Die Düse ist durch die mit einer Feder angedrückte Düsennadel N verschlossen. Das Öffnen der Düse und die Einstellung der Strahlstärke erfolgt durch Anheben des Hebels H. Dadurch wird gleichzeitig das Ventil V freigegeben. Die aufzubringende Lösung befindet sich in dem seitlich eingesteckten Vorratsgefäss T (Inhalt 5 ml). Sie wird auf das mit Glasplatten begrenzte Startband gesprüht. In dieser Form ist die Sprühpistole nur für präparative Zwecke zu verwenden. Wir haben das Gerät so umgebaut und vervollständigt, dass es auch für den analytischen Einsatz geeignet ist. Fig. 1b zeigt die abgeänderte Sprühpistole. Das Öffnen der Düse und damit auch die Feinregulierung der Strichbreite von 0.5–5 mm erfolgt mit der Stellschraube S. Der Hebel H und das Ventil V wurden entfernt und die Führung für den Ventilstift verlötet. Die in organischen Lösungsmitteln leicht quellende Gummidichtung Gd wurde durch eine Teflondichtung Td ersetzt. In die seitliche Öffnung der Pistole ist der neu konstruierte analytische Zusatz Z genau eingepaßst und mit zwei Teflonringen abgedichtet. Die Hülse H1 ist für reines Lösungsmittel vorgesehen. Die Hülse H2 ist mit einem konischen Teflonfutter ausgekleidet, in das Messpipetten verschiedener Grösse dicht eingesetzt werden können. Der Dreiwegehahn Ha ermöglicht das Umschalten von Lösung auf reines Lösungsmittel.

(*b*) *Auftragstisch*

Um ein möglichst homogenes Auftragen zu gewährleisten, wurde ein Auftragstisch konstruiert, der nach dem Prinzip einer mechanischen Kippschwingung arbeitet. Fig. 2 zeigt den Auftragstisch mit der Spritzpistole. Die Dünnschichtplatte wird mit der Klammer K1 auf dem Tisch befestigt. Der Tisch T läuft achtfach kugelgelagert in den beiden Schienen Sch. Er wird durch die Schnecke V gleichmässig vorgeschoben. Die Strichlänge kann durch Auswechseln der Vorschubschnecken variiert werden. Der Motor M ist stufenlos regelbar. Nach Erreichen der grössten Auslenkung wird der Tisch durch die Feder E in die Ausgangsstellung zurückgeschnellt. Ein Zurückprallen des Tisches wird durch einen Magneten verhindert. Dieser befindet sich, auf der Abbildung nicht sichtbar, an der Unterseite des Tisches. Die Spritzpistole wird mit der Klammer K2 am Block B festgeklemmt. Sie lässt sich mit den Stellschrauben S1, S2 und dem Gewinderad G in alle drei Raumrichtungen verstellen. Die Stellschraube S3 dient zur Fixierung des Armes A auf den Führungsschienen F. Zum Herausnehmen der Platte lässt sich die Sprühpistole mit ihrer Halterung hochklappen.

ARBEITSWEISE

Eine nach dem Volumen der aufzutragenden Lösung ausgewählte Messpipette wird in die Hülse H2 gesteckt und durch eine mit einer Kanüle versehene Pipette (Aussendurchmesser der Kanüle kleiner als lichte Weite der Messpipette) bei geschlossener Düse etwa 60 μl über die Nullmarke gefüllt. Dann wird die Druckgaszufuhr geöffnet. Die Gaszuleitung ist verzweigt. Ein Anschluss führt direkt zur

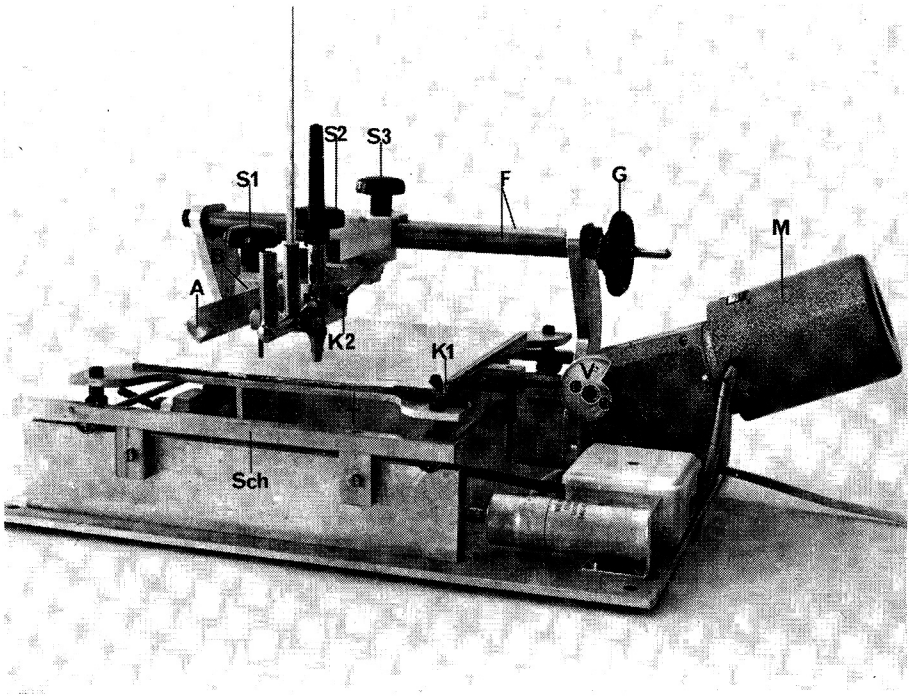


Fig. 2. Auftragsgerät für die analytische Dünnschichtchromatographie. Beschreibung siehe Text.

Sprühpistole. Der zweite Anschluss wird auf die Messpipette gesteckt, um eine konstante Auslaufgeschwindigkeit zu erreichen. Der optimale Betriebsdruck beträgt 400 mm Wassersäule (Reduzierventil: Saturn I-Regulus II, Fa. Dräger, Lübeck). Nun wird mit der Stellschraube S die Düse geöffnet. Nachdem das Totvolumen von etwa 50 μl ausgefüllt ist, lässt man den Spiegel der Lösung langsam auf die Nullmarke absinken und verschliesst die Düse wieder. Die Dünnschichtplatte wird in den Auftragstisch eingelegt und das Sprühgerät mit Hilfe der Stellschrauben S1, S2, G und S3 auf die richtige Position eingestellt. Nach Einschalten des Schneckenantriebs wird die Düse geöffnet und die gewünschte Strichbreite einreguliert. Durch den Gasstrom trocknet das Lösungsmittel sofort ab. Ist die bestimmte Substanzmenge aufgetragen, wird die Düse verschlossen und die Spritzpistole kann mit dem Gewinderad G in eine neue Position gebracht werden. Zum Wechseln der Lösung werden das Sprühgerät und die Pipette nach ihrer Entleerung mit Lösungsmittel durchgespült, durchgeblasen und mit neuer Lösung gefüllt. Als Lösungsmittel wurden bisher Flüssigkeiten bis zur Viskosität des Wassers verwendet.

Stehen von einer Lösung nur geringe Mengen zur Verfügung, so wird die Pipette bei geschlossenem Dreiwegehahn genau bis zur Nullmarke gefüllt. Das Totvolumen wird aus der reines Lösungsmittel enthaltenden Hülse H1 gefüllt. Nach Umschalten des Dreiwegehahns wird die Düse geöffnet und die gewünschte Lösungsmenge kann in das Totvolumen einfliessen und wird nach erneutem Umschalten des Hahns aus dem Gerät verdrängt.

ANWENDUNGSBEISPIEL

Je 50 und 100 μl eines Farbstoff-Testgemisches (Desaga-Testgemisch für die Dünnschichtchromatographie) wurden punktförmig und strichförmig mit einer Mikropipette aufgetragen. Daneben wurden die gleichen Mengen mit dem Auftragsgerät aufgebracht. Fig. 3a zeigt den Start vor dem Lauf. Fig. 3b zeigt das Farbstoffgemisch nach einer Laufstrecke von 8 cm in Benzol.

Als praktische Beispiele seien hier eine Methode zur quantitativen Bestimmung von Gangliosiden und eine Lipoidbestimmungsmethode angeführt[6,7].

RESULTATE UND DISKUSSION

Während in Fig. 3 das mit der Hand aufgetragene Farbstoffgemisch noch keine Trennung zeigt, ist das Gemisch auf dem mit der Maschine aufgetragenen Teil der Platte bereits in scharfe Banden aufgetrennt. Die Möglichkeit der scharfen Auftrennung von Mischungen in gleichmässig ausgebildete Substanzzonen macht das Gerät daher vor allem für die densitometrische Auswertung von Dünnschichtchromatogrammen geeignet. Die heute noch vielfach übliche punktförmige Auftragsweise ist dem strichförmigen Auftragen in vielerlei Hinsicht unterlegen. Die bei der Chromatographie von Substanzen häufig auftretende Schwanzbildung kann beim Übergang vom punktförmigen zum strichförmigen Auftragen oft vermieden oder zumindest stark zurückgedrängt werden. Vermutlich spielen hierbei Konzentrations- und Überladungseffekte eine grosse Rolle.

Bei der densitometrischen Auswertung von Dünnschichtchromatogrammen zeigt sich ein weiterer Nachteil des punktförmigen Auftragens: Neben der Farb-

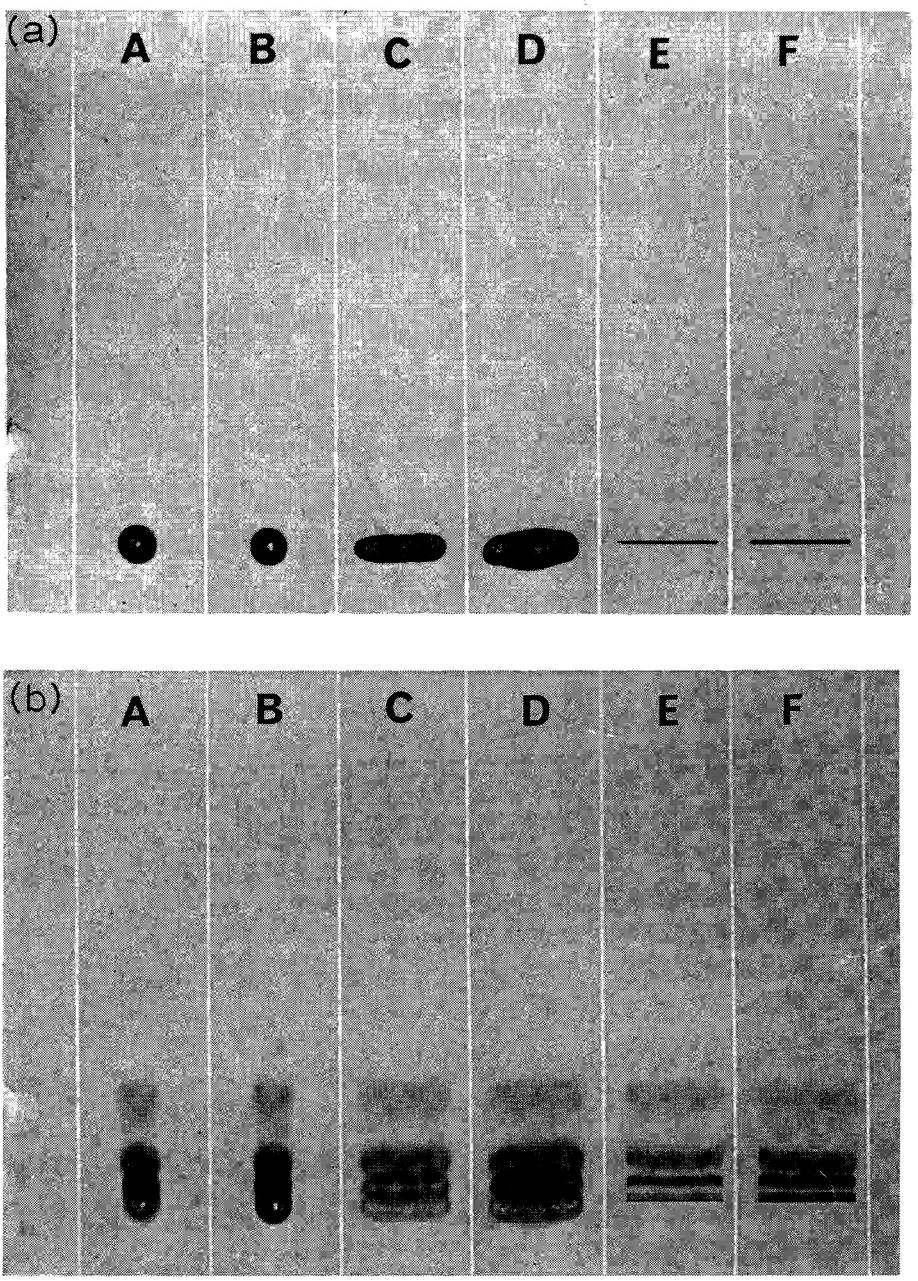


Fig. 3a und b. Dünnschichtchromatographische Auftrennung eines Farbstoffgemisches (b) nach manuellem und maschinellem Auftragen (a). Das verwendete Farbstoffgemisch der Fa. Desaga, Heidelberg, besteht aus *p*-Dimethylaminoazobenzol, Sudanrot G und Indophenolblau (1:1:1) und wird aus benzolischer Lösung aufgetragen (1 mg Gemisch pro ml). Adsorbens: Merck Kieselgel G. Schichtdicke: 375 μ. Laufmittel: Benzol. Steighöhe: 8 cm. (A) 50 μl manuell punktförmig aufgetragen. (B) 100 μl manuell punktförmig aufgetragen. (C) 50 μl manuell strichförmig aufgetragen. (D) 100 μl manuell strichförmig aufgetragen. (E) 50 μl maschinell strichförmig aufgetragen. (F) 100 μl maschinell strichförmig aufgetragen.

intensität ändert sich auch die Fleckengrösse mit der Konzentration. Hinzu kommt noch, dass die Flecken infolge des Konzentrationsabfalles am Rand und wegen der Schwanzbildung nicht homogen sind. Der Messspalt erfasst unterschiedlich grosse Segmente, die ausserdem noch starke Konzentrationsschwankungen aufweisen. Damit verliert das Lambert-Beer'sche Gesetz seine Gültigkeit[8,9] und das Verhältnis von Extinktion (ausgedrückt als Gesamtextinktion des Flecks durch die vom Densitometerschreiber registrierte Peakfläche) zur aufgetragenen Substanzmenge ist nur innerhalb eines sehr kleinen Bereichs annähernd konstant. Soll ein grösserer Konzentrationsbereich erfasst werden, so ist das, wie verschiedene Autoren zeigten[10,11], nur mit Hilfe von komplizierten mathematischen Beziehungen möglich. Diese Schwierigkeiten lassen sich nach unseren Erfahrungen weitgehend vermeiden, wenn man die Substanzen nicht punktförmig, sondern strichförmig aufträgt und eine Strichlänge wählt, die stets um etwa 20 % über der Länge des Messspaltes liegt[6,7]. Dadurch bleiben die störenden Randzonen ausgeblendet. Der Messspalt erfasst nur weitgehend homogene, im Verhältnis zur Strichbreite schmale Zonen, deren Einzelextinktionen über den gesamten Fleckenbereich addiert werden. Das Lambert-Beer'sche Gesetz ist bei diesem Verfahren in guter Annäherung erfüllt.

Beim manuellen Auftragen entsteht das Startband durch enges Aneinanderreihen einzelner Lösungstropfen. Es wird infolge des ungleichen Abstandes und der unterschiedlichen Grösse der Tropfen unregelmässig. Ausserdem wird beim Auftragen von Hand die Schicht leicht verletzt, wodurch das Fliessmittel bei der Chromatographie verschieden schnell nachströmt. Aus diesen Gründen werden die Flecke verzerrt und für eine densitometrische Auswertung ungeeignet.

Diese Nachteile werden mit dem Auftragsgerät vermieden. Durch den periodischen Vorschub der Dünnschichtplatte unter der Sprühpistole (3–5 Perioden pro μl Auftragslösung) entstehen homogene Startbänder. Die Schicht wird selbst bei Auftragsmengen von 1000 μl (Lsm. Chloroform–Methanol, 2:1) nicht verletzt.

Ein Nachteil des Gerätes ist sein Totvolumen von 50 μl. Will man nicht nach der etwas umständlicheren Nachspülmethode (Vgl. S. 3) arbeiten, so verliert man bei quantitativen Arbeiten etwa 70 μl der Lösung, da der Totraum mit etwa 20 μl durchgespült werden sollte.

Bei den Auftragsmengen sollten 3–4 μl nicht unterschritten werden, da sonst die Pipetten- und Ablesefehler zu gross werden. Durch entsprechende Verdünnung lassen sich jedoch auch kleinste Substanzmengen auftragen. Mit dem Gerät sollten keine gesättigten Lösungen aufgetragen werden, um ein Abscheiden der Substanzen an der Düse zu vermeiden.

DANK

Herrn Prof. Dr. H. JATZKEWITZ danken wir für grosszügige Förderung und Unterstützung dieser Arbeit.

Für wertvolle technische Hilfe sind wir den Herren J. BRANDL, R. SCHWARZ und W. PASTOR zu Dank verpflichtet.

ZUSAMMENFASSUNG

Es wird ein automatisches Auftragsgerät für die Dünnschichtchromatographie

beschrieben. Die gelösten Substanzen werden als schmaler Strich (0.5 mm oder mehr) homogen und ohne Verletzung der Schicht aufgesprüht. Das Gerät erlaubt es, im analytischen (Auftragsmengen ab 3–4 μl) oder wahlweise im präparativen Massstab zu arbeiten. Es bringt wesentliche Vorteile beim Auftragen auf Platten, die quantitativ-densitometrisch ausgewertet werden sollen.

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CHROM. 3348

THE DETERMINATION OF BASE RATIOS OF VERY SMALL SAMPLES OF RIBONUCLEIC ACID USING THIN LAYER CHROMATOGRAPHY

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SUMMARY

A technique is described for the quantitative estimation of guanine, adenine, uridylic acid, cytidylic acid and thymine by separation on thin layers of cellulose, elution and spectrophotometric analysis. The method is highly reproducible both for authentic standards and for the estimation of base ratios in plant nucleic acid hydrolysates.

INTRODUCTION

The determination of the base composition of ribonucleic acid (RNA) is usually carried out by the separation of the products of RNA hydrolysis by paper chromatography[1,2], paper electrophoresis[3], a combination of electrophoresis and thin layer chromatography[4] or on strong anion exchange resins[5]. However, these methods in their standard forms are not suited to the accurate analysis of small samples (less than 100 μg RNA).

There have been several reports of rapid one-dimensional separation of bases of nucleic acids on thin layers of cellulose[6–10] and Silica Gel G[6,7], but despite its potential superiority over paper chromatography, both in sensitivity and in the cleanness of separation of the spots[7,11], there is no available method for the quantitative thin layer chromatographic base analysis of RNA. This would require a particularly clear separation of the spots, as was obtained by HOLGATE AND GOODWIN[10] using an isopropanol–HCl–water (65 : 17.2 : 17.8, v/v/v) solvent system on cellulose plates.

This paper describes the development of a similar solvent system for the quantitative analysis of the base composition of small samples of RNA (15–75 μg). Careful attention to standardization at all stages of the procedure is essential but, taking these precautions, the method is very reliable and gives 95–100 % reproducibility both using authentic standards of purine and pyrimidine derivatives and with acid hydrolysates of plant nucleic acids.

METHODS

Thin layer chromatography was carried out on glass plates (20 cm square) with

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a 0.2 mm cellulose layer (Whatman CC41 Chromedia) and isopropanol–HCl–water (68:16.4:15.6, v/v/v) as the solvent[1]. The solvent was made up by weighing the solutions in the proportions 53.5:19.3:15.6, w/w/w).

The preparation of the plates

20 g CC41 and 100 ml Analar grade 2 *N* HCl were stirred in an M.S.E. Top Drive macerator at top speed for one minute. The slurry was filtered using Whatman No. 1 paper in a Buchner funnel and the cellulose pad washed with 100–200 ml glass distilled water. The wet pad was weighed (composition *ca.* 1 g water/1 g cellulose) and resuspended in glass-distilled water by mixing in the M.S.E. macerator at top speed for 15 min, using a ratio 40 g pad material/*ca.* 80 ml water* (3 min at top speed is adequate if a Servall Omnimixer is used).

The plates were wiped with alcohol just before spreading to free them completely from grease and dust. The plates were spread using a Shandon Spreader (Shandon Scientific Co., Ltd.) with a 0.2 mm gap and left to dry for 10 min at room temperature (18–20°) followed by 3 h at 40°. Both the time and temperature for drying are critical for subsequent good separation of the spots. The plates were kept over $CaCl_2$ or silica gel in a desiccator at room temperature for not more than two days before use.

Plates were spotted with 10–50 μl samples (50–250 mμ moles) of purine and pyrimidine derivatives using a Hamilton microsyringe. Adenine and guanine from B.D.H.** and uridylic acid (disodium salt) and cytidylic acid (mixed isomers of the nucleoside 2′ and 3′ monophosphoric acids***) were used as standards. The spots were dried in a stream of cool air. The plate was first given a preliminary run in 0.1 *N* HCl§ (Analar grade) at room temperature until the solvent had run to a distance of *ca.* 4 cm from the origin (*ca.* 10 min). This facilitates the separation of guanine and adenine. The plate was air-dried and then run in the same direction, in isopropanol–HCl–water (68:16.4:15.6, v/v/v) for 3–4 h at 40° in a previously equilibrated tank (Shandon Universal TLC Chromatotank). After 4 h the solvent front was 3–5 cm from the top of the plate.

Removal of the spots from the plates

The plates were dried in a stream of cool air and the spots removed immediately. (If the spots were left on the plates overnight and eluted the following day quantitative reproducibility of duplicates was not obtained.) The spots were viewed under a U.V. lamp (254 mμ emission band) and a rectangle marked round each spot with a dissecting needle (Fig. 1).

In preliminary experiments an equal amount of cellulose was removed with each sample and used as a blank. The whole operation was carried out in a draught-free room. Single-edge flexible razor blades were used to scrape off the samples which were collected onto squares of black smooth surfaced paper and transferred to 5 ml conical centrifuge tubes. 3–4 ml of the acid of appropriate normality (see Table I) for

* Whatman Chromedia CC41 is not consistent in texture and different batches may require small changes in maceration time and in the weight of Chromedia CC41 needed/100 ml glass-distilled water.

** British Drug Houses, Poole, Dorset.

*** Sigma Chemical Co. Ltd., 12 Lettice Street, London.

§ Standardized grade HCl should not be used as it contains impurities which interfere with the chromatography.

each spot was added and each suspension mixed on a Whirlimixer (Scientific Industries, International Inc. (U.K.) Ltd.). The covered tubes were left to stand at + 2° overnight, the precipitate removed by centrifugation at 2650 × *g* for 20 min and the supernatant carefully removed with a fine Pasteur pipette for optical density determinations. Measurements were made using cells of 1 cm path in a Hilger Uvispek spectrophotometer. The wavelengths and factors used for calculating μmoles/ml of each component are given in Table I.

Hydrolysis of the RNA samples

Hydrolysis was carried out in closed Pyrex tubes in 1.0 *N* HCl (Analar Grade) for 1 h at 100°. 1–10 mg RNA were hydrolysed in 0.25 ml *N* HCl and 10–40 μl of the hydrolysates applied directly to the TLC plates.

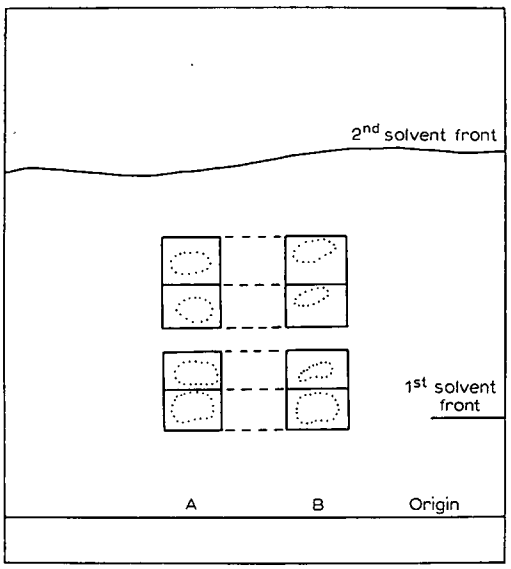


Fig. 1. A tracing of the U.V. absorbing spots on a TLC plate after separation of purine and pyrimidine derivatives from acid hydrolysis of RNA. (A) 10 μl of a mixture of guanine, adenine, uridylic acid and cytidylic acid standards. (B) A hydrolysate of a fraction of ribosomal RNA from leaves of *Vicia faba* L. var. Royal Dwarf Fan. 1st solvent: 0.1 *N* HCl; 2nd solvent: Isopropanol–HCl–water (68:16.4:15.6, v/v/v).

TABLE I

ULTRAVIOLET ABSORPTION DATA ON PURINE AND PYRIMIDINE DERIVATIVES

Substance	*Normality of HCl*	*Absorption max (mμ)*	*Reference wavelength (mμ)*	$\Delta E^{1}_{1\ cm}$ *μ mole/ml between* λ_{max} *and* λ_{ref}
Guanine	1.6	249	290	8.15[12]
Adenine	0.1	262.5	290	12.64[12]
Cytidylic acid	0.01	278	300	10.7[12]
Uridylic acid	0.1	260.5	280	6.94[14]
Thymine	0.1	265	290	6.93[13]

RESULTS

The separation of the two purines, adenine and guanine, and the pyrimidine nucleotides, cytidylic and uridylic acids, was always very clean and a tracing of one chromatogram is shown in Fig. 1. The R_F values are given in Table II. The spots were distinct and never overlapping. The solvent system can also be used for the separation of DNA derivatives. Thymine has an R_F value of 0.75 in a mixture and 0.745 when alone, *i.e.* it runs very close to the position of uracil in the RNA samples but well-separated from the other spots. Optimum separation was obtained when the material was applied as a 10 μl spot. Streaking occurred if the spot contained more than 250 mμmoles of material.

TABLE II

R_F VALUES OF PURINE AND PYRIMIDINE DERIVATIVES OF HCl HYDROLYSIS OF NUCLEIC ACIDS

Solvent: isopropanol–HCl–water (68:16.4:15.6, v/v/v)

Substance	R_F
Guanine	0.33
Adenine	0.42
Cytidylic acid	0.60
Uridylic acid	0.74
Thymine	0.75

TABLE III

QUANTITATIVE ESTIMATIONS OF PURINE AND PYRIMIDINE DERIVATIVES

Triplicate samples of each standard compound applied as a 10 μl spot. The method is described in the text.

Substance	*mμmole/sample*		*Recovery* (%)
	*Applied to plate**	*Recovered*	
Guanine	180, 180, 184 → 181	179, 183, 188 → 183	101
Adenine	192, 196, 196 → 195	188, 192, 188 → 189	97
Uridylic acid	136, 136, 136 → 136	136, 134, 140 → 137	100
Cytidylic acid	200, 200, 192 → 197	197, 199, 199 → 198	100

* The amount of material applied to the plate was calculated by dissolving triplicate samples in 4 ml of elution acid of appropriate concentration and calculating the concentration of substance in the sample using the values given in Table I.

Quantitative recoveries

Triplicate samples (0.1–0.2 μmole) of each standard were applied to the plates and taken through the complete procedure to test its reproducibility. The controls were samples of the standards in 4 ml of the appropriate elution acid (Table I). From the difference in absorption at the maximum and at a reference wavelength the amount of material applied to each plate could be calculated. A comparison of the amount of material applied to the plate and the amount recovered from it is shown in Table III. For guanine, uridylic acid and cytidylic acid the recoveries were always of the order of 100 % but the adenine values were usually in the region 96–98 %. By using samples in triplicate the occasional loss of material during one of the transfer stages could be readily detected. Thus repetition of the assay, which would be necessary with duplicates could be avoided. In several preliminary experiments, readings of the eluted spots were taken against a "cellulose" blank from the plate and against an acid blank. The readings against the acid were more consistent on every occasion so this procedure was adopted.

A comparison was also made to see if the same degree of reproducibility could be obtained when four spots, each containing a simple standard compound, were applied on top of each other and run together. The comparison with each component run singly is shown in Table IV. The % composition giving the base ratios for the artificial mixture and for the single spots were very similar. Thus the presence of more than one derivative does not appear to interfere with the accurate assay of any component. The analyses of the same mixture on different occasions were highly reproducible.

TABLE IV

COMPARISON OF ESTIMATION OF NUCLEIC ACID DERIVATIVES WHEN APPLIED (A) AS SINGLE* SPOTS AND (B) AS A MIXTURE

Substance	*mμmole/sample*		*Moles % of each base*	
	A	*B*	*A*	*B*
Guanine	46	51	18	18.3
Adenine	73	78	28.5	28
Uridylic acid	64	72	25	25.4
Cytidylic acid	73	78	28.5	28

* Each compound was applied as six 10 μl spots which were scraped off and combined in 4 ml of elution acid for spectrophotometric assay.

The analysis of authentic RNA hydrolysates

The method was next used to analyse HCl hydrolysates of RNAs from leaves of *Vicia faba* L. The separation of the components of a hydrolysate of leaf ribosomal RNA is shown in Fig. 1B. (The RNA sample was prepared by phenol extraction according to DYER AND LEECH[15].) The separation of guanine, adenine, uridylic acid and cytidylic acid is clean and comparable with the separation of standards shown in Fig. 1A. Spots were eluted and assayed as for the standards and the results of one analysis are shown in Table V. Four 10 μl samples of the hydrolysates were run on

TABLE V

ANALYSIS OF AN HCl HYDROLYSATE OF LEAF RIBOSOMAL RNA

In each experiment three samples of the hydrolysate were run and similar spots eluted and combined in 4 ml of the appropriate acid for spectrophotometric assay.

Exp.	*mμ moles/sample*				*Base ratios*			
	Guanine	*Adenine*	*Cytidylic acid*	*Uridylic acid*	*G* :	*A* :	*C* :	*U*
26/27	164	152	142	122	28.3	26.5	24.5	21.2
28	168	149	138	128	28.8	25.6	23.7	22.0
29	164	144	135	121	29.1	25.5	23.9	21.9
Mean:	165	148	138	124	28.7	25.9	24.0	21.7

each occasion and combined to give one reading in Table V. Each successive assay was completed a week after the previous one and, as can be seen from Table V, successive assays of the same material gave consistent results.

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CHROM. 3364

DETERMINATION OF SERUM AND BODY FLUID PROTEINS BY MEMBRANE CHROMATOGRAPHY ON NITROCELLULOSE FILTERS: SYNPOR, SARTORIUS AND MILLIPORE

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(Received December 14th, 1967)

SUMMARY

Different types of nitrocellulose membrane filters, *viz.* Synpor, Sartorius and Millipore, were tested as a carrier medium for chromatography and electrophoresis of proteins. All the membranes displayed a characteristic strong adsorptivity towards proteins which could be prevented by an impregnation with neutral detergents (*e.g.* the Tweens). Some important differences in chromatography on various types of membranes were also found and discussed.

Rapid quantitative protein determinations could be obtained by radial membrane chromatography of solutions containing concentrations of 0.001–6 % of various proteins.

In a previous paper[1] we described a simple technique for the rapid quantitative determination of very small amounts of protein (*e.g.* 1 μg/1 μl) by radial chromatography on nitrocellulose membranes (Synpor 6) (former name: HUFS) at pH 3.7. The areas, which were well formed, sharp circular spots of the adsorbed proteins, were proportional to the protein concentration. In the experimental programme it was further observed that Millipore HA membranes were also suitable for the same purpose[2], and with Sartorius 11011 membranes (former name: MF 10) it seemed possible to determine serum proteins even at a neutral pH[3].

The present investigation was undertaken to compare the applicability of the various types of nitrocellulose membranes to protein determinations in native serum, in cerebrospinal fluid, various preparations of modified serum[4] and modified globin[5]. Special attention was given to the analysis of very low protein concentrations, down to 1 mg %. Some other properties of the different membranes were also studied, *e.g.* their impregnation with detergents preventing the adsorption of proteins.

MATERIALS AND METHODS

Native human and rabbit serum, human cerebrospinal fluid, bovine serum modified by heat and formaldehyde[4] and carboxymethylated human globin[5] were used as standard model samples.

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

The following membranes were used: Synpor 1-9 (Chemapol, Prague), Millipore RA, HA, VC (Millipore s.a., Paris) and Sartorius 11001-11011 (Göttingen). It was usually necessary to wash the membranes in boiling water before use to permit regular development of the spots[1]. This operation could be omitted in most cases with Sartorius membranes 11001-11006, and some batches of 11011. Acetate buffer, pH 3.7[1], and citrate buffer, pH 7.0, were used to develop the chromatograms. Radial chromatography of the more concentrated (*e.g.* 1–6 %) protein solutions, application of 1 μl volumes of the samples, rinsing of the capillary, staining with 0.01 % nigrosine or 0.05 % amidoblack 10 B in 3 % trichloracetic acid and quantitative evaluation of the protein spots was carried out as described earlier[1,3]. Self-filling capillaries calibrated up to volumes of 50 μl were used for the analysis of the very dilute protein solutions. The simple arrangement shown schematically in Fig. 1 was found most convenient for the automatic application and development of protein samples on the membranes. The development was performed by application of 1–2 volumes of the buffer using another capillary. Dry strips (about 3 × 5 cm) of Whatman No. 1 filter paper were used to allow further spreading of the developing buffer from the 2 × 2 cm strips of the nitrocellulose membrane. It was advantageous to slightly wet the contact between the membrane and the paper (*cf.* Fig. 1) with the buffer just before chromatography. No closed chromatographic chamber was necessary for these experiments.

Separate control determinations of the protein concentrations of the standard solutions were done by the Kjeldahl method, the Folin method and by refractometry[6].

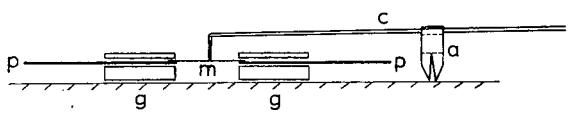


Fig. 1. The method of application and radial chromatography of diluted protein solutions. c = Self-filling glass capillary; a = adhesive paper tape stabilizing the vertical orientation of the capillary; m = membrane; p = filter paper; g = glass slides.

RESULTS AND DISCUSSION

It was found, generally, that all the different types of intact nitrocellulose membranes under investigation adsorbed the proteins investigated in a similar way, However, the areas of the spots formed by the same amounts of the proteins were different on different membranes (Fig. 2). Generally, larger spots were formed at a greater speed on membranes having larger mean pore sizes[1]. The quality of the spots (their symmetry, sharpness of the contours, reproducibility) differed, too. Some types of membrane were not of any use for accurate determinations (*e.g.* Synpor 1 and 2).

When analyzing undiluted or slightly diluted native serum, very good results were achieved at pH 3.7 on Synpor 5,6,8, and Millipore RA, HA, VC membranes and on all the Sartorius membranes. The deviation of the mean was between 3–5 % on different membranes. Modified serum and modified globin (which precipitate at pH 3.7) could be analyzed accurately at pH 7.0 on the same types of membranes as mentioned above. Native serum could only be determined quantitatively, at pH 7.0, on Sartorius 11011 filters; however, in this case the area of the spot was not quite homogeneous. On the other types of nitrocellulose membrane, chromatography of serum at neutral and slightly alkaline pH led to the formation of at least two sharp

circular zones, the inner corresponding to the globulins, the outer to the albumin fraction. The latter phenomenon, which seems to be of both theoretical and practical interest, is being investigated in detail and will be referred to later.

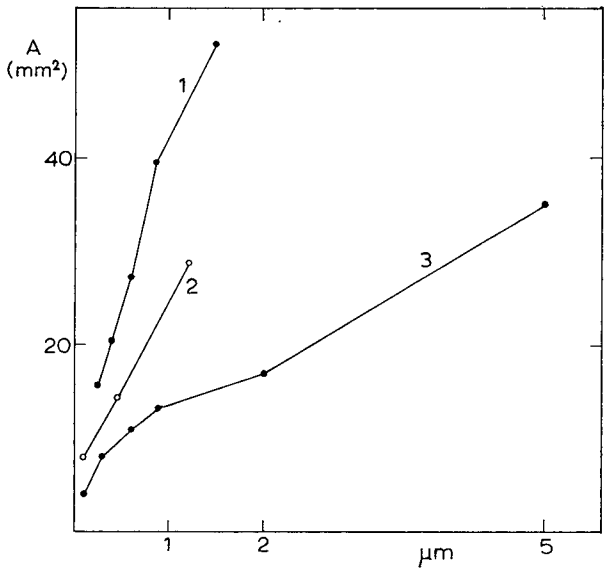


Fig. 2. Areas of protein spots on different types of nitrocellulose membranes. 1 = Synpor; 2 = Millipore; 3 = Sartorius. About 1 μl of 1% human serum (diluted by acetate buffer, pH 3.7) was applied by the same capillary (*cf.* ref. 1). A = Areas of the spots; μm = mean pore sizes of the membranes, according to the manufacturer's data.

The calibration curves shown in Figs. 3 and 4 indicate that the proposed simple technique permits accurate and rapid estimations of proteins also in the very low concentrations which are encountered, for example in cerebrospinal fluid (liquor), in pathological urine, or in contaminated water. Centrifugation or filtration to remove small inhomogeneities in those fluids was not necessary here—they remained fixed at the start. The volume of the solution to be analyzed may be chosen for a given series of samples and a given type of membrane over a wide range in order to achieve protein spots of a convenient size (*i.e.* with a diameter of about 5–15 mm). The samples applied in larger volumes (*i.e.* of the order 10^{-2} ml) were adjusted to pH 3.7 by adding the acetate buffer before chromatography to ensure better reproducibility. When this step was omitted and samples were applied without previous adjustment of the pH, the protein spots sometimes had two well formed concentric areas, the inner being distinctly darker after staining. It was assumed that the inner area probably corresponded to a second protein layer deposited over the first one which was fixed directly to the membrane. This assumption seemed to be confirmed by the fact that a linear calibration curve was only obtained by adding both areas for each concentration.

The areas of the protein spots were generally greater when measured on the wet membranes than on the dry ones. The difference (about 5–10 %) was due to a regular

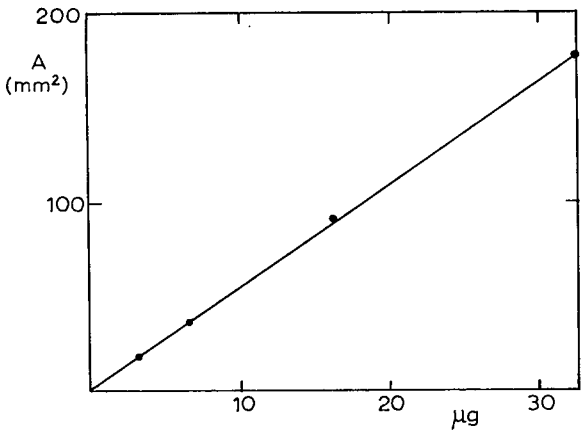


Fig. 3. Calibration curve of very dilute rabbit serum. Rabbit serum diluted by the acetate buffer, pH 3.7, was applied in 50 μl volumes on Sartorius 11001 membranes (mean pore size 5 μm). Chromatography took about 6 min.

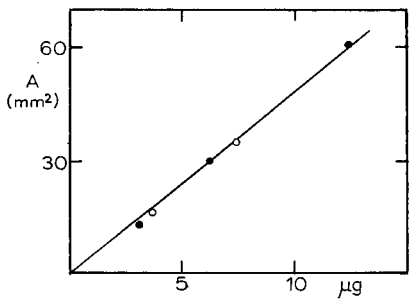


Fig. 4. Calibration curve of human cerebrospinal fluid. Cerebrospinal fluid diluted 2 and 4 times respectively, was applied in 20 μl and 40 μl amounts, respectively, on Sartorius 11001 membranes. ● and ○ correspond to two different patients. Chromatography took about 3 min.

shrinkage of the membranes during drying. The change was "reversible" on wetting the dry chromatogram repeatedly. Thus, two different linear calibration curves can be obtained, one for wet and one for dry membranes.

Furthermore, there were great differences (up to 30 %) when the same spots were measured on the front and the back of the chromatogram. These differences were negligible on Sartorius 11001. On those membranes, where the front and back are easily recognized (*e.g.* Synpor 6 membranes have a glossy surface on one side) it was convenient to use only one side for the calibration and protein determination (*cf.* ref. 1). However, on other membranes, the most accurate results were obtained by calculating the average value from the areas measured on both sides of the dry membrane.

In the studies on the impregnation of the membranes with neutral detergents, it was found that Tween 20, 40, 60 and 80 all had an equal effect in preventing the adsorption of proteins on all types of nitrocellulose membranes tested. Microelectrophoresis of proteins was carried out on the impregnated membranes as was described earlier[7]. Good results were obtained on membranes having pore sizes about 0.2–0.4 μm.

ACKNOWLEDGEMENTS

The authors wish to thank Ing. J. DOSTÁL (VCHZ Synthesia, Uhříněves, ČSSR), Mr. JOLLIVET (Millipore s.a., Malakoff, France) and the "Sartorius Membranfiltergesellschaft" (Göttingen, Germany) for samples of various nitrocellulose membranes, and Doc. HANZAL (Neurological Clinic, Prague) for supplying samples of cerebrospinal fluid.

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CHROM. 3359

BIOCHEMISTRY OF SPHINGOLIPIDS

XIV. IDENTIFICATION OF SPHINGOSINE BASE DEGRADATION PRODUCTS ORIGINATING FROM DIFFERENT ACID HYDROLYTIC CONDITIONS

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(Received December 4th, 1967)

SUMMARY

A systematic paper chromatographic technique for the separation and identification of sphingosine bases and their degradation products formed under different acid hydrolytic conditions is described.

INTRODUCTION

Observations that various degradation products of sphingosine bases were formed during acid hydrolysis of the intact sphingolipids indicated the lability of the parent compounds to these reagents.

During the last few years several authors have studied the nature of these by-products[1–4]. CARTER *et al.*[1] identified 3-O-methyl ethers and WEISS[2], 5-O-methyl ethers of sphingosines. KARLSSON[5–8] observed several by-products, which appeared to be due to allylic rearrangements or to dehydration of sphingosine and related isomers.

In our previous publications[9,10], the effect of different hydrolytic conditions on the sphingosine base content was discussed.

In the present communication the identification of the degradation products formed in these reactions is explored in more detail.

EXPERIMENTAL

Materials

Sphingolipids were isolated from human brain tissue by mild alkaline hydrolysis and chromatography on Florisil and DEAE-cellulose columns. Gangliosides were removed from the initial lipid mixture according to SUZUKI[20]. The purity of all fractions was tested by paper chromatography[11].

Hydrolytic conditions

Aqueous methanolic HCl (AMH), modified aqueous methanolic HCl (MAMH),

10% aqueous HCl with 20% of methanol (AH) and methanolic sulphuric acid (MSA) reagents were used in the same manner as described previously[9,10]. The reaction mixtures were diluted with water and after alkalization the sphingosine bases and their degradation products were extracted with diethyl ether. The ethereal phase was washed with water and dried over sodium sulphate.

The substances isolated from all hydrolytic procedures were converted to their DNP-derivatives[12].

Chromatography

Separation into classes. The DNP-derivatives were separated by preparative chromatography on Whatman No. 3 paper impregnated with silica gel in the following systems:

(1) Petroleum ether (b.p. 60–90°)–diethyl ether (65:35)[10]
(2) Petroleum ether (b.p. 60–90°)–diethyl ether (50:50)
(3) Chloroform.

Separation of individual compounds. The individual classes of DNP-derivatives were further subfractionated by a two-dimensional combination technique on silica gel paper impregnated with sodium tetraborate[10] or on a reversed-phase partition system[10].

Detection. The spots of all DNP-derivatives were detected in ultraviolet light (254 nm).

Semiquantitative determination. After detection, the spots were outlined accurately in pencil, cut out, and eluted with methanol. The eluates were measured against a blank sample at 350 nm.

RESULTS AND DISCUSSION

Fig. 1 shows the separation of crude DNP-derivatives of sphingosine bases and their degradation products obtained from the hydrolytic procedures described above. As can be seen from this figure, MSA-hydrolysis gives the highest yield of O-methyl ethers of sphingosines.

Further characterization of 3-O-methyl ethers and 5-O-methyl ethers is illustrated in Fig. 2. The schematic representation of the two-dimensional techniques is shown in Figs. 3 and 4.

According to KARLSSON[5–8] it is possible to distinguish about eight by-products originating from sphingosines as a result of acid hydrolysis (Table I). It is evident that each type of such hydrolysis gives a somewhat different qualitative and also quantitative profile of these products. This spectrum depends closely on the reaction conditions.

As can be pointed out from the results of GAVER AND SWEELEY[13], hydrolysis of sphingolipids under anhydrous acidic conditions leads to the formation of O-methyl ethers in yields of 50% or more. Although no reagent was found that completely eliminated the formation of these derivatives, the presence of water effectively lowered the production of the ethers to a considerable extent with a concomitant increase in the yield of sphingosine bases[13,14]. Our present findings confirmed these results (Figs. 1 and 5).

It follows quite clearly from our experiments that in all the acid hydrolyses

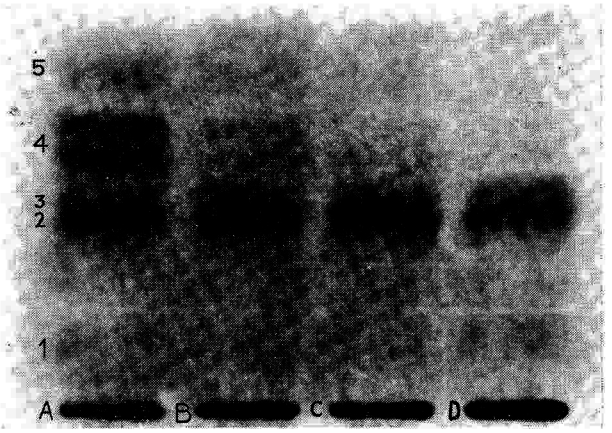


Fig. 1. Separation of DNP-derivatives of sphingosine bases and their degradation products isolated after different acid hydrolytic procedures on human brain sphingolipids. Paper: Whatman No. 3 impregnated with silica gel; solvent system: petroleum ether (b.p. 60–90°)–diethyl ether (50:50); detection: U.V. light (254 nm). A = MSA; B = AMH; C = MAMH; D = AH (for abbreviations see the text). 1 = 1,5-Dihydroxy monoenoic derivatives; 2 + 3 = sphingosines (*erythro* and *threo* isomers) + dihydrosphingosines; 4 = 5-O-methyl ethers; 5 = 3-O-methyl ethers + dienoic derivatives.

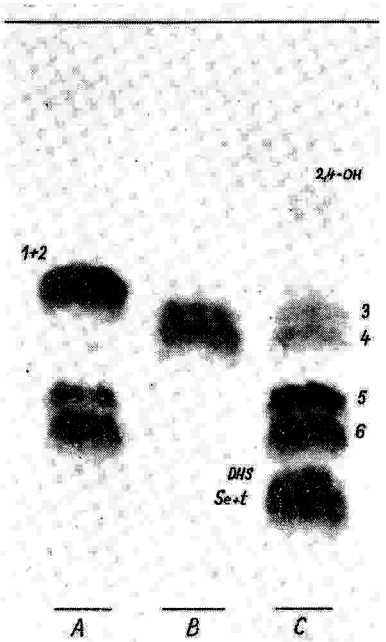


Fig. 2. Separation of DNP-derivatives of sphingosine bases and their degradation products isolated after MSA-hydrolysis of human brain sphingolipids. Paper: Whatman No. 3 impregnated with silica gel; solvent system: petroleum ether (b.p. 60–90°)–diethyl ether (65:35); detection: U.V. light (254 nm). A = Standard mixture of dienoic derivatives (1+2), 5D-O-methyl ethers (5) and 5L-O-methyl ethers (6); B = 3D-O-methyl ethers (3) and 3L-O-methyl ethers (4); C = MSA hydrolysate (DHS-dihydrosphingosines, Se + t = *erythro* and *threo* sphingosines); 2,4-OH = 2,4-dinitrophenol.

studied, the concentration of 5-O-methyl ethers formed by allylic rearrangements is much higher than of 3-O-methyl ethers. This is in agreement with the findings of WEISS[2] (Fig. 6). 5-L-Methoxy isomers predominate in all cases (Fig. 6).

The formation of 1,5-dihydroxy monoenoic derivatives is relatively small except in AH-hydrolysis where about 18 % was found.

Only traces of dienoic products were identified in AMH, MAMH and MSA

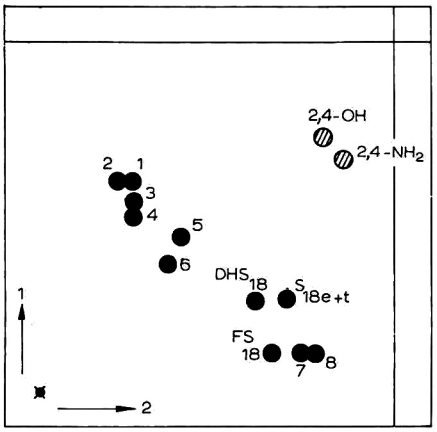


Fig. 3. Two-dimensional combination of adsorption and reversed-phase partition paper chromatography of DNP-derivatives of sphingosine bases and their degradation products. Paper: Whatman No. 3 impregnated with silica gel; solvent systems: 1st dimension: petroleum ether (b.p. 60–90°)–diethyl ether(65:35); 2nd dimension: methanol–tetralin–water (90:10:10; upper phase) after impregnation with tetralin; detection: U.V. light (254 nm). 1 = Dienoic(*cis,trans*)-derivatives; 2 = dienoic(*trans,trans*)-derivatives; 3 = 3D-O-methyl ethers; 4 = 3L-O-methyl ethers; 5 = 5D-O-methyl ethers; 6 = 5L-O-methyl ethers; 7 = 1,5D-dihydroxy monoenoic derivatives; 8 = 1,5L-dihydroxy monoenoic derivatives; S_{18e+t} = C_{18}-sphingosine (*erythro* and *threo* isomers); DHS_{18} = C_{18}-dihydrosphingosine; FS_{18} = C_{18}-phytosphingosine; 2,4-OH = 2,4-dinitrophenol; 2,4-NH_2 = 2,4-dinitroaniline.

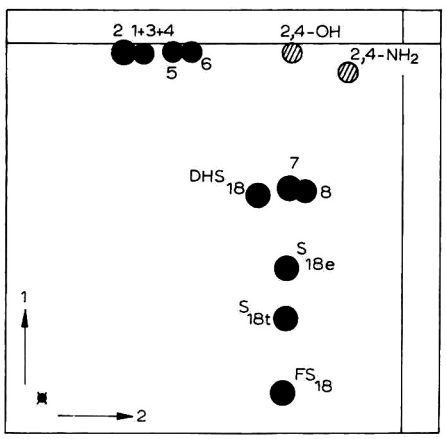


Fig. 4. Two-dimensional adsorption chromatography of DNP-derivatives of sphingosine bases and their degradation products. Paper: Whatman No. 3 impregnated with silica gel and 0.05 *M* $Na_2B_4O_7$; solvent systems: 1st dimension: chloroform–methanol (99:1); 2nd dimension: methanol–tetralin–water (90:10:10; upper phase) after impregnation with tetralin; detection: U.V. light (254 nm). Designation of spots is as in Fig. 3.

TABLE I

MAIN DEGRADATION PRODUCTS OF C_{18}-SPHINGOSINE (1,3-DIHYDROXY-2D-AMINO-4-*trans*-OCTADECENE) FORMED AS A RESULT OF VARIOUS ACID HYDROLYSES[7]

Designation	*Degradation product*
1	1-Hydroxy-2D-amino-3,5(*cis*,*trans*)-octadecadiene
2	1-Hydroxy-2D-amino-3,5 (*trans*,*trans*)-octadecadiene
3	1-Hydroxy-2D-amino-3D-methoxy-4-*trans*-octadecene
4	1-Hydroxy-2D-amino-3L-methoxy-4-*trans*-octadecene
5	1-Hydroxy-2D-amino-5D-methoxy-3-*trans*-octadecene
6	1-Hydroxy-2D-amino-5L-methoxy-3-*trans*-octadecene
7	1,5D-Dihydroxy-2D-amino-3-*trans*-octadecene
8	1,5L-Dihydroxy-2D-amino-3-*trans*-octadecene

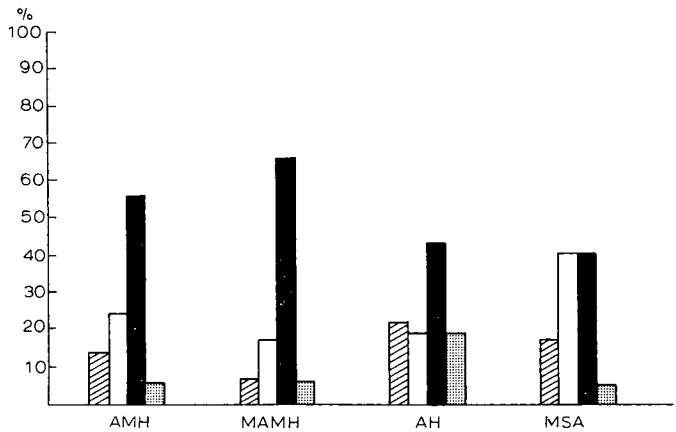


Fig. 5. Average percentage of sphingosine bases and their degradation products after various hydrolytic procedures of human brain sphingolipids. AMH, MAMH, AH and MSA, see text. (▨) Dienoic derivatives + 3-O-methyl ethers; (□) 5-O-methyl ethers; (■) sphingosines (*erythro* and *threo* isomers) + dihydrosphingosines; (▩) 1,5-dihydroxy monoenoic derivatives.

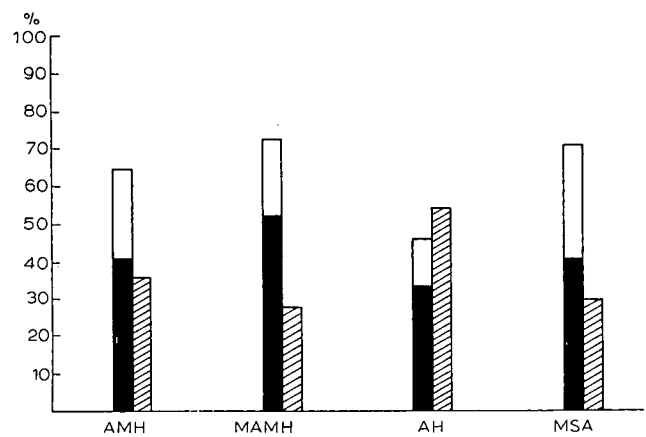


Fig. 6. Distribution of O-methyl ethers after various hydrolytic procedures of human brain sphingolipids. (□) 5D-O-Methyl ethers; (■) 5L-O-methyl ethers; (▨) 3-O-methyl ethers (D + L) + dienoic derivatives.

hydrolytic procedures. In AH-hydrolysis the amounts are higher, but no definite conclusion could be given, since the separation from the 3-O-methyl ether fraction was incomplete.

It has been admitted by numerous authors that during acid hydrolysis of N-acylsphingosines the inversion from *erythro* to *threo* configuration occurs. The *threo* isomer of sphingosine has been isolated, *e.g.* by SEYDEL[15] from aqueous methanolic acid hydrolysates of cerebrosides and by other authors[16,17].

We have found that all acidic hydrolytic conditions lead to considerable formation of *threo* isomer, although its concentration in the intact sphingolipids (*e.g.* sphingomyelins) is negligible (Fig. 7)[18].

In general, when acid hydrolysis has to be used to free the sphingosine bases from sphingolipids, two main problems arise. Firstly, the commonly employed hydrolytic procedures give low yields of bases[9]. Secondly, all these reactions lead to the formation of a lot of undesirable degradation products.

For these reasons the enzymatic hydrolysis of sphingolipids by a specific enzyme system combined with subsequent alkaline hydrolysis is preferable for the precise identification of the sphingosine base profile[7,18].

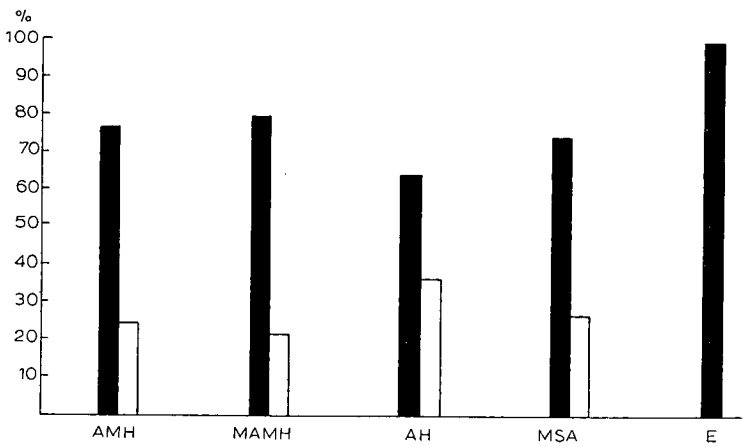


Fig. 7. Formation of *erythro* and *threo* isomers of sphingosines after various hydrolytic procedures of human brain sphingomyelins. E = Enzymatic cleavage with phospholipase C followed by alkaline hydrolysis[9]; (■) *erythro* sphingosines; (□) *threo* sphingosines.

Paper chromatographic techniques were found to be very useful for the separation and characterization of the natural bases as well as their degradation products.

Further techniques for the characterization of the above mentioned substances (*e.g.* chromatographic separation of oxidation products etc.) have been developed in the authors' laboratory, details of which are given in ref. 19.

ACKNOWLEDGEMENT

The authors thank Dr. K. A. KARLSSON (University of Gothenburg) for the samples of some compounds that we used as standards.

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CHROM. 3357

SELECTIVITY OF POLYACRYLAMIDE AND DEXTRAN GELS FOR SIMPLE CATIONS AND ANIONS*

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SUMMARY

Selectivity of Bio-Gel P-2 and P-100, and Sephadex G-10 gels for simple cations was shown by column chromatography of alkali and alkaline earth chlorides and nitrates, and Mn^{2+}, Tl^{+}, and Ag^{+} nitrates. When eluted with water, some of the cations were completely separated. Anion selectivity was demonstrated by chromatography of F^{-}, Cl^{-}, NO_3^{-}, and SO_4^{2-}, as sodium salts. Electrolyte behavior on these gels is discussed in terms of hydrated ionic size, adsorption, and chemical interaction.

INTRODUCTION

Polyacrylamide gels and dextran gels are used primarily for separating macromolecules of different size, and for desalting solutions of macromolecules. They have been successfully applied to the estimation of molecular weights[1–5] and molecular radii[3,6–10], particularly in the molecular weight range of 10,000 to 200,000. More recently, highly cross-linked gels with small pore sizes, on the order of hydrated ion sizes, have become available. Studies of electrolyte behavior on these gels have been very limited, although technical literature[11] mentions fractionations of alkali halides.

Observation of the separation of metal ions on columns used for desalting transfer ribonucleic acid solutions led to this study of electrolyte behavior as related to ion size. Both polyacrylamide and dextran gels were found to exhibit selectivity for several small cations and anions. The separation mechanism appears to involve chemical reactivity as well as size of the solute species.

EXPERIMENTAL

Materials

Reagent grade chemicals were purchased from the usual sources.

Polyacrylamide gel materials, Bio-Gel P-2 and P-100, with molecular weight exclusion limits of about 1600 and 100,000, respectively, were obtained from Bio-Rad Laboratories and from Calbiochem.

Sephadex G-10, a dextran gel with a molecular weight exclusion limit of about

* Research sponsored by the United States Atomic Energy Commission under contract with the Union Carbide Nuclear Corporation.

700, and Blue Dextran 2000, a water-soluble polymer with an average molecular weight of 2,000,000, were purchased from Pharmacia Fine Chemicals, Inc.

Procedures

The dry gel material was stirred into distilled water and allowed to swell for at least 24 h. The gel was then poured into a column of measured volume and washed with several column volumes of water. The columns were jacketed for temperature control. The void volumes of the columns were determined by measuring the elution volumes of Blue Dextran 2000. In each run, the salt solution was added to the top of the column and allowed to drain into the bed. The salts were then eluted with distilled water. In some cases a pump was used to maintain a constant flow rate. Fractions of approximately 5 ml were collected, and the volumes were measured. Effluent fractions were analyzed for metal ions by flame spectrophotometry, and for anions by standard techniques.

RESULTS

Polyacrylamide gel chromatography

A solution containing Li^+, Na^+, K^+, Mg^{2+}, Ca^{2+} chlorides was chromatographed on a 2.5 × 223 cm column of Bio-Gel P-2. The cations were eluted with water, and the order of appearance in the effluent fractions was K^+, Na^+, Li^+, Mg^{2+}, Ca^{2+} (Fig. 1a). Certain pairs of ions, for example Na^+ and Ca^{2+}, were completely separated, and all were sufficiently resolved to indicate a definite order of selectivity of the gel for the ions.

The same elution order for Na^+, Li^+, Mg^{2+}, and Ca^{2+} was observed on a column of Bio-Gel P-100 (Fig. 2); however, the peaks were not separated as well as on the Bio-Gel P-2 column. Although all the ions should be able to permeate the P-100 gel equally well, they still showed different retentions on the column. As expected, the peaks are all shifted to significantly higher elution volumes, near a column volume.

Chromatography of a solution containing Na^+, Cs^+, Mn^{2+}, Tl^+, and Ag^+ nitrates on a Bio-Gel P-2 column gave essentially complete separation of some of these cations (Fig. 1b). The alkali metal ions were eluted first, completely separated from the Mn^{2+} and Tl^+, which were eluted together. The elution volume of Ag^+, eluted last, was greater than a column volume.

The higher elution volume of Na^+ as the nitrate salt, compared with the chloride, suggested anion selectivity. This anion selectivity was demonstrated by chromatography of F^-, Cl^-, SO_4^{2-}, and NO_3^- (as sodium salts) on a Bio-Gel P-2 column (Fig. 1c). The order of elution was F^-, SO_4^{2-}, Cl^-, NO_3^-. The Cl^- and NO_3^- were almost completely separated from each other and from F^- and SO_4^{2-}. The F^- and SO_4^{2-} were resolved into distinct peaks, but they were not separated.

Tetramethylammonium iodide was chromatographed in solution with K^+ and Li^+ iodides on a 2.5 × 240 cm column of Bio-Gel P-2 at room temperature. These cations were well resolved; the elution order was $N(CH_3)_4^+$, K^+, Li^+. The elution volumes, measured as volume at maximum concentration, were 0.75, 0.87, and 0.95 (fraction of a column volume) for $N(CH_3)_4^+$, K^+, and Li^+, respectively. The elution volumes for K^+ and Li^+ were about 0.13 column volume higher in the iodide solution than in the chloride solution, indicating the selectivity for iodide over chloride.

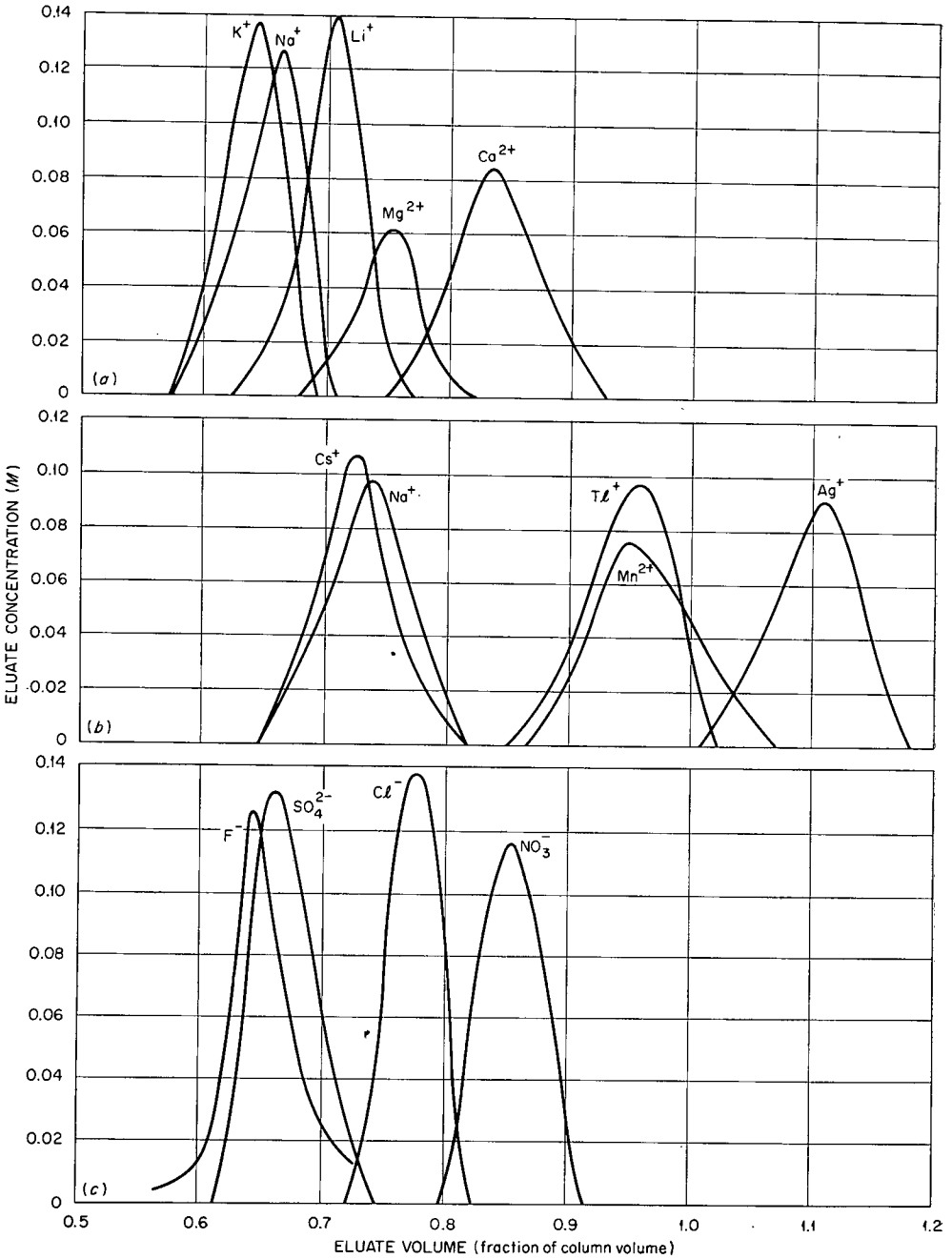


Fig. 1. Polyacrylamide gel chromatography of simple ions. Bio-Gel P-2 eluted with water. (a) 0.05 *M* Mg^{2+}, 0.1 *M* each Li^+, Na^+, K^+, Ca^{2+}, as chlorides, in 100 ml of solution; elution rate, 2 ml/min; temperature, 5°; column, ~ 2.5 × 223 cm; column volume, 1141 ml. (b) 0.1 *M* each Na^+, Cs^+, Tl^+, Ag^+, Mn^{2+}, as nitrates, in 100 ml of solution; elution rate, 2 ml/min; temperature, 5°; column, ~ 2 5 × 223 cm; column volume, 1141 ml. (c) 0.1 *M* each F^-, Cl^-, NO_3^-, SO_4^{2-}, as sodium salts, in 14 ml of solution; elution rate, 0.5 ml/min; temperature, 23°; column, ~ 1.0 × 235 cm; column volume, 210 ml.

Sephadex Gel chromatography

Selectivity by Sephadex G-10 for simple cations and anions is shown in Fig. 3.

A solution of Li^+, Na^+, Mg^{2+}, and Ca^{2+} chlorides was chromatographed on a 1.0 × 235 cm column of G-10 at room temperature. The Mg^{2+} was eluted first, partially separated from Li^+, Na^+, and Ca^{2+}, which eluted together (Fig. 3a).

A solution of Na^+, Cs^+, Tl^+, and Ag^+ nitrates was chromatographed under similar conditions (Fig. 3b). The alkali metals were eluted first. Elution of Tl^+ required more than a column volume. A small amount of Ag^+ was eluted with the Tl^+. Most of the Ag^+ was retained on the column, and would not elute with water.

The anions, SO_4^{2-}, F^-, Cl^-, and NO_3^- (sodium salts) were well resolved on a Sephadex G-10 column (Figure 3c). The elution order was SO_4^{2-}, F^-, Cl^-, NO_3^-.

DISCUSSION

The total column volume, or bed volume, may be expressed as:

$$V_t = V_o + V_i + V_g = V_a + V_g$$

where V_o is the void or interstitial volume, available to all solutes; V_i is the additional volume within the gel; $V_a = V_o + V_i$ is the total aqueous volume, available to solutes small enough to enter the pores; V_g is the volume of the gel matrix inaccessible to the

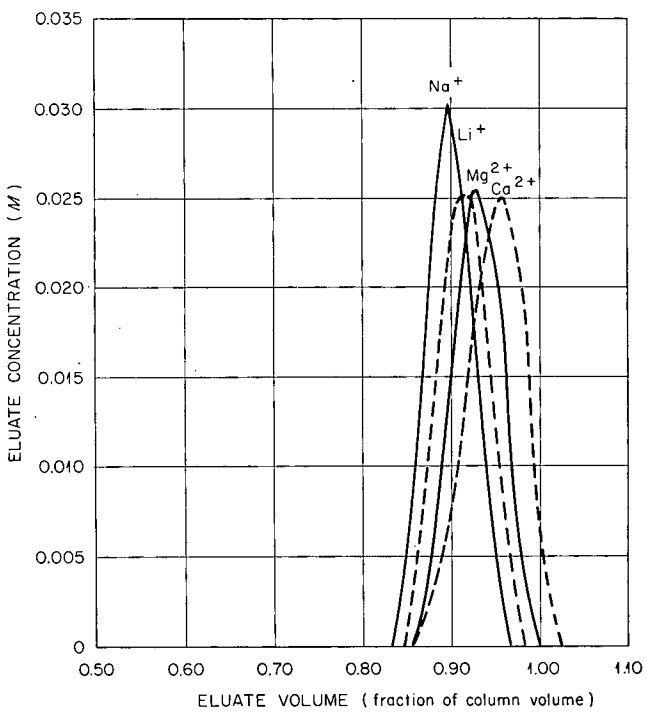


Fig. 2. Polyacrylamide gel chromatography of simple ions. Bio-Gel P-100 eluted with water. 0.1 *M* each Li^+, Na^+, Mg^{2+}, Ca^{2+}, as chlorides in 20 ml of solution; elution rate, 0.6 ml/min; temperature, 23°; column, ~ 2.5 × 211 cm; column volume, 1079 ml.

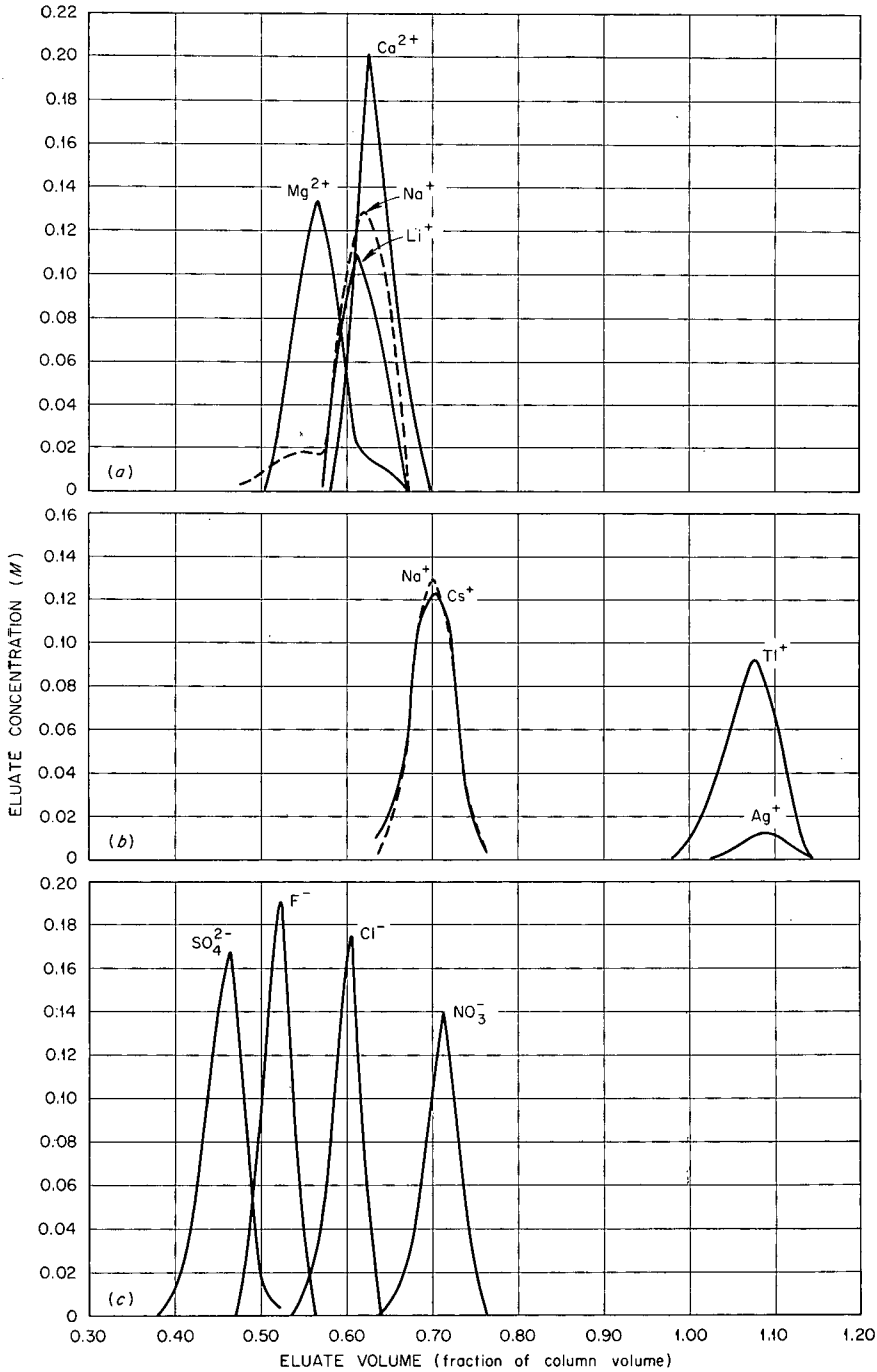


Fig. 3. Dextran gel chromatography of simple ions. Sephadex G-10 eluted with water. (a) 0.1 *M* each Li^+, Na^+, Mg^{2+}, Ca^{2+}, as chlorides in 15 ml of solution; elution rate, 0.45 ml/min; temperature, 23°; column, ~ 1.0 × 235 cm; column volume, 210 ml. (b) 0.1 *M* each Na^+, Cs^+, Tl^+, Ag^+, as nitrates in 15 ml of solution; elution rate, 0.45 ml/min; temperature, 23°; column, ~ 1.0 × 235 cm; column volume, 210 ml. (c) 0.1 *M* each F^-, Cl^-, NO_3^-, SO_4^{2-}, as sodium salts in 15 ml of solution; elution rate 0.45 ml/min; temperature, 23°; column, ~ 1.0 × 235 cm; column volume, 210 ml.

solute. The elution volume, V_e, is the effluent volume at maximum concentration of solute. Three types of behavior may be considered to describe the elution position of the ions from the gels. First, if the freely mobile ions have complete access to the inner volume of the gels, then the elution volume would equal V_a. Secondly, partial exclusion from the gels would result in elution volumes between the void volume (generally about 0.4 V_t) and V_a. Thirdly, interaction of the ions with the gels might cause the elution volume to be greater than V_a. Each of these types of behavior has been observed on both gels, at least in the case of cations. It appears, therefore, that the selectivity of the gels for cations and anions is based on chemical reactivity or physical adsorption as well as size. The chemical reactivity probably includes both ion exchange and complexation of some ions, for example, Ag^+, with the gels.

It is generally agreed[9,12–14] that separation of macromolecules by gel permeation chromatography is based on size differences of the solute molecules, although different parameters have been used to characterize this "size" and quantitatively relate it to column behavior. Several investigators have successfully correlated the elution volumes of large, organic solutes, especially proteins, and their molecular weights[2–5,8,15] and their Stokes radii[3,6–10]. It is difficult to apply these parameters to electrolytes. Molecular weights of dissociated salts are ambiguous. Stokes radius has doubtful meaning when applied to ions with radius less than about 5 Å in aqueous solution, since the water cannot be considered a continuous medium and the hydrated ions are not sufficiently larger than the water molecules to satisfy viscous flow conditions[16–18].

There is considerable disagreement in the literature as to the *absolute* size of hydrated ions, but there is general agreement on the *relative* sizes of many hydrated ions. Attempts to correlate the elution volumes of the ions studied with reported values[17,18] for hydrated radius and other size-related parameters, for example, diffusion coefficients, equivalent conductance, and apparent molal volume, have met with little success. Indeed, if ion size is the only determining factor in the selectivity, then the ions should show similar behavior on both the polyacrylamide and dextran gels, with the larger ions being eluted first. The anions do show similar, though not exactly the same, behavior. The cations apparently do not. According to NIGHTINGALE[18], the sizes of the hydrated anions decrease in the order SO_4^{2-}, F^-, NO_3^-, Cl^-, and the sizes of the cations decrease in the order Mg^{2+}, Ca^{2+}, Li^+, Na^+, K^+ and Mn^{2+}, Na^+, Ag^+, Tl^+, Cs^+. Clearly, the elution orders are not consistent with the larger ions being eluted first. Cation selectivity was observed even on Bio-Gel P-100, which presumably includes solutes with molecular weights as high as 100,000, so that differences among these ion sizes should be negligible. Calculation of the diameter of cation–anion pairs by adding hydrated ionic sizes gives values which are in the range estimated for the pore sizes of P-2 and G-10 gels, permeable to solutes with molecular weights up to about 1600 and 700.

The data illustrate that in addition to size considerations, charge effects and chemical interaction cannot be neglected when considering solute behavior on these gels.

The gels offer a unique method for separating certain cations and anions in which the only added reagent is water. Additional variables to be studied which may influence the separations are temperature, pH, ionic strength, and presence of other cations and anions.

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Notes

CHROM. 3390

The influence of electrolytes on the gas–liquid chromatography of hydrocarbons and alcohols

The recent literature records examples of the use of electrolytes dissolved in the stationary phase to produce unusual resolutions in gas chromatography. For example, alkenes and dienes show remarkable retention times with a stationary phase containing silver nitrate[1–3]. Amines and hydrazines can be separated on polyethylene glycol treated with KOH[4–6]. In the first case the silver ions exert a specific interaction with the double bonds, in the second case the base inhibits the formation of hydrogen bonds. However, effects of the addition of electrolytes to the partition liquid are not limited to specific chemical interactions. In fact, the presence of an electrolyte can cause a new type of interaction to take place: ion–molecule of the solute and ion–molecule of the solvent. The consequence of these physical and chemical phenomena is a considerable variation in the solubility of non-electrolytes in solvents containing various dissolved electrolytes[7].

Experimental

The effect of lithium chloride dissolved in Carbowax 400 on the retention times of *n*-alkanes, alcohols, and benzene was examined and related to the change in the stationary phase containing the dissolved electrolyte. We used a Fractovap GV chromatograph (C. Erba, Milan) equipped with a filament detector and steel columns (1–2 m length, 6 mm diameter).

TABLE I

EFFECT OF ADDED LiCl ON RETENTION TIMES WITH RESPECT TO BENZENE

	Carbowax 400	*Carbowax 400 + LiCl (0.186 M)*	*Carbowax 400 + LiCl (0.766 M)*
n-Hexane	0.08	0.08	0.07
n-Heptane	0.16	0.15	0.13
n-Octane	0.31	0.29	0.26
n-Nonane	0.59	0.55	0.49
n-Decane	1.10	1.03	0.90
Methanol	1.22	1.46	2.16
Ethanol	1.54	1.75	2.40
1-Propanol	2.92	3.20	4.20
Benzene	1.00	1.00	1.00
V_g Benzene	68.9	63.8	53.1

The support was 60–80 mesh silanized Chromosorb P treated with a stationary phase (20 % by weight) containing, in separate experiments: 0.186 and 0.766 molal salt concentrations. We employed helium as a carrier gas at a flow rate of 1 c.c./sec (at 0° and 760 mm Hg). The results (see Table I) are recorded as ratios of the retention times to that of benzene at 78°. In the same table, for comparison, the ratios obtained on Carbowax 400 without added electrolyte are recorded. (The behavior of the standard, benzene, is shown as specific retention volumes V_g.)

When the salt concentration in the stationary phase was increased benzene showed a decrease in solubility. A more remarkable decrease is shown by the alkanes and is reflected in the decreasing retention values. Since the effect of added salt is greater for alkanes than for benzene an inversion in the elution order of the benzene–decane pair occurs.

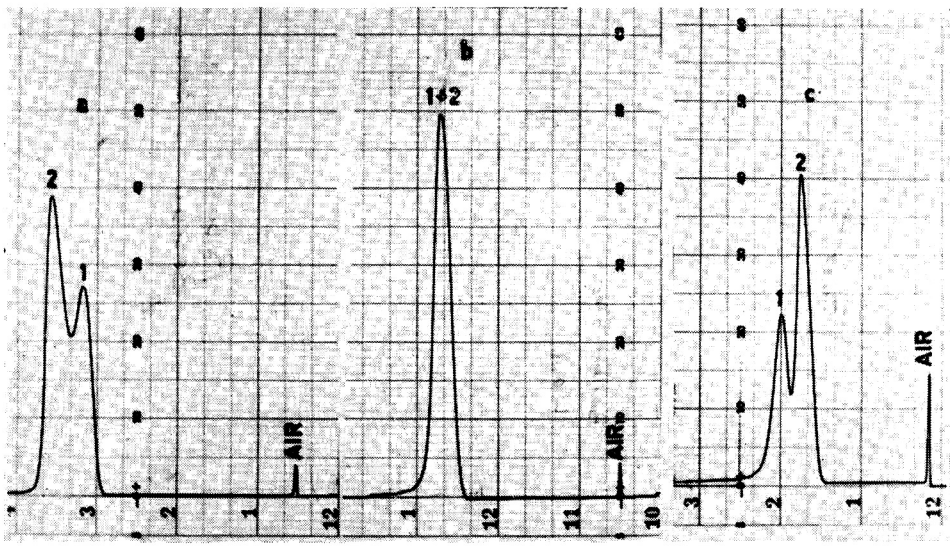


Fig. 1. Chromatograms of a mixture of benzene (1) and decane (2) at 78°. (a) Carbowax 400; (b) Carbowax 400 + LiCl (0.186 M); (c) Carbowax 400 + LiCl (0.766 M).

Fig. 1 shows chromatograms of the benzene–decane elution from Carbowax columns without electrolyte (a) and with LiCl at the two concentrations stated above (b,c). The ratio, 1.03, of the retention times at the lower salt concentration produces a virtually simultaneous elution of the two substances, while at the higher concentration (retention time ratio = 0.90) there is a clear separation again but with an inversion of the elution order, decane (b.p. = 174°) before benzene (b.p. = 80.1°). Alcohols show the opposite behavior in that they become more highly retained with increase of added salt; the increased ratio causes an increase in the retention volume when compared to the saltless phase.

There could be considerable use of this phenomenon since it provides the

research worker with a new parameter by which means the elution order of a variety of materials can be changed virtually at will.

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CHROM. 3405

Gas chromatographic analysis of hydroxydichlorophenoxyacetic acids*

Recently, certain hydroxydichlorophenoxyacetic acids have been reported to be metabolites of 2,4-dichlorophenoxyacetic acid (2,4-D) in plants[1] and microorganisms[2] in a reaction which has been named the "NIH shift" by later workers[3]. We found higher diazoalkanes useful in the preparation of derivatives for gas chromatography of phenolic compounds because they have greater phenol-alkylation activity than diazomethane and their activity is further increased by boron trifluoride catalysis[4]. An important feature of the above procedure is that derivatives may be made from each of several diazoalkanes and that set chosen which gives the best resolution by GLC. The higher diazoalkanes, therefore, offer advantages over conventional procedures for phenolic acids in the GLC analysis of hydroxydichlorophenoxyacetic acids.

Experimental apparatus

An F & M gas chromatograph, Model 400, equipped with a flame ionization detector and a 2 m × 5 mm I.D. glass column, packed with SE-30 (15 %, w/w) on Chromosorb W (60–80 mesh), was operated isothermally at 190°. The injection port and detector were maintained at 235° and 240°, respectively, and the helium carrier flow was 60 ml/min.

Materials

Diazoalkanes were prepared in ethyl ether without distillation[4]. The following acids were prepared: 6-hydroxy-2,4-dichlorophenoxyacetic acid (6-OH-2,4-D)[5];

* Adapted from Ph. D. thesis of senior author.

5-hydroxy-2,4-dichlorophenoxyacetic acid (5-OH-2,4-D)[6]; and 4-hydroxy-2,5-dichlorophenoxyacetic acid (4-OH-2,5-D)[2]. POWELL[1] supplied 4-hydroxy-2,3-dichlorophenoxyacetic acid (4-OH-2,3-D).

Methods

One-milliliter portions of methanolic solutions containing the phenolic acids (2.5 mg/ml) were treated with 3 ml of diazomethane or 1-diazopropane in ethyl ether. Sufficient diazoalkane was added to maintain a yellow color. After 30 min, 0.05 ml of 0.7 % methanolic boron trifluoride was added and the solution kept yellow by the addition of diazoalkane for an additional 30 min. The samples were then concentrated under a stream of dry nitrogen and adjusted to 1.0 ml with methanol for injection into the gas chromatograph. The smallest detectable quantity was 100 ng for 2,4-D and 250 ng for the hydroxydichlorophenoxyacetic acids.

Results and discussion

The sensitivity of detection of 6-OH-2,4-D was sometimes lower than the other hydroxydichlorophenoxyacetic acids possibly because this compound lactonizes at room temperature[7]. This compound should be hydrolyzed immediately before making up standard solutions in order to minimize lactonization.

The preparation of volatile derivatives is necessary for the analysis of hydroxy-acids by gas chromatography. Solutions of diazoalkanes in ether are useful in alkylating phenolic and carboxylic hydroxyls. This method will rapidly alkylate both types of hydroxyl groups at room temperature.

By making two sets of derivatives with different diazoalkanes, it is possible to separate the previously mentioned hydroxydichlorophenoxyacetic acids. Use of only one of the reagents did not produce derivatives of all four compounds which would separate under the conditions used during the course of this work as shown in Table I. The derivatives of 5-hydroxy-2,4- and 4-hydroxy-2,5-dichlorophenoxyacetic acid when treated with diazomethane were inseparable. This was also true when 6-hydroxy-2,4- and 4-hydroxy-2,3-dichlorophenoxyacetic acids were treated with 1-diazopropane. Identification was possible by treating separate samples of the four hydroxy-acids with each of the diazoalkanes as the same two compounds were not inseparable in each set of derivatives.

TABLE I

RETENTION TIMES (min) OF DERIVATIVES OF STANDARDS

Standard	*Methyl ester*		*Propyl ester*
2,4-D	4.0		6.5
	Methyl ester, ether	*Methyl ester, propyl ether*	*Propyl ester, ether*
6-OH-2,4-D	6.7	9.4	16.4
5-OH-2,4-D	8.1	10.9	19.8
4-OH-2,3-D	10.2	13.5	16.0
4-OH-2,5-D	8.0	11.4	21.5

The hydroxydichlorophenoxyacetic acids described here may be prepurified from plant material by the TLC system of THOMAS *et al*[1] for GLC. Some phenoxyacetic acids have been found to form methyl esters under relatively mild conditions[8]; we have found these phenolic acids to form methyl esters in methanol in the absence of any catalyst. When these phenolic acids are eluted from the TLC plate with methanol and the volume then reduced, each forms appreciable amounts of the methyl ester. Subsequent treatment with 1-diazopropane results in a mixture of propyl and methyl esters of the propyl ethers giving split peaks and poorer separation, as is indicated by the retention times of the methyl ester propyl ethers in Table I. This difficulty may be avoided by eluting with acetone. Recovery from TLC was greater than 90 % for all of these phenolic acids except 6-OH-2,4-D. Only 75 % of this compound was recovered, possibly due to facile lactonization.

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CHROM. 3407

Gas chromatographic determination of impurities in vinyl chloride

Monomers of very high purity are extremely important in polymer formation since it is well known that the quality of the monomers has a great influence on the polymerisation and on the characteristics of the polymer obtained. It is therefore necessary to know the quality and the quantity of the impurities present, taking into account the fact that their presence is strictly related to the purity of the starting materials and to the type of process used to produce the monomers.

Vinyl chloride, one of the most important monomers, is mainly produced nowadays by two processes, *viz.* synthesis from acetylene and hydrochloric acid, and cracking of 1,2-dichloroethane. In the first process, the impurities present in the acetylene used are a determining factor, while the acetylene obtained from calcium carbide is practically pure, and that obtained by cracking of hydrocarbons is relatively impure due to the presence of paraffinic hydrocarbons, particularly ethylenic and acetylenic hydrocarbons.

Hydrochloric acid may also contain other impurities if it is a by-product of other processes, while that obtained by direct synthesis is practically pure. In the cracking of dichloroethane the formation of vinyl chloride, which is carried out at a high temperature, is accompanied by secondary reactions, which give rise to saturated and unsaturated chlorinated hydrocarbons. The two processes are often integrated with the aim of using the hydrochloric acid obtained as a by-product in the dichloroethane cracking process; typical impurities of both the processes can therefore be found in the finished product.

Today, however, highly efficient purification methods make it possible to obtain a monomer in which the impurities are very low and therefore difficult to detect by the usual analytical methods. Gas chromatography, therefore, for both its high separating power and its high detection sensitivity, has a fundamental importance as a means of controlling the quality of vinyl chloride.

MIKKELSEN[1] described a gas chromatographic method which uses two columns in series, two thermal conductivity detectors and a condensation trap. The first column is connected to one of the detectors and is used to separate the low-boiling impurities and the greater part of the vinyl chloride; the high-boiling impurities are collected in the cooled trap and then separated in the second column and detected with the second detector. LAZARIS[2] studied the composition of the impurities in commercial vinyl chloride obtained from the hydrochlorination of acetylene produced from calcium carbide.

We have developed a direct one-step method for the analysis of the low- and high-boiling impurities in commercial vinyl chloride, obtained both by hydrochlorination of acetylene (produced by cracking of methane) and cracking of 1,2-dichloroethane. We used tricresyl phosphate as liquid phase and a flame ionization detector, obtaining a detection sensitivity of 0.2 p.p.m. for the impurities eluted before and of some p.p.m. for those eluted after the vinyl chloride.

Experimental

The impurities listed in Table I can be found in the raw vinyl chloride produced

by the two above-mentioned processes; many of them can also be found in the corresponding commercial product, their presence depending on the effectiveness of the purification.

In the case of the impurities, the presence of which was already known, we went on to the addition of pure products and to gas chromatographic analyses on stationary phases with different polarities. In the case of other impurities, we undertook their preliminary enrichment by distillation of raw vinyl chloride, their separation from the enriched fractions by preparative gas chromatography, and their identification both by the retention times on stationary phases of different polarity and by infrared and mass spectrometry.

In order to achieve a complete separation of all the low- and high-boiling impurities in a single operation, chromatographic analyses with variable amounts of different partition liquids (dinonyl phthalate, Carbowax, triallyl citrate, Ucon, Tween 80, Epikote 828, tricresyl phosphate) on different supports were carried out using columns of different lengths and diameters.

The best results were achieved with a 10 m long column packed with tricresyl phosphate on Chromosorb P, which accomplishes the best separation between vinyl chloride, vinylacetylene and ethyl chloride. The column efficiency measured from the

TABLE I

RELATIVE RETENTION TIMES OF IMPURITIES IN VINYL CHLORIDE ON TRICRESYL PHOSPHATE

Peak No.	*Component*	*Temperature*	
		50°	*35°*
1	Ethane, ethylene	0.06	0.05
2	Acetylene	0.17	0.15
3	Propane	0.20	0.17
4	Propene	0.25	0.22
5	Isobutane	0.38	0.34
6	Propadiene	0.52	0.48
7	*n*-Butane	0.57	0.53
8	Butene-1, isobutene	0.65	0.64
9	Propyne	0.70	0.68
10	Butene-2, *trans*	0.85	0.77
11	Methyl chloride	1.00	0.96
12	Butadiene-1,3	1.00	1.00
13	Vinyl chloride	—	—
14	Vinylacetylene	1.94	2.05
15	Ethyl chloride	2.39	2.48
16	2-Chloropropene-1	2.79	2.91
17	Vinyl bromide	3.14	3.28
18	2-Chloropropane	3.89	4.17
19	1-Chloropropene-1, *cis*	4.08	4.37
20	1,1-Dichloroethylene	4.45	4.78
21	1-Chloropropene-1, *trans*	5.07	5.52
22	1-Chloropropane	6.23	6.88
23	Diacetylene	6.88	7.66
24	3-Chloropropene-1	6.88	8.14
25	1,2-Dichloroethylene, *trans*	8.48	9.60
26	Dichloromethane	9.68	11.02
27	2-Chlorobutane	10.50	12.05
28	2-Chloro-butadiene-1,3	11.18	12.85
29	1,1-Dichloroethane	12.25	14.22

1,1-dichloroethylene peak is about 6,500 theoretical plates. Working at a temperature of 50° the methyl chloride–butadiene-1,3 and diacetylene-3-chloropropene-1 pairs, which appears as a single elution peak, remain unresolved (see Fig. 1). However, even these components can be resolved and determined on performing the analysis at a temperature of 35° without varying any other parameter (see Fig. 2). As a result of the lower temperature, the separation of vinylacetylene from vinyl chloride is improved; the sensitivity of detection is almost unaffected for the components until 1,1-dichloroethylene, and becomes slightly lower for the higher boiling components.

Owing to the difficulty of handling the sample and finding a suitable internal standard, a method of direct calibration was adopted for the quantitative determination; an accurately measured and constant quantity of sample (1.5 ml) is always injected and the calibration curves of concentration (p.p.m.) *vs.* area are plotted by means of calibrated mixtures of the various impurities in vinyl chloride; they are linear over a wide range of concentrations.

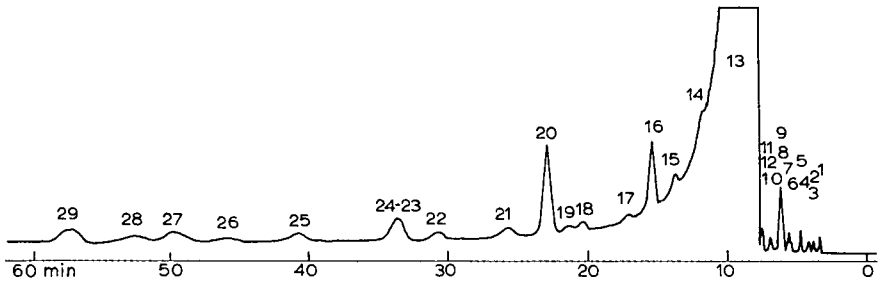


Fig. 1. Gas chromatogram of a synthetic mixture of impurities of vinyl chloride on tricresyl phosphate at 50°. The peak numbers correspond to the components reported in Table I.

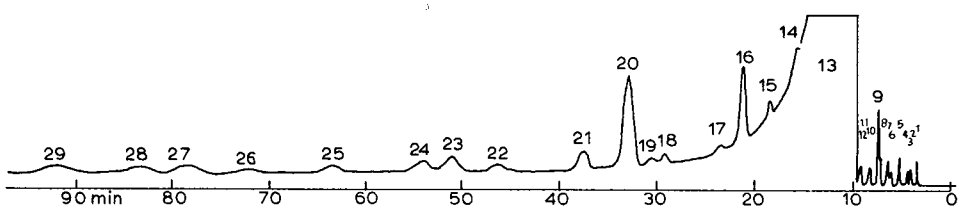


Fig. 2. Gas chromatogram of the same mixture as in Fig. 1, but at 35°.

In the impurity determination by this procedure the error is ± 10 %, which depends almost exclusively on the inaccuracy of the amount of sample injected. Table I reports the detected impurities and their relative retention times with respect to butadiene-1,3 (T_R = 1.00) at the temperature of 50° and 35°.

Fig. 3 shows the gas chromatogram of a commercial vinyl chloride obtained at 35°.

Instrumentation and operating parameters. A C. Erba Fractovap model C (type AID/f) gas chromatograph, equipped with a flame ionization detector, was used in our experiments. The recorder was a Leeds & Northrup Speedomax model G, 2.5 mV f.s., 1 sec f.s. and a chart speed of 12.7 mm/min. The gas sample (size 1.5 ml) was

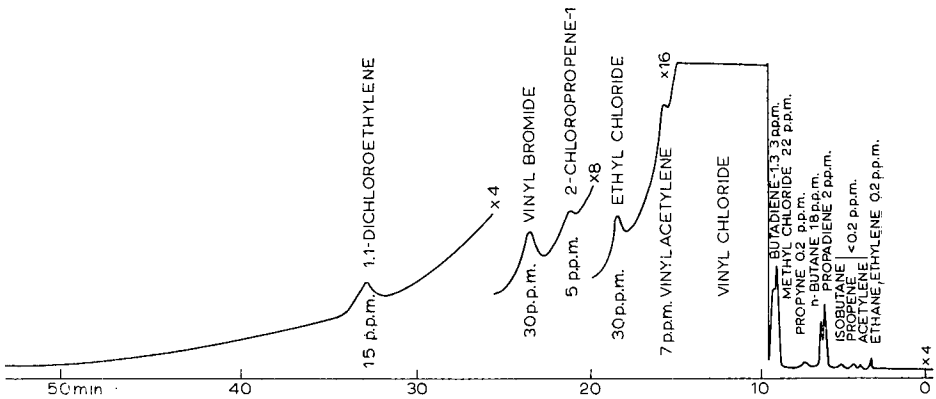


Fig. 3. Gas chromatogram of a commercial vinyl chloride, obtained at 35°.

injected with a 2.5 ml gas-tight syringe (Hamilton mod. 1002 N). The operating parameters were:

Column: 15 % (w/w) tricresyl phosphate on Chromosorb P 40–50 mesh, 10 m × 3.6 mm I.D. nylon tube (Nyloflow pressure tubing, type H, of Polypenco Inc.).

Column temperature: 50° or 35°.

Detector temperature: 120°.

Injection port temperature: 50°.

Carrier gas: nitrogen at a flow rate of 50 ml/min measured at 35°.

Hydrogen flow rate: 60 ml/min.

Air flow rate: 340 ml/min.

Sampling. The vinyl chloride is injected in gas form. The gasification of the liquid sample (at 2.5 atm at 25°) is carried out in a 500 ml glass gas-sampling tube with two tension clip stopcocks, one of which is connected to a PVC tubing closed by a Hoffman clamp. The sampling tube and the PVC tubing are evacuated by a vacuum pump, the free stopcock is cooled with dry ice and filled with liquid sample. By turning the stopcock, about 2 ml of liquid sample are allowed to enter the gas-sampling tube and thus gasify. Then the needle of the syringe is introduced into the PVC tubing, the syringe is carefully washed and afterwards 1.5 ml of sample is drawn out and injected into the gas chromatograph.

Discussion

When the gas chromatographic method suggested above is used, a practically complete separation and determination of the impurities, which can be present in a commercial vinyl chloride, is achieved in one step. This method is applicable to monomers produced both by hydrochlorination of acetylene and by cracking of 1,2-dichloroethane, thus obtaining a complete picture of the purity.

The analysis at 35° is carried out as a control of the end product, while the one at 50° could be conducted to control the various purification stages. A limitation of this method, which, however, cannot be overcome, concerns the determination of acetaldehyde and methanol. These components can be separated and determined using other stationary phases supported on Chromosorb W; but at the same time a

great number of pairs are not separated. When Chromosorb W is used as a support for tricresyl phosphate, the acetaldehyde is eluted together with vinyl bromide as a single peak and in addition the low-boiling components are not clearly separated. When Chromosorb P is used, it is eluted as an asymmetric peak between vinyl bromide and 2-chloropropane and its retention time is dependent on its concentration; furthermore, because of the effect of the adsorption on the support, the small quantities of acetaldehyde present in commercial monomers are not detectable. The same thing occurs in the case of methanol which, when Chromosorb W is used as a support and the analysis in performed at a temperature of 50°, is eluted after diacetylene as an asymmetric peak; also, because it has a smaller response to the flame ionisation detector than acetaldehyde, it can only be detected if it is present in an even greater quantity.

The sample was injected with a gas-tight syringe and not with a gas-sampling valve (which would have given a better reproduction of the quantity injected) because the latter has the greater dead spaces which cause a larger band spreading of the sample so that the separation efficiency results decreased. On the other hand, when the sample is injected with the syringe the reproducibility of the quantity injected is lower. The greatest error obtained, which is $\pm$ 10 % and can be almost exclusively attributed to the operator and to the type of syringe used, is acceptable due to the low contents to be determined.

In order to reduce the troubles at the detector which are due to the corrosion caused by HCl (produced by the combustion of the vinyl chloride) the detector was constructed in Hastelloy C and Teflon; but even normally constructed commercial detectors can be used since corrosion can be reduced to a negligible level by means of thorough and periodic cleaning.

An analysis time of 36 min at 50° and of 52 min at 35° (which corresponds to the elution time of diacetylene) is sufficient for the analysis of a commercial monomer of medium purity.

The reproducibility of the retention times is excellent; even after a year of continuous use of the column no appreciable variation was noticed.

At present the method offers the following main advantages: simplicity and facility of analysis (even by not particularly experienced personnel), high speed performance, and good separation of practically all the impurities present in vinyl chloride.

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CHROM. 3408

Zur Methodik der säulenchromatographischen Bestimmung von Ornithin und γ-Aminobuttersäure

Im Rahmen einer grösseren Arbeit, die sich mit der Untersuchung der freien Aminosäuren in Gerstenmalzwürzen befasst, sollte versucht werden, neben den für die hier benutzte Apparatur nach HANNIG[1] angegebenen Standardaminosäuren, auch noch eventuell vorhandene zusätzliche Aminosäuren zu bestimmen.

Seit Erscheinen dieser Beschreibung hat die Herstellerfirma* diese Apparatur jedoch modifiziert. Die Säulenfüllung besteht nunmehr aus einem (von der Lieferfirma leider nicht genauer definierten) sphärischen Aminalharz, das eine wesentliche Verkürzung der Säulen und damit der Versuchsdauer ermöglicht. Die angewandten Puffer entsprechen dabei in Zusammensetzung und pH ebenso wie die Säulenlängen den von der Firma Beckman für den "Unichrom" angegebenen Werten[2]. Für die Bestimmung von zusätzlichen Aminosäuren bot sich daher bei der basischen Säule die von SPACKMAN, STEIN UND MOORE[3] entwickelte und von der Firma Beckman[2] modifizierte "physiologische" Methode zur zusätzlichen Prüfung, vor allem auf das eventuelle Vorhandensein von γ-Aminobuttersäure, Ornithin und Colamin an.

Für orientierende Vorversuche diente die Eichlösung "Amino acid calibration mixture type 1" von Beckman, der dann jeweils die noch zusätzlich zu bestimmende Aminosäure beigefügt wurde.

Bei diesen Vorversuchen zeigte es sich, dass, bedingt durch die starke Verkürzung der Säule (12 cm gegenüber 50 cm bzw. 29 cm), im Vergleich zu den angeführten Untersuchungen[2,3], eine starke Zusammendrängung der Peaks erfolgte, so dass hier Ammoniak von Colamin überlagert wurde und Ornithin von γ-Aminobuttersäure (Fig. 1). Das Erscheinen eines Peaks an dem Platz für Ornithin und γ-Aminobuttersäure bei Versuchen mit Malzwürze machte es notwendig, zu klären, ob es sich hier um ein Gemisch der zwei Aminosäuren handelte oder nur um eine von beiden.

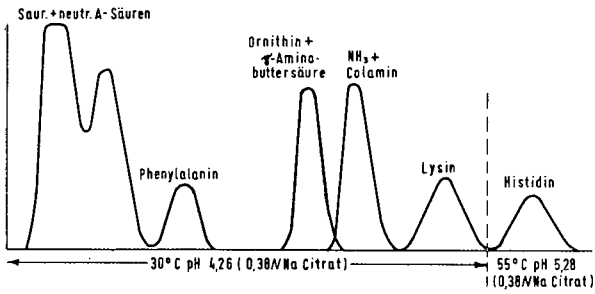


Fig. 1. Auftrennung der basischen Aminosäuren nach SPACKMAN, STEIN UND MOORE[3].

Da WÜNSCH[4] fand, dass Ornithin beim normalen Lauf Lysin überlagert, was auch eigene Versuche bestätigten, wurden zunächst einmal die Werte für Lysin in einer Malzwürze beim normalen und beim physiologischen Lauf, der ja eventuell vorhandenes Ornithin vom Lysin abtrennen musste, verglichen. Das Ergebnis zeigte

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kaum eine Veränderung der Lysinmenge (6.45 cm^2 gegenüber 6.8 cm^2), obwohl beim physiologischen Lauf an der Ornithin und γ-Aminobuttersäure zugeordneten Stelle ein grösserer Peak erschien (5.15 cm^2).

Eine weitere Möglichkeit zur Aufklärung dieser Frage erschien noch durch die Anwendung der Ornithinbestimmung nach WÜNSCH[4] gegeben. Dazu erfolgte zunächst eine Voruntersuchung mit Eichsubstanzen, wobei die Zugabe von Ornithin, γ-Aminobuttersäure und auch Colamin erst einzeln zur Definierung ihrer Standorte zugegeben wurde. Colamin blieb auch hier auf dem Ammoniakpeak, dagegen zeigte sich eine deutliche Abtrennung von Ornithin, das an der von WÜNSCH angegebenen Stelle erschien, und γ-Aminobuttersäure, die unmittelbar nach den saueren und neutralen Aminosäuren auftrat.

Fig. 2 zeigt das sich bei gleichzeitiger Zugabe aller drei Aminosäuren ergebende Bild.

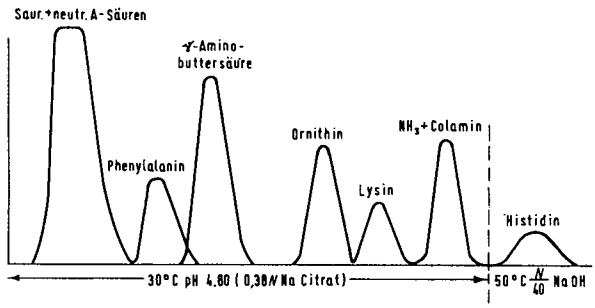


Fig. 2. Auftrennung der basischen Aminosäuren nach WÜNSCH[4].

Eine nun nach dieser Methode durchgeführte Untersuchung der Malzwürze zeigte dann, dass der fragliche Peak praktisch ausschliesslich aus γ-Aminobuttersäure besteht.

Zusammenfassend lässt sich also sagen, dass, für die gegebene Apparatur, die Methode nach WÜNSCH zur Bestimmung von Ornithin und γ-Aminobuttersäure geeigneter ist, da sie eine eindeutige Trennung und Bestimmung der beiden Aminosäuren ermöglicht. Colamin jedoch konnte nach keiner der beiden Methoden abgetrennt werden.

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CHROM. 3393

Simplification of sample loading in continuous automatic amino acid analysis

Some recent papers on automatic amino acid analysis are concerned with acceleration and simplification of the analysis. DUS *et al.*[1] carried out 24 analyses continuously on one single column of a modified Beckman/Spinco analyser. The Technicon Corporation have developed an instrument where even 40 samples can be analysed continuously according to the method of EVELEIGH AND THOMSON[2]. In the meantime in our laboratory we finished the construction of an automatic amino acid analyser capable of chromatographing six preloaded sample injectors, successively, on the same column.

Because the basic principles of the instrument are very similar to commercial ones only a brief description of the complete instrument will be given. Those parts and methods which are essentially different will be described in more detail. The sample loading device in particular is remarkably different from other data published and this device appears to be a very important simplification.

Description of the instrument

Flow scheme. A flow scheme for the automatic amino acid analyser is shown in Fig. 1. One of six buffer solutions, selected by automatically or manually setting the rotary valve, is pumped to the sample injector unit. By means of a double rotary valve, the buffer flows through one of the sample tubes to the column. The eluent is mixed with ninhydrin, supplied by the ninhydrin pump, then heated in the reaction bath, the light absorption is measured in the colorimeter (Multi-Channel Absorptiometer LKB 5900 A), and after passing a flow meter, it flows to a drain tube.

Sample selector valves. The sample selector valves are 6-port valves and they can be automatically or manually turned to any port signalled by a timer or push button, respectively.

The Delrin plugs in the valve housings (Carpenter Stainless Steel Cb.) are

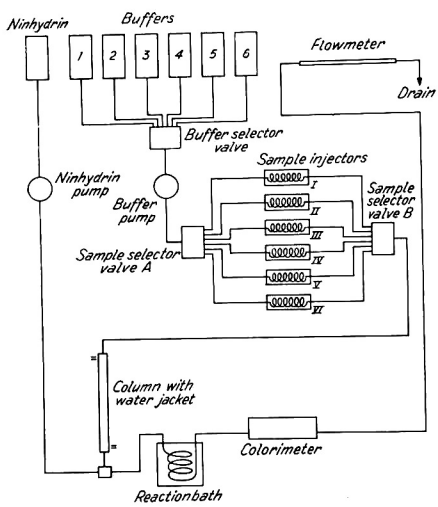


Fig. 1. Flow scheme.

connected by one drive shaft in order to rotate the plugs simultaneously when the motor is started.

The central port of the rotary valve A is connected by teflon tubing (I.D. 0.7 mm, O.D. 1.5 mm) to the buffer pump, the central port of the rotary valve B is connected by teflon tubing to the column plug. The other ports of the valves are connected with inlet and outlet tubing of sample injectors, respectively. All the seals are made with silicone rubber O rings.

Sample injectors. The six sample injectors of the amino acid analyser can be seen in Fig. 2. A cross section of the sample injector is shown in Fig. 3. The main parts are a plunger, a plunger housing (Carpenter Stainless Steel Cb.) and a teflon coil containing the sample to be analysed. The plunger has two longitudinal bores and at the end of each a transverse bore, as is seen in the figure. The plunger is closed at one end with a serum cap. The plunger housing is built up from six different segments connected by two bolts and nuts. To assure tight sealing under high pressure, silicone rubber O rings were used.

All bores in the segments and plunger have diameters of 1 mm. The teflon coil has an inner diameter of 0.7 mm, and an outer diameter of 1.5 mm. The length of the coil is dependent on the amount of sample to be loaded. About 2.67 m has a content of 1 ml. When the sample injector is in the line of the buffer flow the buffer pushes the sample into the column by way of the small space left between plunger and plunger housing. At the end of the analysis the rotary valves are turned to another sample injector and the first one can be reloaded.

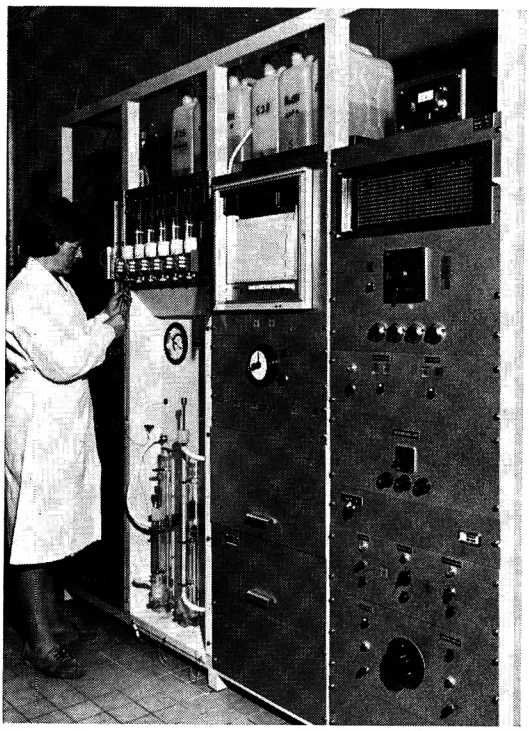


Fig. 2. CIVO automatic amino analyser.

Loading of sample injectors. The loading of sample injectors is very easily carried out by pushing in the plunger so that the transverse bores are in line with the teflon coil of the sample injector. By means of a syringe, the needle of which is pushed through the serum cap into the plunger bore, the sample, preceded by a small air bubble, is pressed into the coil, pushing the buffer present into the drain tubing. As soon as the air bubble reaches the drain tubing, the sample tube is completely filled with sample solution and the plunger is drawn out again. When the syringe is now filled with water, the bores of plunger and drain tubes can be washed without removing the sample solution. The sample injectors were tested under pressures up to 500 p.s.i. The serum cap has only to resist the pressure required to put the sample into the teflon coil, which is sufficiently low.

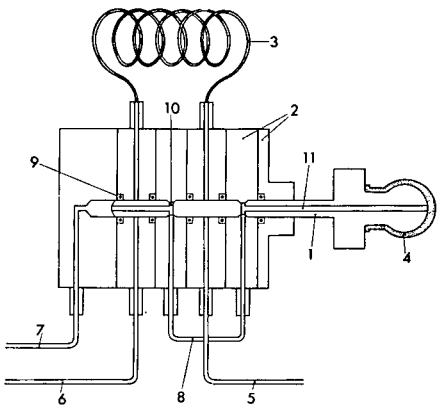


Fig. 3. Sample injector. 1 = Injector plunger (Carpenter Steel Cb); 2 = plunger housing segments; 3 = sample coil (Teflon I.D. 0.7 mm, O.D. 1.5 mm); 4 = serum cap; 5 = outlet tubing to rotary valve B (Teflon I.D. 0.7 mm, O.D. 1.5 mm); 6 = inlet tubing to rotary valve A; 7 = drain tubing; 8 = connection tube to plunger bores; 9 = silicone rubber O ring; 10 = transverse bore of plunger; 11 = longitudinal bore of plunger.

Adjustable column plug. The adjustable column plug (applied to LKB high pressure columns) is somewhat different from that used by DUS *et al.*[1] In contrast to the method used by these authors, we fitted the teflon filter into the plug. This filter is only necessary to prevent resin from entering the teflon tubing. Impurities from buffer and sample are filtered by suitable filters placed in the buffer line to the adjustable plug.

Electrical control system. Because it is beyond the scope of this paper to give a detailed description of the programmer unit composed of 40 relays, we will confine ourselves to remarking that 15 different functions can be independently programmed by means of a programming switch board (Fig. 2). For instance each port of the six-port buffer-change rotary valve represents one strip on the timer, thus enabling the buffer to be changed in any sequence at any selected time. At the end of each run the same programme will be repeated as many times as sample injectors are loaded.

Elution programme. As a consequence of the flexibility of the analyser it is possible, for example, to analyse protein hydrolysates with the two-column system described by BENSON *et al.*[3] as well as by the one-column system of DUS *et al.*[1]. The

column panel has places for two columns, and it is possible to switch manually, by means of a rotary valve, so that each one in turn is in the line of the buffer and reaction coil.

BENSON's method[3] was used with some modification. The regeneration and equilibration of long columns are integrated steps in the elution programme. With the same buffer pump, citrate buffers pH 3.25 and pH 4.25, and 0.2 *N* NaOH and finally citrate buffer pH 3.25 can be pumped in this sequence. Six short column runs can be completed during the day, five long ones overnight.

Discussion

Volume of sample injectors. It is possible to calculate from the internal diameter of the tubes the approximate length required for a desired volume, *e.g.* 1 ml. The coil is filled with a calibration mixture of amino acids and the amino acid constants are calculated from the chromatogram. A small deviation of the volume from 1 ml is not important because unknown samples are analysed with the same sample injector for which the calibration was carried out. Coils of equal length have not exactly the same volume, therefore in order to avoid different amino acid constants for each sample injector coil, a correction factor was calculated for each coil of the same length. The correction factors ranged from 0.99 to 1.01, the same deviation as found by MURDOCK[4] between volumes of equal lengths of teflon tubing.

Applicability of the sample loading system. First we followed the 4 buffer elution programme of DUS *et al.*[5] on 55 cm columns. Unfortunately we did not have the opportunity of testing the system with AA 15 resin of Beckman/Spinco. Only the PA 28 resin was used, and a steadily increasing back pressure and shrinkage of the resins occurred. It was almost impossible to maintain a small dead space between resin and plug without manual correction by turning the adjustable plug. Buffers 3 and 4 (normalities 1.4 and 0.7 respectively) in particular, cause shrinkage of the resin. A further disadvantage of the method is the jump in the base line occurring after phenylalanine.

The automatic sample injector appeared to be very successful in the two column programme, using Bio Rad[6] Aminex A4 spherical resin for the long column and Beckman/Spinco AA 27 spherical resin for the short column.

Separation and determination of ε-dinitrophenyl-lysine on Amberlite IR 120 columns according to SLUMP[7] and tryptophan determinations with Sephadex G 25 columns appeared to be possible (to be published).

Comparison with other sample injectors. DUS *et al.*[1] have used sample tubes which are connected directly with the ports of the rotary valves. The disadvantage of this method is the necessity of disconnecting the Swagelok union every time the samples have to be loaded. The same disadvantage probably occurs with the device described by MURDOCK *et al.*[4]. Moreover the last mentioned sample injectors cannot resist back pressures of more than 100 p.s.i. A completely different sample loading system was recently developed by EVELEIGH[2] and has been used in the commercial instruments of the Technicon Corporation. Here the buffer lines are automatically disconnected before loading of samples.

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CHROM. 3398

Chromatography of isomeric methylene interrupted methyl *cis,cis*-octadecadienoates

I. Argentation thin layer chromatography

Argentation chromatography (recently reviewed[1]) on thin layers or columns has become one of the more valuable separation techniques available to lipid chemists. Though a wide variety of applications in the lipid field have been described, comparatively few reports of studies with model compounds are available and the full potential of the method has not been determined. Two reports of the chromatographic behaviour of isomeric methyl octadecenoates on thin layers of silica gel impregnated with silver nitrate have recently appeared[2,3]. The isomeric methylene-interrupted methyl *cis,cis*-octadecadienoates[4] (*i.e.*, methyl *cis,cis*-2,5-octadecadienoate to methyl *cis*-14,17-octadecadienoate) have now been examined in a similar manner.

Experimental

Silica Gel G (Merck) layers (0.25 mm) containing silver nitrate (10%) were activated by heating at 110°for 2 h. Plates were developed in unlined tanks with hexane–diethyl ether (9:1) as solvent system and visualised by charring with sulphuric acid (10%) at 150°.

Results and discussion

As might be expected, the migration pattern of the isomers conforms to a sinusoidal curve (Fig. 1). The 2,5-isomer has a remarkably high R_F value in comparison to other members of the series. The 3,6- to 6,9-isomers can be separated but the

remainder conform to a gentle curve with a slight but real drop at the 12,15-isomer. A similar pattern has been found with mono-enoic esters[2,3] though the curve is more marked and better separations can be achieved.

A similarly high R_F value has been obtained for methyl *cis*-2-octadecenoate[2] contrary to the predictions of MORRIS *et al.*[3] However, some other factor must be operating in this case as even on untreated silica plates, this isomer migrates ahead of methyl stearate[5,6]. This also pertains to methyl *cis, cis*-2,5-octadecadienoate.

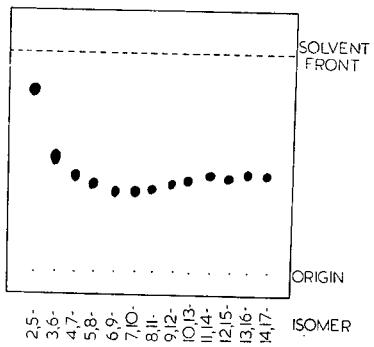


Fig. 1. Argentation thin layer chromatogram of the complete series of methylene-interrupted methyl *cis,cis*-octadecadienoates.

GUNSTONE *et al.*[2] have explained the differences in migration rates of the isomeric mono-enes on the basis of a dual interaction between the polar ester groups with the silica and the double bonds with silver ion. The extent of this would depend on the conformation of each molecule which would vary with the position of the double bond. To account for the distinctive behaviour of *cis*-2 isomers, however, some other factor must operate and a solvent effect may have to be taken into account. Methyl *cis,cis*-2,5-octadecadienoate has unusually high critical solution temperatures[4] in comparison to other members of the series and this may reflect differences in other solution properties. Any explanation must be speculative, however, until more detailed physical measurements on the series are made.

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CHROM. 3412

Dünnschicht-Chromatographie von Carbonsäuren an Cellulose

Verschiedene Methoden zur Trennung von Carbonsäuren durch Dünnschicht-Chromatographie (DC) nach STAHL[1–6] wurden beschrieben: BRAUN UND GEENEN[7], PETROWITZ UND PASTUSKA[8], LYNES[9], TING UND DUGGER[10] sowie RAJAGOPAL, SARASWATHY, SUBBARAM UND ACHAYA[11] verwenden Kieselgel-Schichten; SCHWEIGER[12], BANCHER UND SCHERZ[13], DITTMANN[14], HIGGINS UND VON BRAND[15], LEHMANN UND MARTINOD[16], MYERS UND HUANG[17], BAYZER[18] sowie RASMUSSEN[19] arbeiten mit Cellulose-Schichten. DANCIS, HUTZLER UND LEVITZ[20], HÄKKINEN UND KULONEN[21] und WHITFIELD[22] trennen Ketosäure-2,4-dinitrophenylhydrazone; RONKAINEN[23] trennt Methylester von Ketosäure-2,4-dinitrophenylhydrazonen; THOMPSON UND HEDIN[24] trennen Carbonsäure-2,4-dinitrophenylhydrazide; RINK UND HERRMANN[25] chromatographieren Rhodanin-Derivate von Ketosäuren. BACHUR[26] beschreibt verschiedene Nachweisverfahren der getrennten Säuren; COPIUS-PEEREBOOM[27] hat eine Übersicht über die DC von Carbonsäuren geschrieben.

Zweck dieser Arbeit ist die Beschreibung des Verhaltens einer grösseren Zahl biochemisch interessanter Carbonsäuren bei DC an Cellulose.

Material

Streichgerät für konstante Schichtdicke (Desaga); Cellulosepulver MN 300 (Macherey, Nagel & Co.); Amylalkohol zur Fettbestimmung nach GERBER, Ameisensäure 98–100 % z.A., *n*-Butanol zur Chromatographie, *sek.*-Butanol zur Chromatographie, Ammoniak-Lösung min. 25 % z.A. (alles E. Merck AG).

Methode

15 g Cellulosepulver werden in einem Mixgerät *ca.* 30 Sek. mit 90 ml Wasser intensiv verrührt; Glasplatten von 20 × 20 cm^2 Fläche werden durch Lagern in konzentrierter Soda-Lösung entfettet, mit Wasser gründlich gewaschen und nach Trocknen und Abkühlen mit der Cellulose-Suspension beschichtet. Die Platten bleiben zunächst in einem staubarmen Raum 2 Std. bei Zimmertemperatur waagerecht liegen, anschliessend werden sie 4 Std. auf 105° erwärmt. Abgekühlt und aufbewahrt werden die fertigen Platten in einem Exsikkator über Blaugel[28]. Im allgemeinen wurden wässrige Lösungen der Substanzen aufgetragen; Fumarsäure war in 40 %ig. wässrigem *n*-Propanol gelöst; DL-α-Hydroxybuttersäure, Isocitronensäure und Glyoxylsäure wurden als Natrium-Salze verwendet. Die Konzentrationen der Lösungen waren im allgemeinen so gewählt, dass die in Tabelle I angegebenen Mengen in jeweils 1 µl Lösung enthalten waren.

Zur Herstellung der Dinitrophenylhydrazone einiger Ketosäuren wurden zu jeweils 10 mg Ketosäure: 0.5 ml Isopropanol + 0.4 ml Wasser + 0.1 ml Reagenz-Lösung (300 mg 2,4-Dinitrophenylhydrazin in 100 ml 2 *N* HCl) gegeben; diese Lösung wurde direkt aufgetragen.

Chromatographiert wird einmal 10 cm aufsteigend, ohne Kammersättigung, bei Raumtemperatur von *ca.* 23°. Die Proben werden in Einzelmengen von höchstens 0.5 µl unter Zwischentrocknung 17 mm von unteren Rand der Platte entfernt auf möglichst kleinem Fleck aufgetragen.

Sprühreagenzien

Alkalischer Indikator[29]. 40 mg Bromkresolgrün in 100 ml Äthanol gelöst, 0.1 *N* NaOH bis zur Blaufärbung zugegeben; diesen Indikator für Platten nach Entwicklung mit Systemen I oder II verwendet.

Saurer Indikator[30]. 50 mg Bromphenolblau + 200 mg Citronensäure in 100 ml Wasser gelöst; diesen Indikator nach Entwicklung mit System III benutzt.

Natronlauge. 0.5 *N*; Besprühen mit diesem Reagenz erhöht die Empfindlichkeit des Nachweises von 2,4-Dinitrophenylhydrazonen.

Entwickler-Systeme

System I: Amylalkohol–Ameisensäure–Wasser (40:40:2, v/v/v)[12].
System II: *n*-Butanol–Ameisensäure–Wasser (60:10:20, v/v/v)[13].
System III: *sek.*-Butanol–2 *N* wässrige NH_3-Lösung (80:20, v/v)[14].

Ergebnis

Aufgetragene Mengen und gemessene R_F-Werte sind in Tabelle I zusammengefasst. Die Mengen betragen etwa das Doppelte der minimal nachweisbaren Mengen. (Die Indikatoren müssen mehrfach gesprüht werden, alkalischer Indikator häufiger

TABELLE I

NACHGEWIESENE MENGEN, R_F-WERTE UND STANDARDABWEICHUNGEN DER R_F-WERTE

Substanz	*Menge* (*μg*)	*System I*	*System II*	*System III*
Ameisensäure	1.3			0.21 ± 0.00
Essigsäure	1.0			0.21 ± 0.01
Propionsäure	2.0			0.31 ± 0.01
Buttersäure	2.0			0.44 ± 0.01
Isobuttersäure	0.9			0.43 ± 0.02
Valeriansäure	1.4			0.63 ± 0.01
Isovaleriansäure	1.4			0.60 ± 0.02
Capronsäure	1.4			0.80 ± 0.02
Isocapronsäure	1.4			0.79 ± 0.02
Oxalsäure	1.0	0.58 ± 0.02	0.62 ± 0.04	0.04 ± 0.01
Malonsäure	1.5	0.66 ± 0.01	0.79 ± 0.01	0.06 ± 0.02
Bernsteinsäure	2.0	0.74 ± 0.07	0.85 ± 0.01	0.07 ± 0.02
Maleinsäure	3.5	0.73 ± 0.03	0.78 ± 0.02	0.07 ± 0.04
Fumarsäure	1.0	0.77 ± 0.04	0.93 ± 0.01	0.06 ± 0.02
cis-Aconitsäure	4.0	0.54 ± 0.02	0.66 ± 0.02	0 ± 0
(Nebenfleck)		0.65 ± 0.02	0.81 ± 0.02	
Glykolsäure	2.5	0.66 ± 0.03	0.70 ± 0.02	0.20 ± 0.04
Milchsäure	2.5	0.77 ± 0.02	0.84 ± 0.02	0.23 ± 0.04
α-Hydroxybuttersäure	1.9	0.86 ± 0.01	0.93 ± 0.01	0.37 ± 0.04
β-Hydroxybuttersäure	2.5	0.82 ± 0.02	0.90 ± 0.02	0.31 ± 0.02
α-Hydroxyisobuttersäure	2.5	0.85 ± 0.03	0.89 ± 0.06	0.35 ± 0.02
α-Hydroxyisovaleriansäure	1.9	0.90 ± 0.01	1 ± 0	0.48 ± 0.02
α-Hydroxyisocapronsäure	3.8	0.92 ± 0.01	1 ± 0	0.62 ± 0.04
Äpfelsäure	1.0	0.55 ± 0.03	0.64 ± 0.02	0.04 ± 0.01
Weinsäure	1.0	0.35 ± 0.02	0.45 ± 0.03	0.03 ± 0.01

(*Fortsetzung S. 409*)

TABELLE I (*Fortsetzung*)

Substanz	*Menge* (μg)	*System I*	*System II*	*System III*
Citronensäure	0.5	0.42 ± 0.03	0.57 ± 0.04	0.01 ± 0.01
Isocitronensäure	1.9	0.38 ± 0.03	0.55 ± 0.01	0.02 ± 0.01
D-Galacturonsäure	1.5	0.26 ± 0.02	0.27 ± 0.03	0.04 ± 0.03
D-Glucuronsäure	1.5	0.27 ± 0.02	0.29 ± 0.02	0.04 ± 0.03
L-Ascorbinsäure	5.0	0.45 ± 0.04	0.47 ± 0.02	0.15 ± 0.03
Glyoxylsäure	4.5	0.45 ± 0.04 und 0.70 ± 0.02	0.76 ± 0.04	0.04 ± 0.01
Glyoxylsäure-2,4-dinitrophenylhydrazon	10[a]			0.35 ± 0.04
(Nebenfleck)				0.63 ± 0.01
Brenztraubensäure-2,4-dinitrophenylhydrazon	10[a]			0.55 ± 0.06
Lävulinsäure	1.8	0.86 ± 0.03	0.91 ± 0.03	0.33 ± 0.04
Lävulinsäure-2,4-dinitrophenylhydrazon	10[a]			0.66 ± 0.08
α-Ketoglutarsäure	2.5	0.66 ± 0.02	0.78 ± 0.04	0.08 ± 0.03
α-Ketoglutarsäure-2,4-dinitrophenylhydrazon	10[a]			0.26 ± 0.02
Aceton-2,4-dinitrophenylhydrazon	10[a]			0
Schwefelsäure[b]	1.5	0.23 ± 0.02	0.22 ± 0.01	
		0.30 ± 0.02	0.11 ± 0.03	
		0.36 ± 0.02	0.31 ± 0.01	
Phosphorsäure[c]	1.5	0.46 ± 0.02	0.34 ± 0.05	
		0.41 ± 0.02	0.22 ± 0.05	

[a] Bei den 2,4-Dinitrophenylhydrazonen beziehen sich die Mengenangaben auf die Mengen eingesetzter reiner Ketosäure.

[b] Im System I haben alle drei Flecke etwa gleiche Grösse; im System II ist dagegen die Hauptmenge der Substanz bei R_F 0.22 lokalisiert.

[c] In den Systemen I und II sind jeweils die Flecke mit dem kleineren R_F-Wert (0.41 bzw. 0.22) kleiner als die Flecke mit dem grösseren R_F-Wert.

als saurer Indikator.) Die R_F-Werte sind jeweils Mittelwerte von vier Platten. Hinter den R_F-Werten sind deren Standardabweichungen ($n = 4$) angegeben.

Die beiden Flecke, die man mit Glyoxylsäure im System I erhält, sind etwa gleich gross. Man muss wohl an Umlagerung bzw. Disproportionierung während der Chromatographie denken. Der Nebenfleck mit R_F 0.63, der dagegen bei der DC des Glyoxylsäure-2,4-dinitrophenylhydrazons auftritt, ist möglicherweise so zu deuten, dass sich stereoisomere 2,4-Dinitrophenylhydrazone bei der DC trennten.

Die bei der DC von *cis*-Aconitsäure in beiden sauren Entwickler-Systemen auftretenden Nebenflecken kommen wohl dadurch zustande, dass sich ein Teil der Substanz in *trans*-Aconitsäure umgewandelt hat.

Bei anderen Säuren wurden dagegen Nebenflecke beobachtet, die durch teilweise Zersetzung der Substanzen vor oder während der DC erklärt werden müssen: Brenztraubensäure, Phenylbrenztraubensäure, *p*-Hydroxyphenylbrenztraubensäure, Oxalessigsäure und die 2,4-Dinitrophenylhydrazone der drei letztgenannten Ketosäuren. DC dieser Säuren ist unter den hier beschriebenen Bedingungen nicht möglich.

α-Ketobuttersäure, α-Ketoisovaleriansäure und α-Ketoisocapronsäure lassen

sich zwar mit den beiden sauren Systemen I und II zersetzungsfrei chromatographieren, und die 2,4-Dinitrophenylhydrazone dieser drei Ketosäuren lassen sich auch mit System III gut abtrennen; alle Substanzen haben jedoch sehr ähnliche R_F-Werte zwischen 0.87 und 0.98. Deshalb wurden diese Verbindungen nicht in Tabelle I erwähnt.

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Thin-layer chromatography of some monoterpene alcohol trimethylsilyl ethers

Trimethylsilyl (TMS) ethers of alcohols have been extensively used to facilitate gas chromatographic studies since their initial applications to steroids[1] in 1962. Last year, BROOKS AND WATSON[2] introduced the chromatography of TMS ethers on thin layers, steroids again being the stimulus for the innovation. We have examined TMS ethers of monoterpene alcohols in an attempt to resolve some of these alcohols on thin layers without using special plates (*e.g.* containing silver nitrate[3–5] which rapidly darkens) or prolonged or drastic processing (*e.g.* forming dinitrobenzoates[6] or dehydrogenating[7]). The method has been applied to detect citronellol in the presence of geraniol in rose and related oils, and to note the appearance of monoterpene alcohols in steam-distilled or chemically oxidised limonene.

Experimental

Preparation of trimethylsilyl ethers. To a solution of monoterpene alcohol or a mixture containing such substance in 1 ml equal parts dry pyridine and *n*-hexane, 0.1 ml hexamethyldisilazane and one drop trimethylchlorosilane were added. The mixture was shaken for 1 min, then left 10 min at room temperature to settle before spotting on the thin-layer plate.

Thin-layer chromatography. Silica Gel G plates were waterspread 0.25 mm thick and dried at 105° for 30 min. Applied spots of TMS ethers were allowed to lose most of their pyridine odour before putting the plate in the solvent tank, which was lined with absorbent paper. The plate was removed when the solvent had risen 10 cm above the starting line, and sprayed with 2 % vanillin in concentrated sulphuric acid, without heating. Results are given in Table I.

Limonene steam-distillation products. 4 ml limonene was co-distilled with water twenty times in succession, each condensate was extracted with a little *n*-hexane and it was returned to the still nineteen times. The final *n*-hexane extract was dried over anhydrous sodium sulphate and passed through a column of 10 g silica gel. Unchanged limonene was removed with *n*-hexane, and limonene-oxidation products subsequently eluted with pyridine. TMS ethers were formed from a portion of the pyridine eluate.

Limonene chemical oxidation products. 4 ml limonene was warmed with 10 ml 10 % chromium trioxide in glacial acetic acid for 3 min, then water was added and the mixture extracted with a little *n*-hexane. The extract was washed with water till acid-free, then processed as for the final *n*-hexane extract of the steam-distillate above.

Discussion

On ordinary silica gel plates, geraniol and citronellol (dihydrogeraniol) cannot be resolved. By forming their TMS ethers they can be separated on such plates, and their presence or absence noted in rose-type oils. Thus palmarosa oil is distinct in being deficient in citronellol. The formation of TMS ethers for this purpose is relatively simple, and even if the reaction is incomplete, unchanged alcohols remain near the starting line and do not interfere. This is because solvent systems of very low polarity

TABLE I

THIN-LAYER CHROMATOGRAPHY OF MONOTERPENE ALCOHOL TRIMETHYLSILYL ETHERS

Solute	*Colour of sprayed spot*		*Average R_F value with solvent system*	
	Initially	*After 24 h*	*n-Hexane–benzene (1/1, v/v)*	*Petroleum spirit (60–80°)–benzene (4/1, v/v)*
Geraniol	Dark purple	Fading green	0.30	0.16
Dihydrocarveol	Brown	Blue	0.40	0.23
Carveol	Purple	Blue	0.41	0.23
Citronellol	Pale brown	Deep sky blue	0.46	0.33
Menthol	Pale purple	Light blue	0.52	0.34
Linalool	Purple	Dark green	0.64	0.52
α-Terpineol	Grey-green	Blue	0.64	0.54
Fenchol	Slowly brown	Brown	0.67	0.66
Rose absolute	Purple	Green	0.30	
Rose otto	Purple	Green	0.30	
Geranium oil	Brown	Blue	0.46	
Palmarosa oil	Purple	Green	0.30	
Limonene steam distillate	Purple	Blue		0.23
Limonene chemical oxidate	Purple	Blue		0.53
(Original limonene)	(Purple)	(Blue)	(0.70)	(0.60)

are required, as the polarity of the solute alcohols has been much reduced by forming the TMS ethers.

The method does not successfully separate all monoterpene alcohols. Carveol and dihydrocarveol are not separated from each other, nor are linalool and α-terpineol. Nevertheless, it is possible to say that one or both members of each of the above pairs of alcohols is present in the limonene steam-distillation products and likewise in the chemical oxidation products. Whilst the repeated distillation used here is a laboratory manipulation, it does indicate that limonene is subject to oxidation by this treatment, yielding alcohols, and these may well appear as trace artefacts in commercial oils that contain much limonene and which are prepared by prolonged steam distillation of plant material, such as caraway. Citrus oils distilled for research purposes[8] may also have contained such artefacts.

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Fatty acid composition of serum cholesteryl ester fractions isolated by thin-layer chromatography

Paper and thin-layer chromatographic methods have been devised for routine studies of the fatty acid pattern of cholesteryl esters of serum[1,2]. With appropriate solvents the cholesteryl esters can be separated into five well defined bands (Fig. 1).

In an extensive study of the fatty acid changes of serum cholesteryl esters in alcoholics[3] it was desirable to estimate fatty acid composition by a more rapid and simple method than gas–liquid chromatography (GLC). A modified thin-layer chromatographic (TLC) method was developed[3], but before it was applied routinely, the five cholesteryl ester bands were isolated, and their fatty acid composition was investigated.

Experimental

0.5 ml of serum from normal adults[3] was extracted with chloroform–methanol (1:1, v/v). The polar lipids of the extract were removed by passing the total lipids, dissolved in chloroform, through a 1 g silicic acid column. The chloroform eluate was evaporated under nitrogen to a small volume and spotted as a 10 cm long band on a 0.25 mm thick thin-layer plate of Silica Gel G. The plate was developed in *n*-heptane–toluene (60:25, v/v) three consecutive times, as described elsewhere[3]. The cholesteryl ester bands were visualized by spraying the plate with bromthymol blue reagent. The bands were scraped off, transferred to centrifuge tubes, and dried *in vacuo*. Methyl esters of the fatty acids were made by transmethylation with 0.1 *N* sodium methylate in dry methanol at 100° for 2 h. The methyl esters were assayed by GLC, using a Perkin-Elmer Model 880 apparatus, on a 15% diethyleneglycol–succinate

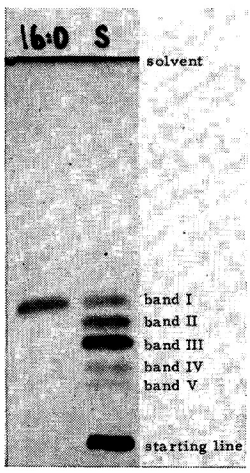


Fig. 1. Thin-layer chromatography of cholesteryl esters in human serum. S = Serum lipids, 16:0 = reference standard. The lipid extract was spotted as a 1.5 cm long line. The plate was developed as described in the text. The spots were visualized by spraying with 50% sulphuric acid and heating at 180° for 30 min.

polyester (DEGS) column, coated on Gas-Chrom Q, 80–100 mesh. The methyl esters were identified as earlier described[4].

Results and discussion

The separation of the serum cholesteryl esters under the present conditions is entirely dependent on the degree of unsaturation of the fatty acids. This is evident from Table I which shows that in each of the five bands there is only one major fatty

TABLE I

PERCENTAGE OF THE FIVE DIFFERENT CHOLESTERYL ESTER BANDS OF HUMAN SERUM ISOLATED BY THIN-LAYER CHROMATOGRAPHY

Fatty acid	*Band*				
	I	*II*	*III*	*IV*	*V*
14:0	3.7	0.8	—	—	—
16:0	86.0	2.6	—	—	0.9
16:1	—	6.0	—	—	—
18:0	8.5	—	—	—	0.5
18:1	1.8	90.6	—	0.3	0.7
18:2ω6	—	—	100.0	2.8	0.8
18:3ω6	—	—	—	7.3	—
18:3ω3	—	—	—	6.2	0.3
18:4ω3	—	—	—	0.3	1.5
20:3ω6	—	—	—	2.8	—
20:4ω6	—	—	—	80.4	2.7
20:5ω3	—	—	—	—	77.5
22:6ω3	—	—	—	—	15.3

acid, namely 16:0, 18:1, 18:2, 20:4, 20:5, respectively. The uniformity in fatty acid composition of each band might explain why it is possible to obtain such a sharp separation between them. Fatty acid compositions of cholesteryl ester bands separated by TLC, have been presented earlier by ZÖLLNER[5] but it was not mentioned how these figures were obtained. He gave the following data for fatty acid composition, arranged according to decreasing R_F value: (I) 16:0 and 18:0, (II) 16:1 and 18:1, (III) 18:2, (IV) 20:3 and 20:4, (V) 20:5, 22:5 and 22:6. Similar but less detailed data have earlier been reported on the fatty acid composition of cholesteryl esters in the plasma of the *Cebus* monkey[7]. Since no quantitative figures were given in the two papers it is not possible to make any detailed comparison. However, the trienes of our preparations were composed mainly of 18:3 of both the linoleate and linolenate families instead of 20:3 and we were able to identify 22:5 only in trace amounts. Overlapping does not exceed 3.4% of the total fatty acid content of any band except for band V (7.4%). This band contains only 2–4% of the total cholesteryl esters of human serum[2,3] and the risk of admixture is thus greater.

These results are valid only for the fatty acid pattern of cholesteryl esters of human serum. The fatty acid composition of the cholesteryl esters are different in other fluids and tissues[6]. Thus, it is necessary to determine the fatty acid composition of the cholesteryl esters from every new source by GLC before the assay of chromatographically separated cholesteryl esters can be used as an index of their fatty acid composition.

Conclusion

Cholesteryl esters were separated into five well defined fractions by TLC. Their fatty acid composition was quantitatively determined by GLC. It was found that each fraction obtained only one major fatty acid, 16:0, 18:1, 18:2, 20:4 and 20:5, respectively, which means that the separation was only dependent on the degree of unsaturation of the fatty acids.

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Délipidation totale des extraits plasmatiques contenant les 17 cétostéroïdes (déhydroépiandrostérone, androstérone, étiocholanolone)*

Pour évaluer les 17 cétostéroïdes plasmatiques, il est indispensable de les séparer des lipides que contiennent les extraits totaux. Cette délipidation doit retirer des quantités importantes de cholestérol libre et estérifié, triglycérides, acides gras, phospholipides et, éventuellement, diglycérides et monoglycérides. Pour la réaliser de nombreuses méthodes ont été préconisées, qui ont toutes des inconvénients: la partition entre solvants (p. ex., méthanol/eau 70:30–hexane 1:1) est fastidieuse et laisse un résidu lipidique non négligeable[1–6]; la précipitation à basse température (−15 °) durant une quinzaine d'heures dans des mélanges complexes à base de méthanol–eau 90:10[7], 70:30[8], 80:20[9] ne débarrasse qu'incomplètement les 17 cétostéroïdes des lipides les accompagnant; le recours à une purification sur colonne de florisil[10–12], d'alumine[13–15], de silicagel[16,17] ou d'Amberlite[18] ne résout pas davantage le problème et introduit, en plus, des pics artefacts dans la chromatographie gazeuse ultérieure[19]; le passage de l'extrait plasmatique sur deux colonnes successives chargées d'un

* Travail du Groupe de Recherches sur l'Athérosclérose (INSERM), Laboratoire du Prof. J. L. BEAUMONT, Hôpital Boucicaut, Paris 15e, France.

support différent (Séphadex G 25 et acide silicique) n'aboutit pas davantage au degré de pureté souhaitable du matériel à chromatographier[19].

ANGELICO et coll.[20] ont préconisé récemment une séparation des lipides tissulaires et des stéroïdes par chromatographie en couche mince. Leur mode opératoire comporte toutefois deux migrations successives: dans la première, les stéroïdes restent sur la ligne de départ avec les phospholipides et, dans la seconde, pratiquée après élution du premier chromatogramme, les 17 cétostéroïdes se séparent des phospholipides.

Dans le présent travail, nous avons étudié les conditions qui permettent de séparer en un seul temps, par chromatographie en couche mince, les 17 cétostéroïdes plasmatiques (déhydroépiandrostérone (DHEA), androstérone, étiocholanolone) de tous les constituants lipidiques après les opérations d'hydrolyse classiques.

Matériel

Appareil automatique d'étalement des couches minces Chemetron.
Plaques en verre de 20 × 20 cm.
Silica Gel H Merck pour chromatographie en couche mince.
Hexane Merck.
Éther éthylique Merck.
Acide phosphomolybdique Merck.
Méthanol Merck.
Seringue Hamilton de 50 μl.
Générateur d'air, réglé à $\pm$ 300 ml/min.
Cuve en verre de 21 × 21 × 12 cm pour contenir les chromatogrammes.
Étuve à 110°.

Méthode

Préparation des plaques. Les plaques sont dégraissées soigneusement au méthanol ou au mélange méthanol–ether éthylique 80:20[21], puis séchées.

On ajoute au Silica Gel H une quantité du mélange méthanol–eau 1:1 telle que 100 g de Silica Gel H soit imprégné de 250 ml du mélange. Les plaques dont l'épaisseur est de 0.7 mm sont laissées à l'air 20 min puis activées durant 1 h à 110°.

On dépose l'extrait à délipider à 1 cm du bord de la plaque et à une distance de 4 cm de ce premier dépôt, on dispose 20 μg de déhydroépiandrostérone et d'etiocholanolone. L'extrait à délipider est solubilisé dans 50 à 100 μl de $CHCl_3$–méthanol 9:1 et l'étalon, dans 20 μl du même mélange.

Les conditions idéales pour déposer les produits à chromatographier nous paraissent être les suivantes: la plaque est déposée sur une surface tiède et à mesure que l'on dépose les gouttes de solvant, on favorise son évaporation par un léger courant d'air, amené par une pipette Pasteur doublement effilée, reliée à un générateur d'air ou d'azote.

Chromatographie. Dans le fond de la cuve à chromatographie, on dispose la phase mobile suivante: éther–hexane–acide acétique 70:30:1, en quantité telle que la surface du liquide recouvre de 1 à 2 mm le silicagel de la plaque, ce qui correspond à un volume de $\pm$ 130 ml de solvant pour une cuve dont les dimensions sont données plus haut. La migration est complète en 35–40 min. Le R_F de la DHEA et de l'androstérone est de 0.33 et celui de l'étiocholanolone est de 0.22.

Révélation des étalons. Après séchage de cinq minutes sous la hotte, on vaporise sur la partie de la plaque contenant les étalons une solution d'acide phosphomolybdique à 10 % dans le méthanol. On peut aussi vaporiser la solution récemment préconisée par TREIHER et coll.[21] et comportant un mélange de *m*-dinitrobenzène et de bleu de tétrazolium en milieu KOH aqueux. La sensibilité de cette méthode est supérieure à celle de l'acide phosphomolybdique (0.5 μg/cm^2) mais les réactifs doivent être fraîchement préparés.

La partie du chromatogramme contenant les extraits plasmatiques est protégée par une plaque de verre.

Élution. La zone correspondant aux étalons est grattée par une lame de rasoir et la surface de verre ainsi mise à nu est rincée au moyen de petites parcelles de papier filtre imbibé d'éthanol, de façon à récupérer les stéroïdes qui restent accrochés au verre. La poudre de silicagel et les morceaux de papier filtre sont mis dans 5 ml d'éthanol ou d'acétate d'éthyle durant 10 min et centrifugés. On répète l'extraction à trois reprises. Le résidu d'évaporation ne contient plus aucune trace de lipides.

Présence d'artefacts à la chromatographie gazeuse. Si le Silica Gel H utilisé contient des impuretés qui se traduisent par des pics parasites en chromatographie gazeuse,

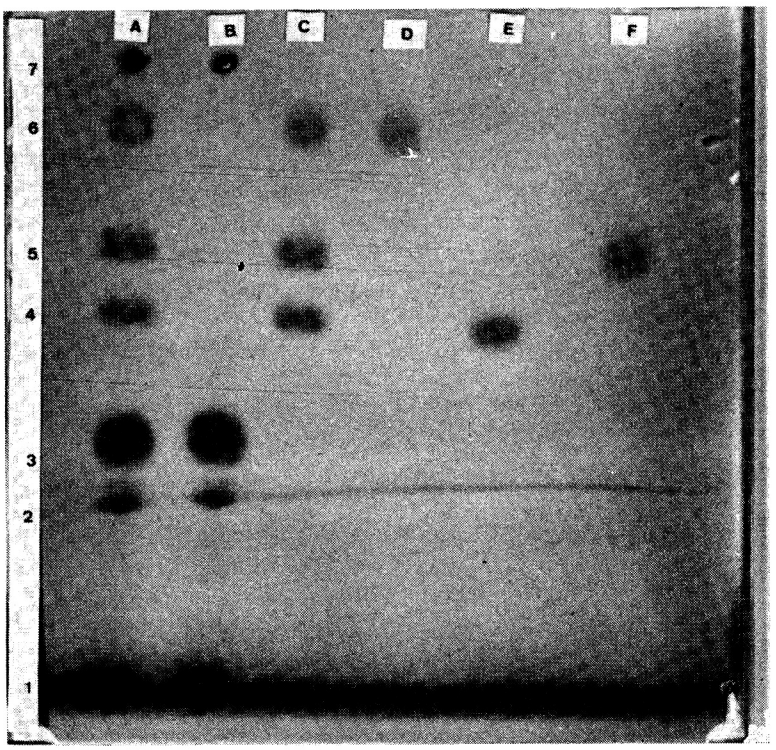


Fig. 1. Délipidation complète des extraits plasmatiques contenant les 17 cétostéroïdes. (A) Sérum normal enrichi par divers stéroïdes. (B) Sérum normal. (C) Mélange d'étalons de stéroïdes. (D) Étalon de 11-céto-étiocholanolone. (E) Etalon de déhydroépiandrostérone. (F) Etalon d'étiocholanolone. (1) Esters du cholestérol. (2) Triglycérides et, légèrement en arrière, acides gras. (3) Cholestérol libre. (4) Déhydroépiandrostérone, Δ^4-androstènedione, androstérone. (5) Étiocholanolone. (6) 11-Céto-étiocholanolone. (7) Phospholipides et, éventuellement, monoglycérides.

on pourra s'en débarrasser par un lavage préalable, selon la méthode de MEYER ET WHITE[22], ou selon celle de REDGWELL ET BIELESKI[23].

Résultats

Les résultats sont reproduits sur la Fig. 1. On y distingue les esters du cholestérol (R_F 1), les triglycérides (R_F 0.72), les acides gras libres, (R_F 0.66), le cholestérol libre (R_F 0.60), la déhydroépiandrostérone, mêlée à l'androstérone et à la Δ^4-androstènedione (R_F 0.36), l'étiocholanolone (R_F 0.30), la 11-céto-étiocholanolone (R_F 0.10) et les phospholipides (R_F 0).

Les R_F des lipides et ceux des 17 cétostéroïdes sont assez différents pour que l'élution de ceux-ci soit faite avec une grande sécurité.

Un tel chromatogramme accepte le dépôt, sur une longueur de 15 cm, d'un extrait plasmatique total qui peut contenir les lipides de plus de 20 ml de plasma.

Remerciements

Nos remerciements vont à Mlle CH. VERDET pour sa collaboration technique et à la firme Organon qui nous a procuré les stéroïdes utilisés dans le présent travail.

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A rapid thin-layer chromatographic method for the anthocyanins in grape varieties

In the course of our studies on the distribution of anthocyanins in various grape varieties, it was necessary to obtain a quick method of separation for the various pigments involved. Although paper chromatography seems to be preferred by most workers in this field, it is a cumbersome and time-consuming method. In view of the success achieved recently by BIRKOFER *et al.*[1], HESS AND MEYER[2], TANNER *et al.*[3], NYBOM[4], ASEN[5] and MORTON[6] whereby anthocyanins were separated using thin-layer chromatography (TLC), we decided to use this technique.

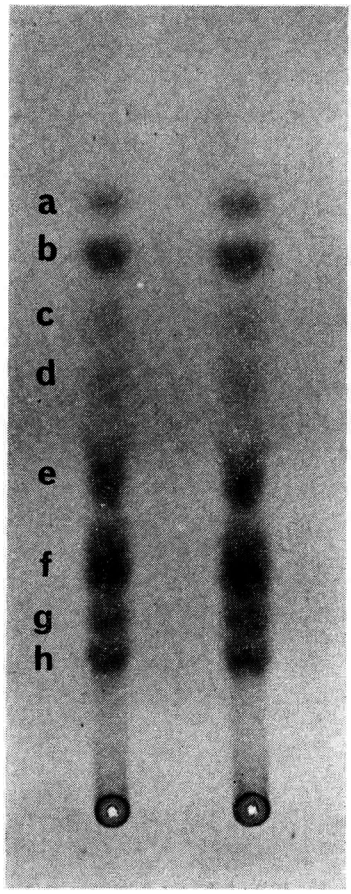


Fig. 1. Separation of anthocyanins from skins of *Vitis vinifera* cultivar Barlinka on TLC plates coated with a 0.3 mm layer of silica gel (Camag D-5). Developing solvent: 1-butanol–ethyl acetate–benzene–formic acid (1:1:1:1, v/v) saturated with solid paraformaldehyde. Concentrations: 1 μl (left) and 2 μl (right) of methanolic anthocyanin extract. The spots are identified as: (a) = Mixture of peonidine-3-monoglucoside acylated with *p*-coumaric acid (pca) and caffeic acid (ca); (b) = mixture of malvidine-3-monoglucoside acylated with pca and ca; (c) = mixture of petunidine-3-monoglucoside acylated with pca and ca); (d) = mixture of delfinidine-3-monoglucoside acylated with pca and ca; (e) peonidine-3-monoglucoside; (f) = malvidine-3-monoglucoside; (g) petunidine-3-monoglucoside; (h) = delfinidine-3-monoglucoside.

TLC was carried out on 10 × 20 cm chromatoplates with 0.3 mm thickness layers of silica gel. To prepare eight chromatoplates, 20 g of silica gel (Camag D-5 for partition chromatography) and 0.5 g of sodium acetate was mixed with 50 ml of distilled water and shaken vigorously for about 2 min. The required thickness layer was obtained with a Camag applicator. Smoothness and uniform thickness of the layer was found to be of importance for satisfactory and reproducible separations. The chromatoplates were dried in the air at room temperature for at least eight hours (preferably overnight). Prior to use the plates were heated for 15 min at 120°. Application of the sample and development followed immediately after cooling of the plates. Chromatograms were developed in a mixture of 1-butanol–ethyl acetate–benzene–formic acid (1:1:1:1, v/v) saturated with solid paraformaldehyde. Development time for a 15 cm shift of the solvent front was approximately 2 h. Fading of the colour of the separated anthocyanins occurred if the development time was more than 2 h. This was probably due to alkaline hydrolysis of the anthocyanins by the sodium acetate present in the thin layer. Since the solvent mixture separated into two phases after standing for a period of 3–4 h it was necessary to use a freshly made up solvent for individual runs. The water content of the chromatoplates was found to be the most critical factor in the successful separation of the anthocyanins in question. Any deviations in the above mentioned procedure resulted in unsatisfactory separations.

Anthocyanins were extracted with 5 % HCl in methanol. In Fig. 1 a separation of anthocyanins obtained from *Vitis vinifera* cultivar Barlinka is depicted. Eight distinct spots which can be divided into two subgroups are visible. Spots were identified by co-chromatography with known standards. The first four spots with the lower R_F-values are the 3-monoglucosides of delfinidine, petunidine, malvidine and peonidine whereas the other four spots are the same anthocyanins acylated with *p*-coumaric and caffeic acids. The success of this method led us to investigate other plant material containing anthocyanins. Various flowers and fruits were investigated and in all cases a satisfactory separation of the above anthocyanins was obtained.

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CHROM. 3383

Thin-layer chromatographic behaviour of urobilinoid pigments

Based on the observations of BIRCH[1], GRAY[2] proposed that the lactam structure was the most probable one for the bile pigments. He also suggested that the keto and enol forms of the urobilinoid molecule are in an equilibrium state under certain circumstances[3] and that their structure might vary according to their chemical environment.

According to KELLER[4], "if a substance exists in two forms which are interconverting and if the rate of conversion is slow relative to the time of development, then two spots can be obtained" by chromatography. In the experiments described here we made use of the above-mentioned fact. Urobilinoid pigments were fractionated into two main fractions by means of thin-layer chromatography thus strongly supporting GRAY's assumption.

Materials and methods

For thin-layer chromatography, chloroform solutions of crystalline *d*-urobilin, *i*-urobilin (urobilin IXα) and stercobilin were used. For comparison, stercobilin dimethyl ester, mesobiliviolin, mesobiliviolin dimethyl ester, bilirubin (Reanal) and bilirubin dimethyl ester were used.

Mesobiliviolin and mesobiliviolin dimethyl ester were prepared according to GRAY and coworkers[5]; the bulk of the product so obtained proved to be the dimethyl ester, and to obtain mesobiliviolin, a subsequent hydrolysis with 0.1 *M* potassium hydroxide in methanol at boiling point for 10 min was employed.

Stercobilin dimethyl ester, bilirubin dimethyl ester and biliverdin dimethyl ester were prepared according to FISCHER[6].

Glass plates 20 × 20 cm were coated with a 250 μ layer of silica gel (Kieselgel G, Merck), the proportion of gel to water being 1:1.5 and were activated for 1 h at 110°.

As solvent systems *n*-butanol–ethanol–water (3:1:1.5)[7], or *n*-butanol–pentanol–water (3.2:2:1.5) were used.

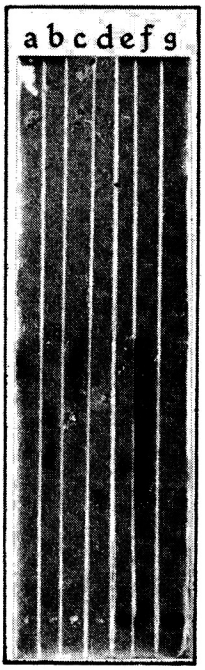
For two-dimensional chromatography the urobilinoids were applied as a spot 15 mm from the edges of the plate, and the solvent level was 10 mm from the edge of the plate. The development was performed at different temperatures (+2–+15°). The spots of urobilinoids were dissolved in chloroform containing 10% methanol and the solutions so obtained were evaporated to dryness. Just prior to measuring absorption spectra the dry residues were dissolved again in either chloroform containing 1% methanol, or 100% methanol, or 0.1 *N* HCl, or 0.1 *N* NaOH, or 0.1 *N* NaOH + 0.1 *N* NaCl.

U.V. absorption spectra were determined with a Beckman DU spectrophotometer, infra-red spectra with a Unicam SP. 200 spectrophotometer in potassium bromide discs.

Results

Chromatography was performed at 4° and when a relatively large quantity of urobilinoid had been applied the separation of the urobilinoids into two spots was clearly visible. When working at a higher temperature (15°) three to four smaller

spots instead of two were obtained on the chromatogram. The R_F values differed greatly from those of other bile pigments, which are supposed to be due to concomitants or byproducts resulting from chromatography (Fig. 1). If the development was not stopped after separation of the spots—in some instances, especially, if cooling was not sufficient—the spot which was nearer to the solvent front increased, and the spot nearer to the start line decreased and finally the two spots reunited. In two-dimensional chromatography, where the spots were separated in the second direction again, four spots were obtained on the chromatogram in all. All the urobilinoids (*i*-urobilin, *d*-urobilin and stercobilin) had the same above-mentioned chromatographic feature. The ratio by which the different urobilinoids separated in the same solvent system, however, was not the same. D-Urobilin separated first (Fig. 2).



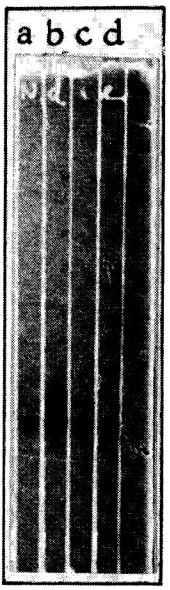


Fig. 1. Thin-layer chromatogram of: (a) mesobiliviolin dimethyl ester; (b) stercobilin; (c) stercobilin dimethyl ester; (d) mesobiliviolin; (e) bilirubin and biliverdin; (f) bilirubin dimethyl ester; (g) bilirubin. Solvent: *n*-propanol–ethanol–water (3:1:1.5).

Fig. 2. Thin-layer chromatogram of: (a) urobilinoid isolated from faeces (further identification was not made); (b) *d*-urobilin; (c) *i*-urobilin; (d) stercobilin. Solvent: *n*-propanol–ethanol–water (3:1:1.5).

The separated spots were isolated in a test tube and the urobilinoids were dissolved from the chromatographic medium in the solvents mentioned. The U.V. absorption spectra of the two spots showed no difference in either solvent. (The spectra in chloroform solution correspond to those referred to in the literature.) Infrared spectra of both spots were determined in KBr discs with a Unicam SP 200 infra-red spectrophotometer (Fig. 3).

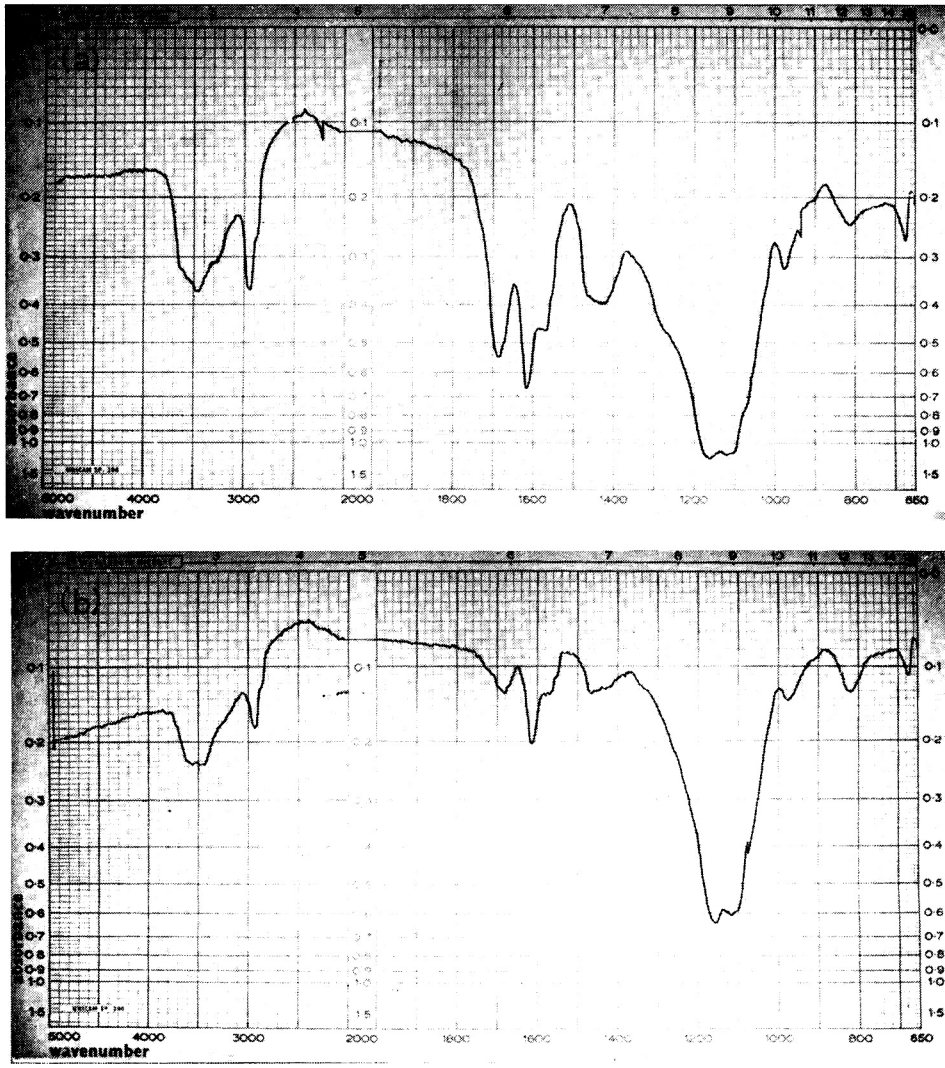


Fig. 3. Infra-red spectra of urobilinoid spots, nearer the start line (a) and nearer the solvent front (b).

Discussion

The results above show that the equilibrium of the different urobilinoid forms changes during chromatography.

According to KELLER[4] chromatography of tautomers may result in "multi-spots". Formation of two spots occurs on condition that the speed of conversion is slow in relation to the time of development. Therefore at low temperature, where transformation of the different tautomer forms is slow, conditions are favourable for the development of separate spots.

The fact that absorption spectra of the two spots failed to show any difference in the same solvent system makes it highly probable that the two spots are tautomers of the same compound, which are always in equilibrium ratio depending on the solvent

system used. Up till now no solvent has been found in which the two separate spots would not interconvert into the equilibrium state. MENDIOROZ[8] described a similar phenomenon while chromatographing bilirubin in chloroform containing ethanol.

The infra-red spectrum of the material nearer to the start line shows a shoulder at about 1270 cm^{-1} on the higher frequency side of the double band between 1200–1100 cm^{-1}, and a weak band at 3280 cm^{-1}, which are absent for the spot nearer to the solvent front. According to several authors[9–11] the amide III band occurs in secondary amides in the range 1304–1200 cm^{-1} which is weaker than either of the amide I or II bands and almost certainly due to a mixed vibration involving OCN and N-H modes.

The double band itself between 1200–1100 cm^{-1}, which is present in both spectra, is assigned to either ring vibrations or hydrogen deformation modes (*cf.* those of pyridine and derivatives[12]). Regarding the weak band at 3280 cm^{-1} the frequency, together with that of the stronger one at 3450 cm^{-1} is much too low to correspond to hydroxyl absorption (*cf.* refs. 13–15), thus both bands may be regarded as strong evidence for a keto-structure. On the other hand the absence of these bands would prove the enolic structure. We may mention that in this case a band differentiation can be observed at about 3600 cm^{-1} which can be assigned to OH stretching vibrations supporting the enolic character of the material nearer to the solvent front. Both spectra show the band at 1600 cm^{-1} which is due to CO absorption but it is greatly reduced in intensity in the latter case.

We may thus conclude that the spot nearer to the solvent front contains the enolic compound mixed to some extent with the keto form. On the other hand the spectrum of the keto form also shows a shoulder at about 3700–3600 cm^{-1}, thus the keto form itself contains a small quantity of the enolic substance.

The crystalline urobilinoids are generous gifts of Prof. C. H. GRAY and Dr. D. C. NICHOLSON which are gratefully acknowledged. The infra-red spectra were determined in the Institute of Organic Chemistry University of Szeged for which our thanks are due to Dr. K. L. LÁNG.

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CHROM. 3384

Sur la séparation du paeonoside et du malvoside au moyen de la chromatographie sur papier et sur couche mince de cellulose

La séparation du malvoside et du paeonoside au moyen de la chromatographie sur papier présente des grandes difficultés. En effet, si l'on compare les résultats obtenus avec les différents solvants de développement, utilisés pour la chromatographie unidimensionnelle descendante, on constate l'identité des R_F des deux diglucosides mentionnés, les différences étant de l'ordre de quelques centièmes, tout au plus 0.04[1–9].

Egalement la chromatographie à deux dimensions n'a pas réussi à les séparer nettement et rapidement[10–12].

Comme nous l'avons déjà signalé[13], notre technique de chromatographie unidimensionnelle multiple sur couche mince de cellulose, basée sur l'emploi des deux solvants (le premier acide acétique 2 %, le second alcohol amylique tertiaire–acide acétique glacial–eau (6:0.1:94)) reste inefficace.

Par conséquent on peut dire que la séparation de ces diglucosides reste un problème analytique non résolu. Dans ces conditions il nous a paru intéressant de rechercher les moyens d'en trouver une solution acceptable.

Méthodes expérimentales et matériel utilisé

Les constatations faites au cours des expérimentations précédentes nous ont permis de supposer que la séparation chromatographique des diglucosides du malvidol et du paeonidol nécessite en premier lieu l'utilisation de solvants peu rapides, permettant l'augmentation de la durée du développement; cette condition est valable pour la chromatographie sur couche mince et pour la chromatographie sur papier.

Chromatographie sur couche mince de cellulose

Appareillage et technique utilisés. Pour effectuer la chromatographie, unidimensionnelle à développement multiple[14], sur couche mince de cellulose nous avons fait appel à l'équipement de la Société DESAGA (Heidelberg), comprenant le dispositif pour préparation des couches de cellulose sans liant (Cellulose MN 300, Société Macherey et Nagel, Allemagne), de 0.5 mm d'épaisseur, sur plaques de 20 × 20 cm et les cuves parallélipipédiques en verre.

Anthocyannes utilisées. L'expérimentation a été faite avec un vin obtenu à la suite de la vinification au laboratoire, sans emploi d'anhydride sulfureux, des raisins rouges du cépage hybride Seyve-Villard 18315 et avec les solutions des chlorhydrates de malvoside (Sté Fluka, Suisse) et de paeonoside. Le paeonoside a été extrait des pétales de fleurs de Pivoine rouge (Paeonia) par macération pendant 44 h dans une solution aqueuse d'acide chlorhydrique à 1 %[15], suivie d'une précipitation à l'aide d'une solution aqueuse saturée d'acétate neutre de plomb selon BOCKIAN, KEPNER ET WEBB[16]. Comme nous l'avons montré[17], ce réactif détermine seulement une précipitation partielle des diglucosides. La régénération du paeonoside était obtenue en traitant des sels de plomb par du méthanol chlorhydrique à 1 %.

Chromatographie sur papier

La chromatographie descendante sur le papier Whatman No. 1 a été exécuté suivant les règles habituelles.

Résultats obtenus

L'étude comparative préliminaire de différents solvants de développement à été faite à l'aide de la chromatographie sur couche mince de cellulose en utilisant le vin rouge de Seyve-Villard 18315.

À la suite de nombreux essais d'orientation, nous avons retenu trois solvants, tous utilisant, comme substance de base, de l'alcool amylique tertiaire ($C_2H_2C(OH)$-$(CH_3)_2$, 2-diméthylbutanol-2) à savoir, l'alcool saturé d'eau ou de solution aqueuse d'acide acétique à 2 % ou de solution aqueuse d'acide chlorhydrique 2 *N*. On prépare ces solvants en mettant en contact, par agitation énergique, les volumes égaux de deux composants, on laisse reposer au moins une nuit (16 h) à 20° et on prélève la phase supérieure. La phase inférieure est utilisée pour effectuer l'humidification du papier dans l'enceinte chromatographique pendant 24 h lors de la chromatographie de partage sur papier.

Au cours de notre expérimentation l'emploi de l'alcool amylique tertiaire saturé d'eau a été restreint, à cause de son efficacité moindre. Par contre, l'utilisation de l'alcool amylique tertiaire saturé d'acide acétique ou d'acide chlorhydrique 2 *N* a permis d'obtenir une séparation nette de ces diglucosides à partir de leur mélange. Le premier développement avec de l'alcool amylique tertiaire saturé d'acide acétique à 2 % dure toute une journée, soit 7 h environ; le deuxième avec le mème alcool saturé d'acide chlorhydrique 2 *N* demande une nuit (16–17 h). La tache du paeonoside se sépare nettement de celle du malvoside et se place au-dessus à une distance suffisante permettant l'élution de chaque tache. Les essais effectués ont montré que la malvoside commercial contiendrait une petite quantité d'une anthocyanne, dont le R_F correspond à celle du paeonoside.

Les résultats obtenus rendent possible la séparation du malvoside et du paeonoside, aprés leur isolement suivant la technique préconisée par nous en 1964 (op. cit. 13). À cet effet il suffit d'extraire à l'aide du méthanol ou de l'éthanol, par exemple, ces diglucosides contenus dans une couche cellulosique correspondante et d'effectuer une nouvelle chromatographie sur couche mince de cellulose en employant les solvants indiqués précédemment.

En ce qui concerne la chromatographie descendante sur papier, les essais effectués ont montré qu'il est également possible d'obtenir, en utilisant, comme solvant de développement, de l'alcool amylique tertiaire saturé d'acide chlorhydrique 2 *N*, une séparation suffisamment nette du malvoside et du paeonoside, mais moins prononcée par rapport à la chromatographie sur couche mince de cellulose, malgré la même durée totale de développement (24 h).

Voici respectivement les R_F du malvoside et du paeonoside: 0.20–0.21 et 0.26–0.30; on remarquera la différence entre les moyennes de 0.08. Toutefois, le pouvoir séparateur de ce solvant est moindre par rapport à celui des deux solvants utilisés pour la chromatographie sur couche mince de cellulose. En effet, le malvoside commercial forme une seule tache au lieu de deux. Quant au pouvoir séparateur de l'alcool amylique tertiaire saturé d'eau, il est complètement nul dans les mêmes conditions.

Conclusion

Les recherches effectuées ont abouti à la première solution du problème de la séparation chromatographique du malvoside et du paeonoside. Toutefois, le caractère préliminaire de notre expérimentation milite en faveur d'autres recherches à entreprendre afin de rendre moins longue la technique de séparation et de dosage du malvoside et du paeonoside.

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CHROM. 3394

A sensitive method for detecting cyanoglycosides on paper and cellulose thin layers

The lack of a suitable detection method for microgram quantities of cyanoglycosides after thin-layer or paper chromatography has been a hindrance to the location of cyanoglycosides.

In the present communication, a procedure is described whereby trace amounts of cyanoglycosides can be accurately located by a modification of the colorimetric method for cyanide of GUILBAULT AND KRAMER[1], after enzymic hydrolysis of the cyanoglycosides.

Experimental

Emulsin. Hopkins and Williams, grade unspecified, 0.1 % solution in 0.02 *M* phosphate buffer, pH 7.4.

Linamarase. Prepared according to the method of COOP[2]. The 20-fold purified enzyme was diluted three times with 0.02 *M* phosphate buffer, pH 7.0.

Solution A. *p*-Nitrobenzaldehyde and *o*-dinitrobenzene solution, 0.05 *M*, in 2-methoxyethanol. This solution remained stable for at least one week in a deep-freeze.

Solution B. Isonitrosobenzoylactone, 0.02 *M*, prepared by the method of WOLF[3], dissolved in solution A. This solution was unstable and was prepared freshly each day.

Method

The TLC plate or paper was given a very light spraying with the appropriate enzyme, taking care to achieve an even and speedy coverage. The plate or paper was then covered with a thin sheet of polythene followed by a 1/4 in. sheet of foam plastic and sandwiched between rigid supporting plates.

The glycosides were allowed to hydrolyse for 30 min. The optimum combination of temperature and time of incubation was found to vary with the different enzyme preparations. In the case of amygdalin-type glycosides, hydrolysis at room temperature was found to give best results, while lotaustralin and linamarin required an incubation temperature of 30° for maximum sensitivity.

Longer incubation times than this were tried (45, 60 and 75 min) but the gains in sensitivity were outweighed by the concomitant disadvantage of diffusion of the spot. For mixtures of glycosides of close R_F value, *e.g.* lotaustralin and linamarin, the diffusion was sufficient to mask the separation.

After incubation the sandwich was unclamped and immediately given a light spraying (*ca.* 1 ml/100 cm^2) with a 10 per cent solution of sodium carbonate. This fixed the HCN and raised the pH of the medium to 8.5, the optimum pH for formation of the purple dye. A final spraying was then given with solution A. Areas corresponding with the glycosides appeared almost immediately as mauve spots reaching maximum intensity after 15 min. Thereafter fading took place, which was accelerated by strong light.

The addition of isonitrosobenzoylacetone to the reagent increased the sensitivity of detection two-fold, but the procedure of spraying was altered. Immediately upon

unclamping, the plate was sprayed with solution B, followed by an exceedingly light spraying (*ca.* 1 ml/300 cm²) of 10% sodium carbonate solution. Glycoside areas appeared immediately almost at their maximum intensity. Spots appearing by this altered procedure were more compact in size but a high background colouration which soon developed masked areas of low concentration.

Both procedures have been used to detect cyanoglycosides separated on paper chromatograms and cellulose thin layers where as little as 0.005 μmoles was detected. The cyanoglycosides could not be detected on silica gel thin layers and only weakly on plates prepared from a mixture of silica gel and cellulose. No interfering substances were observed in several alcoholic extracts of plant tissue containing cyanoglycosides.

The glycosides dhurrin and taxiphyllin gave purple spots which on standing developed brown centres. This is attributed to a separate reaction of *p*-hydroxybenzaldehyde or its derivatives which are produced in the enzymic hydrolysis and may be useful in giving a partial identification of unknown glycosides.

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CHROM. 3421

A method for the differentiation of histone fractions F2(a)1 and F2(a)2 by starch gel electrophoresis

The histones of calf thymus have been separated into four main fractions designated F1, F2(a), F2(b), and F3.[1] These fractions have all been characterized by total and N-terminal amino acid analyses and by starch gel electrophoresis at pH 2.3[2], which effectively separates the four groups. More recently fraction F2(a), the largest of the four groups has been subdivided into F2(a)1 and F2(a)2 by a variety of methods[3–5]. Starch gel electrophoresis at pH 2.3 does not however differentiate between these subfractions, and although it may be possible to separate them at higher pH values this is not desirable since it has been shown that histones, particularly F3, aggregate with increase in pH[6–8]. Also the proteolytic activity associated with the histones rapidly increases as the pH is raised[9].

A simple modification has therefore been made to the method described previously[2], which enables F2(a)1 and F2(a)2 to be separated in starch gel at about pH 2 and which also enables F2(a)1 to be specifically detected in a mixture of histones.

Experimental and results

Preparation of histone fractions

Histone fractions F1, F2(b) and F3 were prepared as described by JOHNS[1] using method 2. Histone fractions F2(a), F2(a)1 and F2(a)2 were prepared as described by JOHNS[5] using methods 1 and 2.

Starch gel electrophoresis

Electrophoresis in starch gel was carried out essentially as described previously[2] except for the following modification. The solution used for making the gel, for sample application and for the electrode vessels was 0.01 *N* HCl, made 0.01 *N* with respect to trichloroacetic acid, the final pH being about 1.8. This addition of trichloroacetic acid to the 0.01 *N* HCl used previously[2], appears to aggregate the histone fraction F2(a)1 sufficiently to prevent it entering the gel, but not to precipitate it since it is still readily soluble in the sample solvent.

The results obtained using fractions F2(a)1, F2(a)2 and F2(a) (*i.e.* F2(a)1 + F2(a)2) are shown in Fig. 1, together with the results using the unmodified system (0.01 *N* HCl only) for comparison. It can be seen that without using the trichloroacetic acid it is impossible to distinguish between F2(a)1 and F2(a)2 and that there

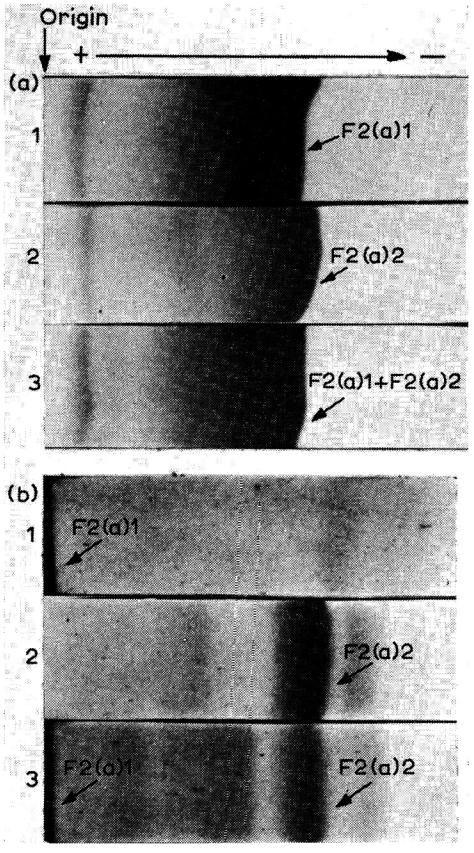


Fig. 1. (a) The electrophoresis of histone fractions in starch gel at pH 2.3 as described by JOHNS, PHILLIPS, SIMSON AND BUTLER[2]. (b) Modified method as described in the text. 1 = F2(a)1; 2 = F2(a)2; 3 = F2(a) (*i.e.* F2(a)1 + F2(a)2).

is no protein remaining at the origin. However with the modified system F2(a)1 aggregates, cannot enter the gel, and therefore remains at the origin where it stains in the normal manner. F2(a)2 migrates easily into the gel and appears to be unaffected by the trichloroacetic acid.

The other histone fractions F1, F2(b) and F3 were also tested under similar conditions and all migrated freely into the gel. Whole unfractionated histone however gave a band at the origin presumably due to its content of F2(a)1.

Discussion

The modification described above now enables the histone fractions F2(a)1 and F2(a)2 to be separated on starch gel at a low pH, and F2(a)1 to be specifically detected in whole histone or in a mixture of histones. The mechanism of the aggregation is not known, but since the other histone fractions do not aggregate under similar conditions it may be connected with the outstanding characteristics of F2(a)1 when compared with the other histone fractions. These are, a very high glycine content (approximately 15 %) and a very low proline content (1.5 %)[10]. The aggregation is unlikely to be due to its high content of arginine (13 %) since the other arginine-rich histone F3 does not aggregate under similar conditions.

It is also of interest to note that in the polyacrylamide gel electrophoresis of histone fractions described by GURLEY AND SHEPHERD[11] F2(a)1 is obviously aggregating and showing much material unable to enter the gel.

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CHROM. 3410

Dünnschichtchromatographische Untersuchungen von Organosiliciumverbindungen

In einer früheren Mitteilung wurde darauf hingewiesen, dass zwischen der Struktur und dem chromatographischen Verhalten einer Reihe von Organosiliciumverbindungen ein bestimmter Zusammenhang besteht[1]. Es wird angenommen, dass bei den Triarylmonohydridsilanen die Arylgruppen und bei den Triarylsilanolen die stärker zur Ausbildung von Brückenbindungen neigenden silanolischen Hydroxylgruppen als Adsorptionszentren wirken. Beobachtet wurde bei Monohydridsilanen, Silanolen und auch bei Siloxanen eine Parallelität zwischen den R_F-Werten der Substanzen und dem Elektronenakzeptorcharakter der in ihnen enthaltenen Arylgruppen. Dieser Charakter wird durch die Substituenten in den Arylgruppen bestimmt. Es zeigte sich, dass eine Verbindung um so stärker adsorbiert wird, je geringer der Elektronenakzeptorcharakter der im Molekül vorhandenen Arylgruppen ist. Die vorliegende Mitteilung soll zeigen, wie sich diese Vorstellungen stützen lassen und welche anderen Faktoren zu berücksichtigen sind.

Kieselgel der Firma VEB Chemiewerk Greiz-Dölau wurde wieder als Sorptionsmittel in 500 μm starker Schicht auf Glasplatten und eine Lösung von Jod in Chloroform zum Anfärben der Substanzen auf dem Chromatogramm benutzt. Mit dieser Anfärbetechnik sind die untersuchten Monohydridsilane noch in Mengen von 1 γ deutlich erkennbar. Bei den untersuchten Silanolen liegt die Nachweisgrenze bei etwa 5 γ. Als Fliessmittel dienten für die Monohydridsilane Petroläther der Siedefraktion 70–80° und für die Silanole Benzol, beide mit Natrium getrocknet.

*Monohydridsilane**

In der Gruppe der Phenylmethylmonohydridsilane $(C_6H_5)_x(CH_3)_{3-x}SiH$ nehmen die R_F-Werte mit wachsender Zahl der im Molekül enthaltenen Phenylgruppen ab. Eine weitere Abnahme des R_F-Wertes ist beim Übergang zum Tetraphenylsilan zu beobachten. Die gleiche Abhängigkeit der Lage der R_F-Werte von der Zahl der Arylgruppen im Molekül ist bei den entsprechenden substituierten Monohydridsilanen nachzuweisen. Diese Beobachtungen sprechen für die Annahme von Arylgruppen als Adsorptionszentren:

$$(C_6H_5)(CH_3)_2SiH > (C_6H_5)_2(CH_3)SiH > (C_6H_5)_3SiH > (C_6H_5)_4Si$$
$$(p\text{-}(CH_3)_3Si\cdot C_6H_4)_2(CH_3)SiH^* > (p\text{-}(CH_3)_3Si\cdot C_6H_4)_3SiH$$
$$(m\text{-}(CH_3)_3Si\cdot C_6H_4)_2(CH_3)SiH^* > (m\text{-}(CH_3)_3Si\cdot C_6H_4)_3SiH^*$$

Die chromatographische Untersuchung isomerer *p*- und *m*- substituierter Monohydridsilane zeigt, dass die jeweilige *p*-Verbindung einen grösseren R_F-Wert hat als die zugehörige *m*-Verbindung. Bisher wurde als einzige Ausnahme von dieser Regel gefunden, dass die beiden entsprechenden Tritolylsilane etwa gleiche R_F-Werte besitzen (siehe auch Tabelle I):

* Über die Darstellung der mit einem Stern versehenen Substanzen berichten G. SCHOTT UND S. KUHLA demnächst.

$(p\text{-}(CH_3)_3Si\cdot C_6H_4)_3SiH$ > $(m\text{-}(CH_3)_3Si\cdot C_6H_4)_3SiH$
$(p\text{-}Cl\cdot C_6H_4)_3SiH$ > $(m\text{-}Cl\cdot C_6H_4)_3SiH$
$(p\text{-}CF_3\cdot C_6H_4)_3SiH$ > $(m\text{-}CF_3\cdot C_6H_4)_3SiH$*
$(p\text{-}F\cdot C_6H_4)_3SiH$ > $(m\text{-}F\cdot C_6H_4)_3SiH$
$(p\text{-}(CH_3)_3Si\cdot C_6H_4)_2(CH_3)SiH$ > $(m\text{-}(CH_3)_3Si\cdot C_6H_4)_2(CH_3)SiH$
$(p\text{-}CH_3\cdot C_6H_4)_3SiH$ ~ $(m\text{-}CH_3\cdot C_6H_4)_3SiH$

TABELLE I

R_F-WERTE EINIGER MONOHYDRIDSILANE*

Sorptionsmittel: Kieselgel D (VEB Chemiewerk Greiz-Dölau). Schichtdicke: 500 μm. Behandlung der Platten: 15 min bei 120°. Fliessmittel: Petroläther der Siedefraktion 70–80°, mit Natrium getrocknet.

X–	$(X\text{-}C_6H_4)_3SiH$		$(X\text{-}C_6H_4)_2(CH_3)SiH$		$(X\text{-}C_6H_4)\cdot(CH_3)_2SiH$
	p-X–	m-X–	p-X–	m-X–	
$(CH_3)_3Si$–	0.70	0.65	0.75	0.70	
Cl–	0.63	0.55			
CF_3–	0.55	0.48			
F–	0.50	0.45			
CH_3–	0.40	0.39			
H–	0.40		0.65		0.75

* Die angegebenen R_F-Werte sind als Richtwerte zu betrachten.

Im Gegensatz zu den aufgeführten Monohydridsilanen wandern $(p\text{-}CH_3O\cdot C_6H_4)_3SiH$ und $(m\text{-}CH_3O\cdot C_6H_4)_3SiH$ in Petroläther nicht.

In der *p*- und in der *m*-substituierten Reihe der Verbindungen vom Typ $(X\cdot C_6H_4)_3SiH$ zeigen die R_F-Werte folgende Abhängigkeit von den Substituenten X an den Phenylgruppen:

$$(CH_3)_3Si\text{–} > Cl\text{–} > CF_3\text{–} > F\text{–} > CH_3\text{–} \sim H\text{–} \gg CH_3O\text{–}$$

Neben der Zahl der Arylgruppen im Molekül bestimmen also die Substituenten an den Phenylgruppen das chromatographische Verhalten der Substanzen. Dieser Substituenteneinfluss kann verschiedener Art sein: (1) Die elektronische Struktur des Adsorptionszentrums Phenylgruppe wird durch den Substituenten im Sinne der in Lit. 1 diskutierten Veränderung des Elektronenakzeptorcharakters dieser Gruppe beeinflusst. (2) Der Substituent bewirkt eine sterische Behinderung der Adsorption. Dieser Effekt ist bei sperrigen Substituenten zu erwarten. (3) Der Substituent enthält selbst ein starkes Adsorptionszentrum.

Eine sterische Abschirmung der Phenylgruppe als Adsorptionszentrum liegt ohne Zweifel bei den trimethylsilylsubstituierten Verbindungen vor. Dadurch ist die aus dem Elektronenakzeptorcharakter nicht zu erklärende Stellung der Trimethylsilylgruppe in der oben angeführten Reihe zu verstehen.

Ein zusätzliches Adsorptionszentrum ist bei den methoxysubstituierten Verbindungen in den freien Elektronenpaaren des Sauerstoffatoms anzunehmen. Eine ähnliche Rolle spielen bei den trifluormethyl-substituierten Verbindungen wahrscheinlich die Fluoratome.

Silanole

Im Gegensatz zur Adsorption der untersuchten Monohydridsilane wird dieser

Vorgang bei den Silanolen von den im Molekül vorhandenen Arylgruppen nur indirekt bestimmt. Die Ergebnisse sprechen für die in Lit. 1 diskutierte Adsorption über die silanolische Hydroxylgruppe. Sie ist im Molekül im Vergleich zu den Arylgruppen das stärkere Adsorptionszentrum. So wandert Diphenylsilandiol im Gegensatz zu Triphenylsilanol in Benzol als Fliessmittel nicht; zwei silanolische Hydroxylgruppen im Molekül verursachen also eine festere Adsorption als eine.

Ferner konnte beobachtet werden, dass die R_F-Werte gleichsubstituierter Arylsilanole mit abnehmender Zahl der Arylgruppen im Molekül abnehmen (siehe auch Tabelle II):

$(p\text{-Cl}\cdot C_6H_4)_3SiOH > (p\text{-Cl}\cdot C_6H_4)(CH_3)_2SiOH$
$(m\text{-F}\cdot C_6H_4)_3SiOH > (m\text{-F}\cdot C_6H_4)_2(CH_3)SiOH$
$(m\text{-}CH_3\cdot C_6H_4)_3SiOH > (m\text{-}CH_3\cdot C_6H_4)_2(CH_3)SiOH$

TABELLE II

R_F-WERTE EINIGER SILANOLE*

Sorptionsmittel: Kieselgel D (VEB Chemiewerk Greiz-Dölau). Schichtdicke: 500 μm. Behandlung der Platten: lufttrocken. Fliessmittel: Benzol, mit Natrium getrocknet.

X–	$(X\text{-}C_6H_4)_3SiOH$		$(m\text{-}X\text{-}C_6H_4)_2\cdot(CH_3)SiOH$	$(m\text{-}X\text{-}C_6H_4)\cdot(CH_3)_2SiOH$
	m-X–	p-X–		
Cl–	0.68	0.61		0.33
F–	0.58	0.54	0.38	
CH_3–	0.50	0.38	0.33	
H–		0.38		

* Die angegebenen R_F-Werte sind als Richtwerte zu betrachten.

In Lit. 1 wurde bereits mitgeteilt, dass bei den untersuchten isomeren Silanolen vom Typ $(X\cdot C_6H_4)_3SiOH$ die *m*-substituierte Verbindung einen grösseren R_F-Wert besitzt als die *p*-substituierte.

Aus diesen Ergebnissen kann auf den Einfluss der substituierten Phenylgruppen auf die silanolische Hydroxylgruppe als Adsorptionszentrum geschlossen werden. Hierbei ist ein Zusammenhang zwischen dem chromatographischen Verhalten und dem Säurecharakter des Silanols zu erkennen. Ein Silanol ist um so stärker sauer, je mehr Arylgruppen es im Molekül enthält und je ausgeprägter der Elektronenakzeptorcharakter der Substituenten in den Arylgruppen ist; bei den chromatographierten isomeren Silanolen ist ferner die *m*-substituierte Verbindung stärker sauer als die *p*-substituierte Verbindung[2]. Aus der relativen Lage der R_F-Werte ist zu erkennen, dass das Silanol um so schwächer adsorbiert wird, je stärker sauer es ist. Allerdings scheinen auch hier andere Effekte, besonders solche, die durch zusätzliche Adsorptionszentren bedingt sind, von Bedeutung zu sein. Diese Einflüsse werden noch näher untersucht.

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News

New apparatus

Supelco, Inc., Bellefonte, Pa. has just made available the VandenHeuvel/Valentini Semi Automatic *TLC sample applicator*. Thin-layer chromatography is widely used to concentrate compounds of interest from complex mixtures of biological origin. The efficiency in locating the exact boundary of the desired components is dependent on the sharpness of separation. Since the capacity of the thin-layer plates is small, it is necessary to use repeated spot loading of dilute solutions to insure both complete separation and the recovery of enough material for subsequent analysis. These repetitive spots should be of uniform size and in a straight line since irregular loading leads to jagged distorted bands. Attempts to apply such samples by hand usually meet with nonuniform results.

The VandenHeuvel/Valentini applicator utilizes three Auto Delivery syringes and consequently can apply up to three different samples on a thin-layer plate simultaneously. The syringes dispense a preset volume at the press of a button. The thin-layer plate is moved horizontally 0.8 mm by turning the knob at the base of the plate. The complete operation of filling the syringes, making adjustments, application of 50 samples (spaced 0.8 mm from center to center) and final rinsing of syringes, can be made in less than 5 min.

For further information apply to the publisher under reference No. Chrom. N-104.

A new series of general purpose laboratory *recorders* is announced by Tracor, Inc. The MT 21 and MT 22 series instruments will be manufactured by Tracor's Westronics subsidiary in Fort Worth, Texas, and distributed exclusively through Tracor's analytical instrument sales and service organization. All the new models include a 1-sec full-scale response over 11 in. calibrated chart, convenient dialomatic selection of five chart speeds plus provision for installation of one or two disc integrators and completely new span and zero options. The MT 21 single-pen system has a new optional plug-in model incorporating switchable span, zero suppression and reverse polarity controls. The ten discrete spans are 1, 5, 10, 20, 50, and 100 mV, 0.1, 0.5, 1, 2, 5, 10 V. Maximum zero suppression is 100 mV, on all millivolt ranges and 100 % full scale on all voltage ranges. The MT 22 dual-pen system includes options for adjustable span and zero suppression of one or both recording channels. Convenience in operation is assured by the Westronics disappearing door, adjustable writing platen and capillary inking system. In addition, the complete recording system is slide mounted and gives ready access to any component.

For further information apply to the publisher under reference No. Chrom. N-107.

A new adaptation of the Coulson conductivity principle (U.S. Pat. 3309845) that gives sensitivity to nanogram quantities of Cl, S, or N even in 10,000 to 1 excesses of other organic materials is announced by Tracor, Inc. The new model C321 Coulson *conductivity system* is now manufactured by Tracor in Austin following the recent acquisition of the Coulson Instrument Company, Palo Alto, Calif. The C321 system includes a pyrolyzer unit used in either oxidizing or reducing modes plus the unique glass electroconductivity detector cell with associated electronics. The products of the reduction or oxidation (hydrogen halides, ammonia or sulfur oxides) are mixed directly with a stream of pure conductivity water in the detector cell. The measured change of conductivity gives a direct, reliable, quantitative indication of Cl, S, or N according to the pyrolyzer system used. The short response time plus rapid recovery of the system makes it ideal for direct coupling to a gas chromatograph for specific detection of Cl, S, or N compounds. In addition to the simplicity and direct response the recovery from accidental overload which takes place in a few seconds gives real advantages compared to previous systems used for this work. The C321 conductivity system is the latest in a succession of highly specific detectors for gas chromatography established by Tracor's Micro Tek subsidiary. The C321 is particularly well suited to operation with the Micro Tek MT 220 chromatograph with which it will find wide application in the measurement of pesticides, amino acids, steroids and any other chemical compounds containing Cl, S, or N. The C321 may also be adapted to most other commercial chromatographs or operated as a self-contained analyzer.

For further information apply to the publisher under reference No. Chrom. N-108.

The new Stone H5 *subambient system* (patent pending) provides the world's first completely automatic DTA programs for heating, cooling, and recycling between any temperature limits from —160° to 300°. The new unit is operated by any LB 202 series recorder controller. The H5 incorporates Stone patented ring type thermocouples of iron constantan in a special sample holder capable of operation at all pressures from 10^{-2} torr to 250 p.s.i.g. Cooling is controlled by boiling liquid nitrogen from the built-in dewar flask with smooth transition from heat to cool programs at any selected temperature. In operation the subambient units maintain the Stone standard performance with maximum ΔT sensitivities of 0.002° per recorder chart division at any program rate from 0.5°/min to 30°/min. Vital new information can be obtained in studies of polymers, food products, biochemicals and many other materials with this automatic subambient programming.

For further information apply to the publisher under reference No. Chrom. N-109.

Announced at ALTEX and to be shown at LABEX Northern are the new *thermionic detectors* to expand the already successful range of analytical gas chromatographs made by W. G. Pye & Co. Ltd. of Cambridge. With the introduction of these detectors 14 models in the Series 104 analytical gas chromatographs are available. Four models have electron capture and four models the new thermionic detector. By changing appropriate modules, instruments most suitable for particular applications can be built easily and economically. A feature of those models having a two-detector head is that each detector can be used independently by replacing the 1:1 splitter with the two connection capillaries supplied. The exceptional performance of all Series 104 chromatographs depends on the basic design of the analyser oven and columns. Temperature stability of the large (18 cm coil diameter) vertically mounted columns (up to 500° this is $\pm$ 0.1°), on-column injection and minimum dead volume are standard features.

Produced in stainless steel the new thermionic detector has been designed for the high sensitivity selective detection of organo-phosphorous compounds and is of advanced proven design incorporating a number of important features. The geometry of the detectors gives a wide range of linear response, and the construction is such that the stability at high temperatures is exceptionally good. Ionic dissociation of a caesium bromide electrode is greatly increased when minute traces of organo-phosphorous compounds are present, and the resulting increase in current is amplified and fed to the potentiometric recorder. Hydrocarbons do not increase ionic dissociation but only cause a small change in thermal ionization; consequently the detector has only a very small response to hydrocarbons.

In order to attain the highest sensitivity, adjustments can be made to the hydrogen flow and/or CsBr electrode position relative to the flame. Detector maintenance has been simplified by careful design, spare electrode tips and special tools for cleaning.

W. G. Pye and Co. Ltd. also announce a new *liquid solvent pump*, a pulse free pump, to meet the need for a reliable, reasonably priced device where applications require constant liquid flows above 0.1 ml/min. The pump, which allows liquid to flow in one continuous stream, consists of a flexible 500 ml polyethylene reservoir enclosed in a steel pressure vessel encased in an outer container. The steel vessel is pressurised via a built-in regulator from any gas supply not exceeding 10.5 kg/cm^2 (150 lb./in.^{2}), such as a compressed air supply or a bottle of either nitrogen or argon. The liquid is forced from the reservoir and through an on/off valve by the pressure applied to the reservoir. The solvent flowrate is a function of the pressure applied to the reservoir which is set by adjusting the pressure regulator and is indicated by a pressure gauge.

The applications of the pump are many and varied and have eliminated the need for either the uncontrollable gravity feed of a liquid or the pulsing action of normal piston type pumps when pulse free constant flow delivery is required. In addition the unit is ideal for reagent addition to external systems or for solvent gradients using a mixing chamber initially filled with a different solvent from that in the pump reservoir.

To complement the highly successful liquid chromatograph detection systems introduced by W. G. Pye Ltd. of Cambridge, comes a series of accessories that include *sample saving glass columns with column connections and syringes* in addition to the recently introduced pulse free pump.

After exhaustive experiments and tests carried out by W. G. Pye on liquid chromatograph column bore sizes, it has been found that 4 mm internal diameter straight glass columns give optimum results for most applications. These columns range from 12 in. to 36 in. in 6 in. steps and, with W. G. Pye column connectors designed to withstand up to 7 kg/cm^2 (100 lb./in.^{2}), can be used with the pulse free pump. With columns that have an internal diameter of 4 mm, on-column injection can be performed, even under dynamic conditions, using the variety of syringes now made available from W. G. Pye. The extremely high-sensitivity and low noise characteristics of the detection systems allow minute samples (0.1 p.p.m. relative to solvent) to be easily detected and displayed on a recorder as a sharp chromatogram with a steady baseline. This attribute of the detection systems can be used to great advantage by analysing these small samples which are separated with greater ease, efficiency and higher resolution than is possible with larger samples. An added advantage of small samples is that small bore columns can be used for analytical work, resulting in even better performance and a saving of solvent and packing material.

For further information apply to the publisher under reference No. Chrom. N-110.

Japan Electron Optics Laboratory Co., Ltd., Tokyo, Japan, is engaged in manufacturing a wide range of *JEM-brand electron microscopes* from the 1000 kV high voltage instrument to low cost 30 kV type.

The JEM-200, a recently developed instrument with an accelerating voltage of 200 kV, exhibits penetrating power 60 % higher than that of the conventional 100 kV microscopes, so that it meets the rigorous requirements of solid state researchers and metallurgists. This apparatus is not only compact in size, easy in operation and maintenance but it can accommodate various attachments for a wide application.

The typical main features are:

(a) The electron gun employing the two-stage accelerating system eliminates high voltage discharge. (b) The coaxially-arranged detecting resistors serve to enhance the stability of high voltage. (c) By virtue of the two incorporated two-stage electromagnetic beam deflectors and the electromagnetic type stigmator, alignment and astigmatism correction can be magnetically carried out by a simple knob operation on the control panel. (d) Asymmetrical deflection of the electron beam in each lens is completely eliminated, resulting in an excellent lens axis condition and increasing the resolution.

Principal performance: resolution, 4.5 Å (lattice) or 10 Å (point) guaranteed; direct magnification, 500 ×–150,000 × continuously; accelerating voltage, 100–150–200 kV (two-stage accelerating system); stability, 1×10^{-5}/min.

For further information apply to the publisher under reference No. Chrom. N-98.

Book Reviews

The Gas Liquid Chromatography of Steroids, edited by J. K. GRANT, *Memoirs of the Society for Endocrinology*, No. 16, University Press, Cambridge, Great Britain, 1967, 15 papers are presented, 294 pages, price $ 13.50 (in U.S.A.).

All too frequently, specialized symposia are published in book form and presented to the scientific public as the latest information on a particular subject. A truly adequate review of the field is generally not achieved, because of the rather limited and frequently one-sided selection of participants. Under these circumstances, a wide variety of viewpoints cannot be presented. This can be seen here in the frequent omission of references to significant contributions by a number of investigators, as can be ascertained by reading the references cited in the individual papers.

The volume presented here, despite certain obvious shortcomings, must be rated among the better ones of its type. Several of the chapters are particularly useful both to the beginner in chromatography and to the more advanced chromatographer. For example, the excellent work of ADLERCREUTZ *et al.*, and the rather detailed report by VANDERMOLEN, are a compilation of both old and new data and also contain a number of practical laboratory hints that could be of great value to the beginner. In view of the title of the book, one might question the worth of some of the more theoretical aspects, such as capillary columns, which have not found application in the analysis of steroids. The first chapter glosses over a number of points important in steroid chromatography, and overemphasizes aspects that are not germane.

One must question seriously the value of data confined almost entirely to the separation or quantitative analysis of pure steroids, particularly if they are sufficiently unrelated so that they can be separated by other means of purification. Further, liberties are taken in broadening the application of a technique on the basis of a single experience. For example, the schematic representation for the analysis of testosterone as the heptafluorobutyrate (a relatively simple and sensitive procedure) by no means permits any equally simple analysis of estrogens in the same sample, as this reviewer has found through bitter experience.

Several authors have presented gas chromatograms of generally poor efficiency. Since merging peaks may well resolve with increasing efficiency, preliminary purification may be eliminated in some instances. Thus, the paper by BROOKS may be criticized, as may the presentations of representative chromatograms in the analysis of testosterone heptafluorobutyrates. One of the most interesting chapters, because of its possible promise, is that by THOMAS AND WALTON on the development of what may be more stable and sensitive new derivatives for electron capture work.

In the discussions, the disagreement on the use of gas chromatography in steroid analysis becomes quite apparent. Whether the gas chromatograph in the final analysis is only a black box for the very sensitive detection of a highly purified peak, or whether the high-efficiency column itself, followed by sensitive measurement, becomes one of the primary means of separation, is not yet clear. This reviewer

cannot agree with some of the thoughts offered by VANDERMOLEN on the subject. While PRIMAVESI's opposite views are perhaps oversimplified, I feel that they add some balance to the picture.

The question of gas–liquid radiochromatography is a particularly interesting one, which also appears unsettled.

It is most unfortunate that the paper by JAMES is not published since this reviewer tends to agree with him on the superiority of continuous flow counting over discontinuous counting.

It is also unfortunate that this reviewer, who admittedly has made only minor contributions to the field, was misquoted on his findings in the use of tritiated steroids. He is in full agreement with Dr. SCOTT that tritium exchange does occur on the column and that the labeling of cold material did not result from the holdup of labeled material in the column.

The final chapter by SJÖVALL is of particular interest. Those of us in research clearly realize the enormous potential of the combination of the gas chromatograph with the mass spectrometer. One would expect to see a great deal more material of this type appearing at future gas–liquid chromatography meetings.

There is little doubt that the monograph is of value and should be available to those working in the field. This reviewer is certain that future and perhaps more specialized reviews of a similar nature will keep up-to-date the researcher who wishes to apply GLC to steroid investigations. H. H. WOTIZ (BOSTON)

Chromatographic Reviews, Vol. 9, No. 1, June 1967, edited by M. LEDERER, Elsevier, Amsterdam, London, New York, 1967, 101 pp.

Beginning with volume 9 Chromatographic Reviews appears no longer as an annual volume but in two or more issues per year, in order to reduce publication delay.

The present issue contains three articles:

Peak identification in gas chromatography, by S. G. PERRY, with 177 references. The literature up to 1966 is selectively reviewed, and a critical opinion in the light of practical requirements is given. The proposed methods are subdivided into purely gas chromatographic techniques, methods involving chemical modification of the sample, collection of separated pure components for subsequent examination, and the use of auxiliary detectors.

Thin layer chromatography of amino acids, by G. PATAKI, with 240 references. The separation of the free amino acids and of five derivatives of these compounds is reviewed. Separate attention is also paid to their quantitative analysis. The last 37 references appeared during the preparation of the paper, so that an almost up-to-date coverage of the literature has been obtained.

Chromatography of carbamates, by L. FISHBEIN AND W. L. ZIELINSKY, Jr., with 217 references. The detection, separation and differentiation of pharmaceutical, pesticidal and some miscellaneous carbamates and thiolcarbamates by paper, thin-layer and gas chromatography are dealt with in the sequence of intended use and chemical structure. The literature up to summer 1966 is covered.

The result of this new publishing policy is that literature reviews by experts will reach the reader with a delay of about a year. J. PYPKER (Utrecht)

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CHROM. 3406

THE RESOLUTION OF A MIXED ALKYL ETHER AND MONOGLYCERIDE SYSTEM BY GAS–LIQUID CHROMATOGRAPHY

THE IDENTIFICATION OF MONOGLYCERIDE CONTAMINATION IN ALKYL ETHER PREPARATIONS

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SUMMARY

The resolution of a mixed system of alkyl ethers and monoglycerides, as their trimethylsilyl ether derivatives, has been studied by gas–liquid chromatography on several different types of liquid phase.

Individual monoglyceride and alkyl ether derivatives up to C18:0 were resolved on non-polar columns in under 25 minutes and best separations were achieved on JXR methyl silicone liquid phase. Semi-polar and polar columns did not separate saturated and mono-unsaturated TMS derivatives of either alkyl ethers or monoglycerides.

Contamination detected in alkyl ether samples prepared by acid hydrolysis of the glycerogalactolipid fraction of sheep brain has been identified as monoglyceride.

Recently, a detailed study on the gas–liquid chromatography (GLC) of the trimethylsilyl (TMS) and trifluoroacetate (TFA) derivatives of isomeric alkyl ethers has been reported[1] and limitations involved in the preparation and analysis of the isopropylidine, dimethoxy and acetylated forms described in earlier reports on the gas chromatography of lipids of this class[2–4] have been overcome. The TMS and TFA derivatives are formed in reactions which go to completion rapidly at room temperature and require no further purification steps prior to analysis. Also the 1- and 2-isomers of alkyl ethers can be successfully resolved as their TFA derivatives on semi-polar liquid phases[1].

Monoglycerides have also been analysed by GLC as their TMS ethers[5,6] but little work has been reported on the resolution of a mixture of these two closely related groups of lipids by gas chromatography. In connection with a problem of contamination encountered in alkyl ether preparations isolated from the glycero-galactolipid fraction of brain tissue[7], the behaviour of a mixed system of alkyl ethers, characteristic of nerve tissue, and some common monoglycerides has been studied by gas chromatography on several types of liquid phase. The results of this study and also the identification of the contamination observed in alkyl ether preparations as monoglyceride are discussed in this report.

EXPERIMENTAL

*Standards**

Chimyl alcohol (C16:0), batyl alcohol (C18:0) and selachyl alcohol (C18:1) have been identified as the main alkyl ether components of nerve (personal observations) and standards of these lipids were obtained in about 75 % purity (Calbiochem), the impurities in each case being alkyl ethers of longer and/or shorter chain lengths. In some experiments the retention times and values of the C14:0 and C15:0 alkyl ether components of the chimyl alcohol standard have been recorded. Monomyristin (Applied Science Labs.), monopalmitin, monostearin and monoolein (Hormel Institute) were all about 99 % pure. Standards were predominately in the 1-isomer form and resolution of the 1- and 2-isomers of monoglycerides and alkyl ethers has not been studied, having been described by other workers[1,5].

Silylation

Lipids were chromatographed as their trimethylsilyl ether derivatives and the silylation reagent was prepared freshly when required by mixing anhydrous pyridine (distilled over BaO and stored over KOH), hexamethyldisilazane and trimethylchlorosilane in a ratio of 10:2:1, v/v/v[8]. This mixture was well shaken and allowed to stand for 20 min when the precipitate which formed on mixing was removed by centrifugation. The clear supernatant was then used to prepare TMS ethers by the addition of 5 ml of the silylating reagent to 5 mg of each individual lipid standard weighed out in 15 ml centrifuge tubes. Tubes were stoppered, shaken well and allowed to stand at room temperature for 15 min.

TMS ethers were then extracted into hexane as described by WOOD *et al.*[5] and hexane solutions were made up to a final concentration of 2 μg lipid/μl. An aliquot of each standard was then pooled to produce a mixed system which contained all the alkyl ethers and monoglycerides studied as their TMS derivatives.

Gas chromatography

An F and M Model 402 gas chromatograph fitted with a hydrogen flame ionisation detector was used and TMS derivatives of the standards described above were resolved on the four types of liquid phase indicated in Table I. Liquid phases were prepared by the flash evaporation technique and were packed in glass columns, 6 ft. × 1/4 in. O.D. and conditioned prior to use by the usual methods. Carrier gas (helium) flow rate for all columns was 50 ml/min and the ratio of hydrogen: helium: air was 1:2:7. Attenuation and range settings were 2 and 10^2 respectively and the flash heater and detector were set about 30–40° above the operating temperature of the column in use.

In experiments designed to study the relationship between detector response and lipid concentration TMS ethers of chimyl alcohol and monopalmitin were chromatographed directly in pyridine and peaks were quantitated by the method of CARROLL[9]. Other results are the means of data collected over a period of several days.

Acid hydrolysis of diglycerides

To study the hydrolysis products formed from diglycerides treated with acid

* The shorthand nomenclature system of WOOD AND SNYDER[1] is used in this work.

TABLE I

LIQUID PHASES, SUPPORTS AND OPERATING TEMPERATURES OF COLUMNS USED TO STUDY THE RESOLUTION OF ALKYL ETHERS[a] AND MONOGLYCERIDES[a] BY GLC

Liquid phase	*Percentage*	*Support*	*Operating temperature (°C)*
Apiezon L	5	Gas-Chrom Q[b]	240
JXR[c]	5	Gas-Chrom Q[b]	225
XE-60	3	Gas-Chrom Q[b]	185
DEGS	20	Diatoport W[d]	200

[a] As their TMS ether derivatives.
[b] 100–120 mesh (Applied Science Labs.).
[c] Routinely used for alkyl ether analysis.
[d] 80–100 mesh, acid washed.

under conditions similar to those used for glycerogalactolipid degradation, 5 mg samples of synthetic diglycerides, including oleoyl-1-palmitoyl glycerol (about 90 % 1,2-isomer) and oleoyl-1-steroyl glycerol (about 60 % 1,3-isomer) were dissolved in 0.5 ml methanol and hydrolysed with 2 ml 3 *N* H_2SO_4 for 2 h at 100° in sealed tubes. Samples of monopalmitin were treated similarly.

Lipid-soluble hydrolysis products were extracted into diethyl ether which was washed and dried over anhydrous Na_2SO_4. The solvent was then removed in a stream of nitrogen and residual material, dissolved in a small volume of 5 % diethyl ether in hexane, was applied to a 5 g column of silicic acid (Bio-rad, 200–325 mesh) and free fatty acid, diglyceride and monoglyceride fractions eluted according to the method of BARRON AND HANAHAN[10]. Fatty acids were methylated with BF_3–MeOH reagent[11] and methyl esters were extracted into hexane and the extract washed and dried as above.

Fractions were analysed by thin-layer chromatography (TLC) on Silica Gel G in solvent systems of (1), chloroform–methanol (185:15, v/v) to resolve monoglycerides and (2) hexane–diethyl ether (6:4, v/v) to resolve monoglycerides, diglycerides and methyl esters. Standards of monopalmitin, diglyceride and methyl palmitate were chromatographed at the same time. The monoglyceride fraction was further analysed by TLC on plates of Silica Gel G impregnated with 5 % boric acid and developed in chloroform–methanol (98:2, v/v)[12]. Standards of 1- and 2-monopalmitin were chromatographed simultaneously. In all cases lipids were located by sulphuric acid charring.

RESULTS AND DISCUSSION

In studies on the glyceryl ether-containing lipids of nerve tissue gas chromatography has proved to be the most satisfactory technique for the characterisation and quantitation of alkyl ethers. During preliminary work on the glycerogalactolipid fraction of sheep brain[7], alkyl ether preparations isolated from this source by acid hydrolysis were found to contain a component which could not be identified as an alkyl ether. Non-polar columns of JXR methyl silicone are usually used for alkyl ether resolution (Table I). With this system the unidentified component had a retention value of 2.55 (relative to chimyl alcohol), close to but not identical with that

expected for a C19:0 alkyl ether and was also found to account for as much as 11 % of the total sample. Recent work has revealed that alkyl ether preparations from the same source which have been treated with alkali prior to silylation and gas chromatography lack the contamination observed in unsaponified samples and that the C19:0 alkyl ether component occurs in very low concentrations only.

It is unlikely that fatty acid contamination was present since alkyl ether samples are purified prior to analysis by methylation and thin-layer chromatography. Furthermore, under the conditions of gas chromatography used, fatty acid methyl esters are eluted more rapidly than alkyl ethers. Earlier work[7,13] has also revealed that the glycerogalactolipid fraction does not contain long chain fatty acids such as lignoceric acid (C24:0) or hydroxy fatty acids.

It is more probable, however, that alkyl ether preparations were contaminated with monoglyceride, formed by the action of acid on the diacyl glyceryl galactoside component of the glycerogalactolipid fraction. The glycosidic bond in such glycolipids is readily labile under the acid conditions used (3 *N* H_2SO_4, 100°, 2 h) but diglycerides, remaining after release of galactose would be more resistant to acid especially in aqueous medium[14] and quantitative conversion to fatty acids and glycerol is unlikely to be achieved.

Indeed, hydrolysis of synthetic diglycerides with acid revealed that monoglyceride is formed under these acid conditions and can be recovered in the ether-soluble phase following hydrolysis (Fig. 1A). Also of interest was the finding that, following hydrolysis, about 50 % of the original diglyceride could be recovered intact in the ether-soluble extract indicating that conversion of diglyceride to glycerol and fatty acids is only partially achieved under the acid conditions used. Diglyceride recovered following acid hydrolysis of 1,2-diglyceride was mainly the 1,3-isomer (Fig. 1A) indicating that acyl migration occurs readily under these hydrolysis conditions. TLC on borate-impregnated Silica Gel G plates (Fig. 1B) revealed that monoglyceride formed by the action of acid on diglyceride was exclusively in the 1-isomer form. Monoglyceride was also recovered in the ether-soluble phase following hydrolysis of monopalmitin.

The results indicate that monoglyceride is formed as a partial hydrolysis product by the action of acid on diglycerides under the conditions described above. It is likely, therefore, that monoglycerides are also produced during acid hydrolysis of the glycerogalactolipid fraction. Monoglyceride thus formed would be extracted into the ether-soluble extract following hydrolysis along with alkyl ethers and other lipid-soluble hydrolysis products and, having similar chromatographic properties, would be resolved with alkyl ethers during subsequent purification procedures. Alkyl ether samples would therefore contain monoglyceride contamination on silylation and gas chromatography. The presence of monoglyceride could account for the lability of the unidentified contaminant peak to alkali.

It was therefore necessary to study the chromatographic behaviour of monoglycerides under the conditions usually used for alkyl ether analysis. At the same time this work was extended to study the resolution of a mixed alkyl ether and monoglyceride system by GLC on several different types of liquid phase including Apiezon L and JXR (non-polar), XE-60 (semi-polar) and DEGS (polar) under the operating conditions shown in Table I. Retention values, relative to chimyl alcohol, of individual monoglyceride and alkyl ether TMS derivatives are reported in Table II and typical

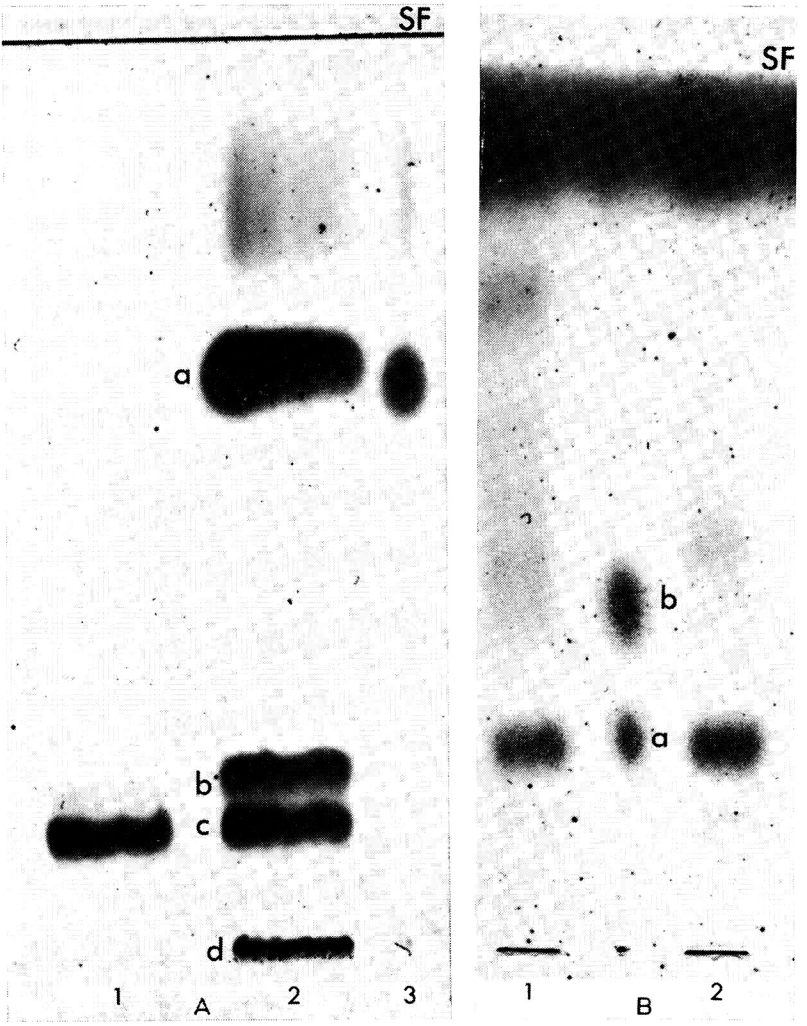


Fig. 1. (A) TLC of 1,2-diglyceride before hydrolysis (1), ether-soluble hydrolysis products (2) and methyl ester standard (3). Hydrolysis products identified as fatty acid methyl esters (a), 1,3-diglyceride (b), 1,2-diglyceride (c), and monoglyceride (d). Developing solvent: hexane–diethyl ether (6:4, v/v). SF = Solvent front. (B) TLC on borate-impregnated silicic acid of monoglyceride fractions (1 and 2) isolated following acid hydrolysis of 1,3- and 1,2-diglycerides respectively and standards of 1-monopalmitin (a) and 2-monopalmitin (b). Developing solvent: chloroform–methanol (98:2, v/v). SF = Solvent front.

separations of the mixed standard on the different liquid phases are shown in Figs. 2a, 2b, 3a and 3b.

In the present study TMS derivatives of alkyl ethers and monoglycerides were prepared with a clear silylation reagent with the precipitate of ammonium chloride, which forms on addition of trimethylchlorosilane to pyridine and hexamethyldisilazane, removed by centrifugation. CARTER AND GAVER[15] have reported that peak areas of TMS ethers of sphingolipid bases formed with the uncentrifuged silylation reagent decreased as time after addition increased while, with the clear centrifuged

TABLE II

RETENTION VALUES OF ALKYL ETHERS[a] AND MONOGLYCERIDES[a] RELATIVE TO C16:0 ALKYL ETHER[b]

Standard	*Apiezon L*	*JXR*	*XE-60*	*DEGS*
C14:0 monoglyceride	0.65	0.73	1.23	1.00
C14:0 alkyl ether	0.50	0.53	0.50	0.50
C16:0 monoglyceride	1.36	1.35	2.43	2.07
C16:0 alkyl ether	1.00	1.00	1.00	1.00
C16:1 alkyl ether	0.92	0.94	[c]	[c]
C18:0 monoglyceride	2.64	2.57	4.70	4.13
C18:0 alkyl ether	2.02	1.88	1.95	2.05
C18:1 monoglyceride	2.31	2.34	4.70	4.38
C18:1 alkyl ether	1.80	1.70	1.94	2.10

[a] As their TMS ether derivatives.

[b] C16:0 alkyl ether derivative resolved in the following average times: Apiezon L, 7.75 min; JXR, 9.5 min; XE-60, 4.25 min; DEGS, 6.5 min.

[c] C16:1 alkyl ether not resolved on XE-60 and DEGS liquid phases.

reagent, reproducible and constant height ratios were obtained up to 2.5 h after derivative formation. The clear silylation reagent was usable for about 6 days after preparation.

Although the silylation reaction for monoglycerides is very rapid[5] a 15 min reaction period was allowed in the present study, after thorough mixing, to ensure solubility of the lipids and complete formation of TMS derivatives. Direct chromato-

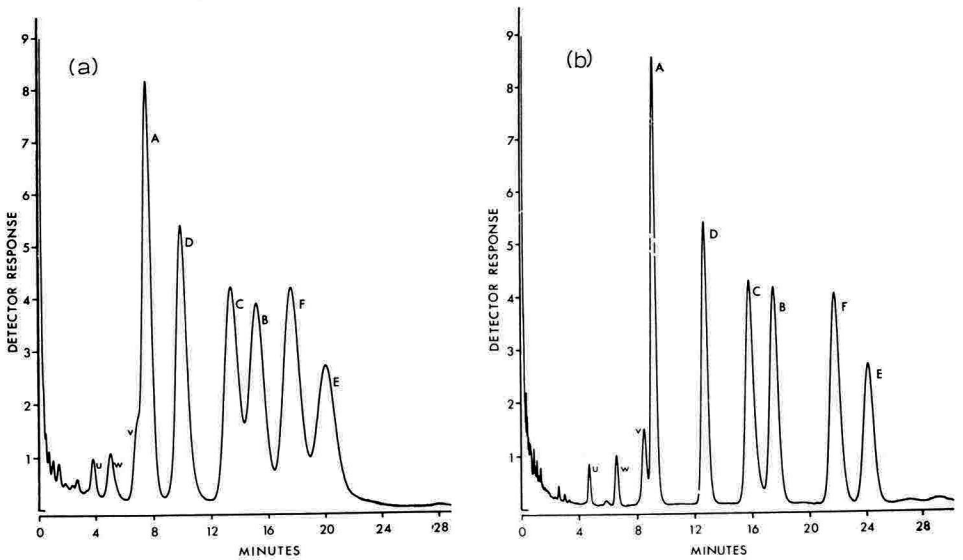


Fig. 2. Typical separations of mixed alkyl ether and monoglyceride TMS ether derivatives on non-polar liquid phases of (a) Apiezon L 240° and (b) JXR methyl silicone 225°. Peaks identified as alkyl ethers: u = C14:0; A = C16:0; v = C16:1; B = C18:0; C = C18:1 and monoglycerides: w = C14:0; D = C16:0; E = C18:0; F = C18:1.

graphy of TMS ethers in pyridine has been widely described but better results in terms of a smaller solvent peak, etc., are obtained from hexane. TMS ethers were therefore chromatographed in hexane and it has been reported[5] that such derivatives stored in hexane at low temperatures are stable for at least four months.

Two types of non-polar liquid phase were used and typical separations of the mixed standard on Apiezon L and JXR columns are shown in Figs. 2a and b. Retention values of the individual components are reported in Table II. The results indicate that non-polar columns are superior to semi-polar and polar liquid phases (Figs. 3a and b) for the separation of the mixed alkyl ethers and monoglycerides studied. Individual monoglyceride and alkyl ether derivatives up to C18:0 were completely resolved on either non-polar phase in about 25 min, peaks showing good symmetry especially on JXR columns. A better separation was achieved on the JXR methyl silicone liquid phase, routinely used for alkyl ether analysis, than on Apiezon L columns, TMS derivatives of monounsaturated alkyl ethers and monoglycerides being more completely resolved from their saturated analogues. As can be seen in Fig. 2b the detector response returned to zero during the separation of the monoolein (F) and monostearin (E) derivatives.

The complete resolution of 18:1 and 18:0 alkyl ethers achieved on JXR columns in this work is in contrast to the partial separation on SE-30 columns reported by WOOD AND SNYDER[1] and while the two types of liquid phase are little different, both being methyl silicone gums, the combination of a silanised support of finer particle size (100–120 mesh) and the slightly longer column may be responsible for the better separation. The same workers have also reported that TMS ethers of 18:2 and 18:1 alkyl ethers are not separated on SE-30 columns. WOOD *et al.*[5], have described the resolution of saturated monoglyceride TMS derivatives from C10:0 to C18:0 on a 2.6 % SE-30 column in under 30 min by temperature programming.

Peaks resolved on Apiezon columns (Fig. 2a) were broader and showed some

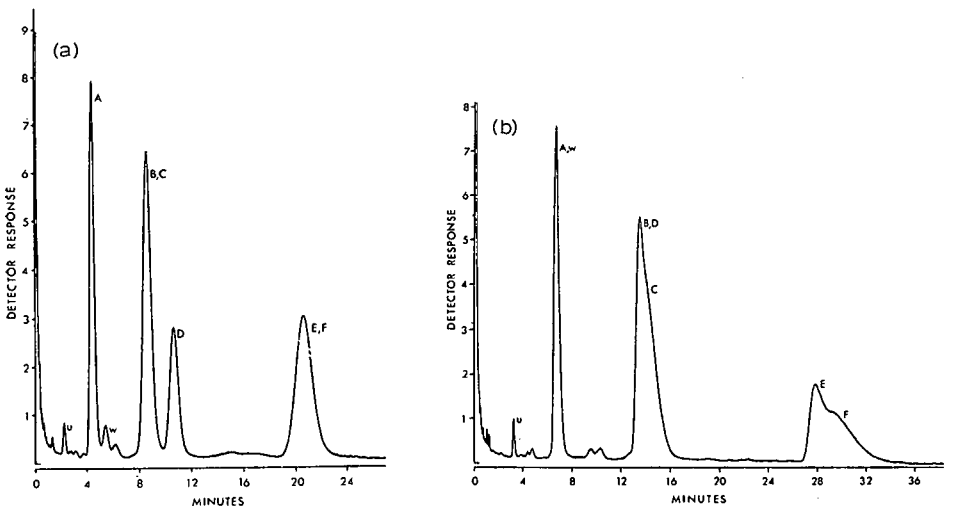


Fig. 3. Typical separations of mixed alkyl ether and monoglyceride TMS ether derivatives on (a) semi-polar XE-60 185° and (b) polar DEGS 200° liquid phases. Peaks identified as alkyl ethers: u = C14:0; A = C16:0; B = C18:0; C = C18:1 and monoglycerides: w = C14:0; D = C16:0; E = C18:0; F = C18:1.

slight tailing compared with the JXR separations (Fig. 2b). Operation of the Apiezon phase at higher temperatures only slightly sharpened the peaks and did not improve the resolution achieved between unsaturated and saturated monoglyceride and alkyl ether derivatives. In agreement with other workers[1] 18:0 and 18:1 alkyl ethers were not well resolved on Apiezon columns.

The results in Table II indicate that TMS derivatives of monoglycerides and alkyl ethers were less successfully resolved on semi-polar and polar liquid phases. The mixed standard was resolved on XE-60 columns (Fig. 3a) in about 22 min but, in agreement with other reports[1], the saturated and unsaturated derivatives of standards were not separated. Isomeric saturated alkyl ethers and monoglycerides were resolved however.

WOOD *et al.*[5], have described the resolution of the TMS ethers of C18:0, C18:1 and C18:2 monoglycerides (1-isomers) on a DEGS liquid phase at 215°. In closer agreement with earlier findings[1], however, DEGS columns used in this study generally failed to separate saturated and unsaturated derivatives of either alkyl ethers or monoglycerides (Fig. 3b) although slightly different retention values were observed for these standards when chromatographed individually (Table II). On DEGS columns the mixed standard was resolved in about 32 min but C16:0 monoglyceride and C18:0 and C18:1 alkyl ethers were eluted in one peak as were the C14:0 monoglyceride and C16:0 alkyl ether components (Fig. 3b).

A plot of $\log_{10}$ retention against the carbon number of the ether or ester linked hydrocarbon side chain for individual alkyl ether and monoglyceride derivatives (Fig. 4a, 4b) shows that for each liquid phase linear results are obtained over the molecular weight range studied. Monoglyceride and alkyl ether derivatives are widely differentiated on XE-60 and DEGS columns (Fig. 4b) while plots for the same standards on non-polar columns (Fig. 4a) lie closer together. With either Apiezon L or

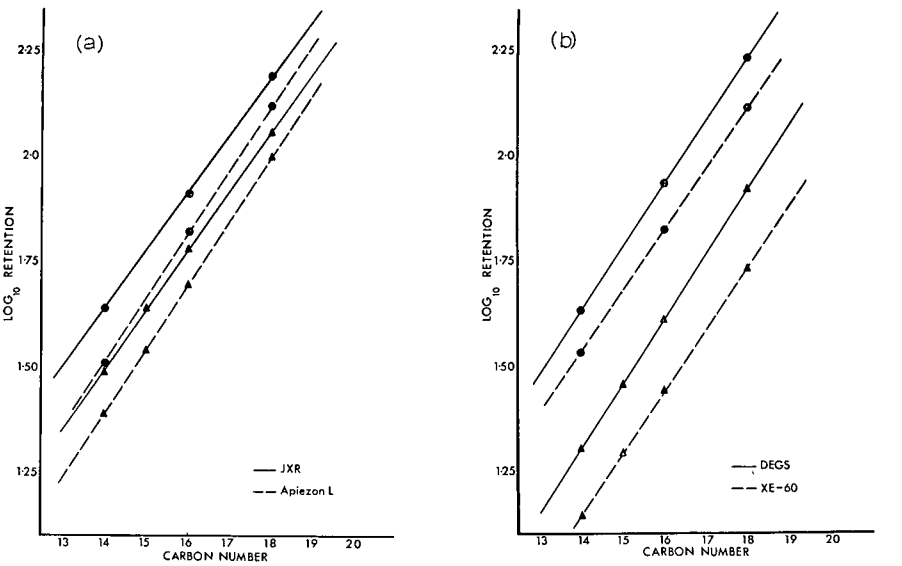


Fig. 4. Plot of $\log_{10}$ retention against carbon number for individual alkyl ethers (▲) and monoglyceride (●) TMS ether derivatives on (a) non-polar liquid phases and (b) semi-polar and polar liquid phases.

JXR liquid phases monoglycerides are resolved in approximately the same time as the respective alkyl ether containing an additional carbon unit in the side chain. Thus, on JXR columns (Figs. 2b, 4a), TMS derivatives of C16:0 monoglyceride and C17:0 alkyl ether, C18:0 monoglyceride and C19:0 alkyl ether, etc., have about the same retention.

These findings are of interest in relation to identification of the contamination detected in alkyl ether preparations described earlier. On JXR columns, the unidentified contaminant peak had a retention value of 2.55 relative to chimyl alcohol, a figure close to that expected for a C19:0 alkyl ether. However, it can be seen from Table II that this figure is in good agreement with that of 2.57 observed for C18:0 monoglyceride analysed under identical conditions while the retention value of a C19:0 alkyl ether resolved on the same system is 2.63 (Fig. 4a). These results indicate further that alkyl ether samples isolated from the glycerogalactolipid fraction of brain tissue contained monoglyceride contamination and, from the GLC studies above, the unidentified peak present in such samples has been resolved as monostearin.

Gas chromatography can be used for the quantitative assay of alkyl ether preparations and on JXR columns a linear response was obtained for the TMS ether derivatives of both monopalmitin and chimyl alcohol over a concentration range up to about 1 μg (Fig. 5). The plot tends to become non-linear at higher concentrations probably due to peak broadening. However, under the conditions of chromatography used injection of samples over 1 μg resulted in an off-scale detector response. In this experiment, as in routine alkyl ether analyses in which very low concentrations of sample are available, TMS ethers were injected in pyridine and the results achieved were similar to those described above, peak resolution and symmetry being as good as with hexane.

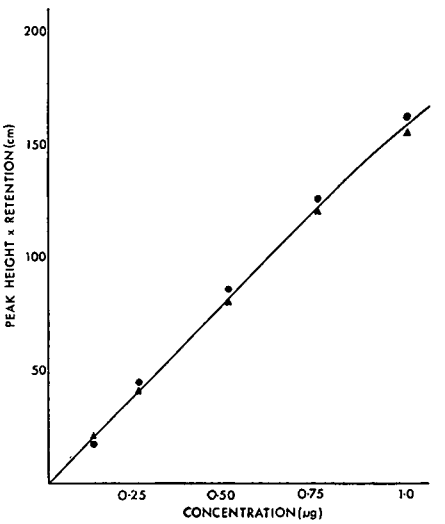


Fig. 5. Plot of concentration (μg) against peak height × retention (cm) for C16:0 alkyl ether (▲) and C16:0 monoglyceride (●) TMS ether derivatives. Lipids were chromatographed in pyridine.

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DETERMINATION OF NEUTRAL SUGARS IN GLYCOPROTEINS BY GAS–LIQUID CHROMATOGRAPHY*

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SUMMARY

A procedure is described for the quantitative hydrolysis, derivatization, and analysis by gas–liquid chromatography of the neutral monosaccharides in glycoproteins. The new liquid phase combination employed gives resolution of eleven alditol acetates and shows a low level of baseline drift during temperature programming. Application of the analysis to a number of different glycoproteins is given.

In the course of studies on the structure of oligosaccharides from glycoproteins, a method was required which would quantitate on a submicrogram scale the individual neutral sugars occurring in protein hydrolysates. The method described below has satisfied this need. It involves hydrolysis of the protein sample with Dow 50 in the hydrogen form, reduction of the sugars to the corresponding alcohols, acetylation with acetic anhydride in pyridine, and gas chromatography of the acetylated alcohols.

Much attention has been given in recent years to the gas chromatographic analysis of carbohydrates, and extensive reviews have been published[1–4]. Of the various potential derivatives, we have chosen to use the O-acetyl glycitols, proposed by GUNNER *et al.*[5,6] and extended by other workers[7–11].

The procedure is quite similar to that recently described by KIM *et al.*[9]. However, there is sufficient difference in the methodology, in the column and operating conditions, and in the time required for analysis to merit presentation of this paper.

PROCEDURE

Apparatus and chromatographic conditions

A Microtek Model 220 gas chromatograph equipped with a temperature programmer and flame ionization detectors was employed. The injector port was modified by the use of a glass insert (8.4 cm × 1 cm O.D. × 0.25 cm I.D.) to reduce the dead volume of the vaporizing chamber. The U-shaped glass column 4 mm I.D. × 1.83 m containing by weight 0.75 % HiEFF-lBP, 0.25 % EGSS-X, and 0.1 % 144–B (phenyl-

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diethanolamine) on 60/80 Gas-Chromosorb Q (Applied Science Laboratory, State College, Pennsylvania, Lot 690-42B).

The conditions for chromatography were:
Column temperature: initial 160°, final 210°.
Program rate: 1.3°/min.
Detector cell temperature: 325°.
Injector temperature: 225°.
Transfer temperature: 235°.
Hydrogen (to detector): 50 ml/min.
Air (to detector burner): 189 ml/min.
Air (to detector purge): 189 ml/min.
Nitrogen (carrier): 40 ml/min.
Chart speed: 30 in./h.

A centrifuged bio-dryer, VirTis Research Equipment, Model 10-310, was used for all concentrations at reduced pressure. The 6 × 50 mm Kimax culture tubes and 5 × 50 mm micro-polyethylene disposable centrifuge tubes were obtained from Arthur H. Thomas Company. Silicone blind hole rubber septa were used to seal hydrolysis tubes.

Reagents

Pyridine, acetic acid, acetic anhydride, methanol, hydrochloric acid, Dowex 1 X8 (HCO_3^-) 200/400 mesh, and Dowex 50 X2 (H^+) 200/400 were "Baker Analyzed" reagents. The pyridine was refluxed for 1 h with ninhydrin, distilled with protection from moisture, and stored over KOH until used. Sodium borohydride was obtained from Fischer Scientific Company. All parent carbohydrates were purchased from Calbiochem and were recrystallized until pure when tested by gas chromatography as described below. The corresponding alditols and alditol acetates were prepared as described by ABDEL-AKHER *et al.*[12] and recrystallized until pure by gas chromatography. Orosomucoid was prepared by the method of BEZKOROVAINY AND WINZLER[13]

Application to glycoproteins

Glycoprotein samples, 0.1 to 3 mg, containing about 0.1 μmole of neutral sugar, are dried in 6 × 50 mm culture tubes under reduced pressure in the bio-dryer. Samples are stored in a desiccator at this stage until they are hydrolyzed. The dried samples are dissolved in 50 μl of H_2O and 50 μl of a 40 % w/v suspension of Dowex 50 X2 (H^+) 200/400 mesh resin in 0.02 *N* HCl is added. The sample tubes are sealed with silicon rubber septa, mixed, and placed in a steam bath for 40 h.

The samples are removed from the hydrolysis bath, allowed to cool, and 50 μl of an internal standard solution containing 0.0277 to 0.2771 μmoles of arabitol, depending on the carbohydrate content, is injected through the septum into the sample. The sample is thoroughly mixed and centrifuged for 2 min at 2800 r.p.m. to sediment sample from the septum and walls of the tube.

The septa are removed, and 0.4 ml of H_2O is added to each tube with thorough mixing. The mixture is transferred with a disposable pipette to columns containing Dowex 1 X8 (HCO_3^-) resin, and allowed to elute into 12 ml siliconized conical centrifuge tubes. These columns are prepared in 6 in. disposable pipettes with a glass wool plug by the addition of 50 μl of a 20 % w/v suspension of Dowex 1 X8 (HCO_3^-)

200/400 mesh resin. A glass wool plug is employed to separate the Dowex 1 X8 (HCO_3^-) from the Dowex 50 X2 (H^+) used in hydrolysis.

The hydrolysis tubes are washed twice with 0.3 ml of water, the washings also being passed through the column. The elution of the column is continued with 1 ml of a 50 % v/v solution of methanol–water, and combined eluates are concentrated to dryness in the bio-dryer under reduced pressure.

The residues in the conical centrifuge tubes are dissolved in 100 μl of water and transferred to a 5 × 50 mm polyethylene disposable centrifuge tube using a 100 μl Lang-Levy pipette. The centrifuge tubes are washed with two 100 μl portions of water and the washings also transferred to the 6 × 50 mm tube. The samples are then concentrated to dryness in the bio-dryer under reduced pressure.

The samples are dissolved in 50 μl of water and treated for 1 h at room temperature with 50 μl of 0.22 *M* $NaBH_4$. Excess $NaBH_4$ is decomposed by the addition of 50 μl glacial acetic acid, and the samples are concentrated to dryness on the bio-dryer.

Borate is removed as the volatile trimethyl borate by the addition of three 100 μl portions of a 1:1000 v/v HCl–methanol solution with concentration to dryness in the bio-dryer under reduced pressure after each addition. The samples are usually held overnight at this stage in a desiccator to insure dryness.

The samples are acetylated for 15 min at 100° with 50 μl of pyridine and 50 μl of acetic anhydride. They are then removed, mixed, and replaced in the steam bath for an additional 15 min. The samples are then cooled and 1 to 10 μl aliquots injected directly into the gas chromatograph. Samples can be kept for at least three months at this stage without decomposition.

RESULTS

Separation of standard monosaccharides

The procedure gives well separated peaks for most of the common sugars from trioses to hexoses. This is shown in Fig. 1. Of the twelve compounds shown in this

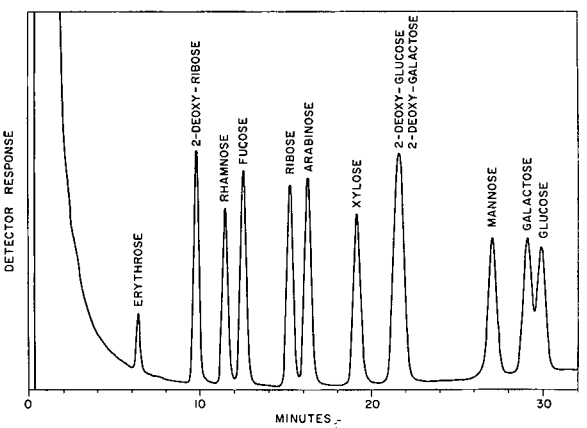


Fig. 1. Gas–liquid chromatography of the alditol acetates produced from a standard mixture of the parent carbohydrates. Liquid phase and column parameters are given in the text.

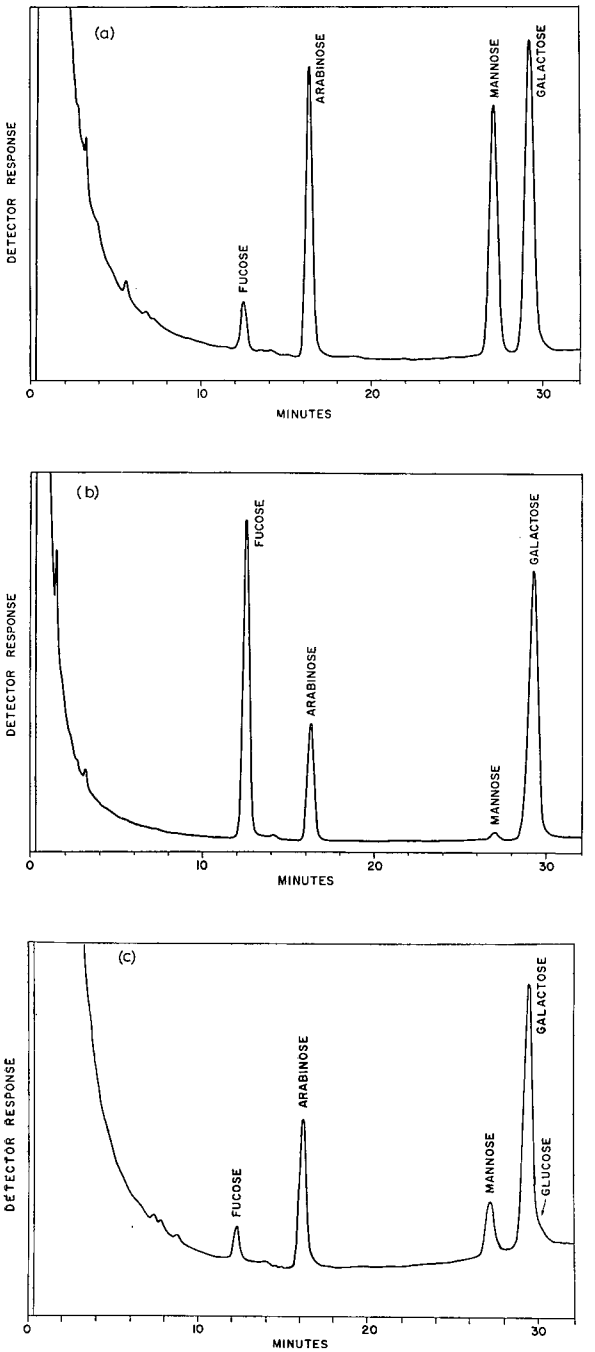


Fig. 2. Gas–liquid chromatography of the alditol acetates of the neutral sugars released after hydrolysis of various glycoproteins. Arabitol was added as the internal standard. (a) Orosomucoid; (b) canine submaxillary mucin; (c) a glycopeptide released by the action of trypsin from intact human erythrocytes. Liquid phase and column conditions are described in the text.

TABLE I

THE CARBOHYDRATE VALUES OBTAINED FROM VARIOUS GLYCOPROTEINS BY RESIN HYDROLYSIS, DERIVATIZATION, AND ANALYSIS BY GAS–LIQUID CHROMATOGRAPHY OF THE ALDITOL ACETATES

Parent carbohydrate	*Orosomucoid (μMoles/mg)*	*Canine submaxillary mucin (μMoles/mg)*	*Glycopeptide released by trypsin from human erythrocytes (μmoles/mg)*
Fucose	0.0453	0.6250	0.0546
Mannose	0.3017	0.0148	0.1108
Galactose	0.3842	0.6983	0.5830
Glucose	—	—	trace

chromatogram the only complete overlap is that of 2-deoxyglucose with 2-deoxygalactose. Separation of glucose from galactose is not complete in this system. Overlap can naturally be expected between pairs of sugars which produce identical alcohols upon reduction, *e.g.* arabinose and lyxose.

The procedure has been applied to a number of glycoproteins. Fig. 2 shows chromatograms obtained with orosomucoid, canine submaxillary mucin, and a glycopeptide released by trypsin from intact human erythrocytes[14]. Table I gives the content of galactose, mannose, and fucose for each of these proteins calculated from the relation:

$$\mu\text{moles/mg} = \frac{(\text{area of } x)\quad(\mu\text{moles of arabitol})}{(\text{area of arabitol})\ (\text{response factor})\ (\text{mg of protein})}$$

Time required for hydrolysis

Orosomucoid, an α-1 acid glycoprotein containing the neutral sugars, fucose, mannose, and galactose, was used to determine the time required for hydrolysis. It was dried in an Abderhalden drying pistol for three days at 76° under reduced pressure, and a weighed amount dissolved in distilled water. Aliquots containing 0.9450 mg were placed in 6 × 50 mm culture tubes and concentrated to dryness on the bio-dryer.

The optimal time for hydrolysis of orosomucoid was determined using two different methods of hydrolysis. One method involved treatment with Dowex 50 X2 (H^+) resin in 0.01 *N* HCl as described under Methods, Fig. 3. The other procedure involved the solution of the dried protein in 6 × 50 mm culture tube in 50 μl of water followed by the addition of 50 μl of 2 *N* H_2SO_4. The tubes were sealed with silicone rubber septa and placed in a steam bath for the appropriate time. Fifty microliters of water containing 0.2761 μmoles of arabitol was injected into each sample and passed through an ion exchange column to deionize the sample. These columns consisted of dispopipettes containing a glass wool plug, 1 ml of a 20 % w/v suspension of Dowex 1 X8 (HCO_3^-) 200/400 mesh resin in H_2O, another glass wool plug, and 50 μl of a 40 % w/v suspension of Dowex 50 X2(H^+) 200/400 mesh resin in H_2O. The hydrolyzed samples were passed through the column and eluted and washed into centrifuge tubes as previously described. The rest of the procedure was that already described.

The results of this hydrolysis (Fig. 4) show that the release of fucose and galactose preceded the release of mannose in both hydrolytic procedures. The destructive

effect of the H_2SO_4 hydrolysis on the sugars is quite apparent, and the quantitative values never rise to the maximum obtainable by Dowex 50 hydrolysis.

Hydrolysis with Dowex 50 shows maximal values reached after about 30 h, with very little destruction with prolonged hydrolysis periods.

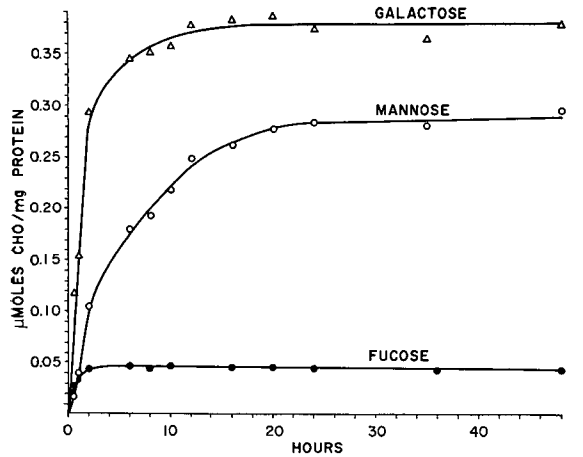


Fig. 3. Liberation of neutral monosaccharides from orosomucoid by hydrolysis with Dowex 50 X2 (H^+) resin in 0.01 *N* HCl and analysis by gas–liquid chromatography.

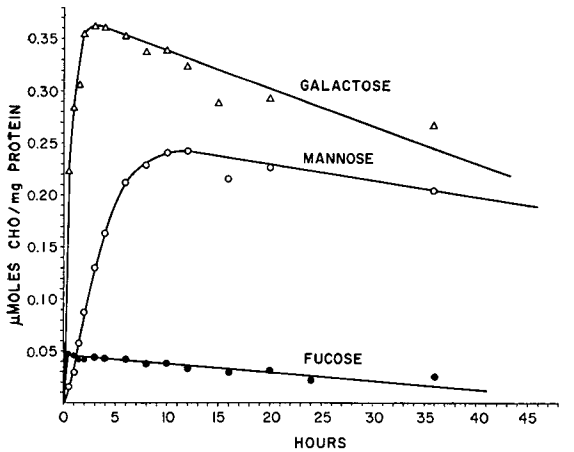


Fig. 4. Liberation of neutral monosaccharides from orosomucoid by hydrolysis with 1 *N* H_2SO_4 and analysis by gas–liquid chromatography.

Determination of response factor

The relative detector response for each monosaccharide was calculated with respect to arabitol. Samples of different carbohydrates were dried for 48 h at 76° under reduced pressure in an Abderhalden drying pistol. Each sample was weighed accurately in a range between 9.5 and 10 mg on a Cahn electrobalance and diluted to 5 ml with water. Twenty microliter aliquots of each sample were combined in a micro

polyethylene disposable tube and concentrated to dryness under reduced pressure. The samples were reduced with 0.11 M $NaBH_4$ and acetylated using the procedure already described.

The relative response factors were calculated using the following equation:

$$\text{Response factor} = \frac{(\text{area of } x)\ (\mu\text{moles arabitol})}{(\text{area of arabitol})\ (\mu\text{moles of } x)}$$

The results for several monosaccharides are shown in Table II. These response factors were used in the calculation of neutral sugars in unknown samples.

TABLE II

RELATIVE RESPONSE FACTORS AND RELATIVE RETENTIONS FOR THE ALDITOL ACETATES

The values were calculated using arabitol pentaacetate as the standard. The retention time for arabitol pentaacetate is about 16 min under the conditions described.

Parent carbohydrate	*Response factor*	*Relative retention*
2-Deoxyribose	0.841	0.593
Rhamnose	1.135	0.702
Fucose	1.089	0.765
Ribose	0.937	0.937
Arabinose	1.000	1.000
Xylose	0.965	1.178
2-Deoxyglucose	1.101	1.317
Mannose	1.182	1.665
Galactose	1.211	1.784
Glucose	1.197	1.839

Accuracy and reproducibility of method on standard sugars

Samples of L-fucose, D-mannose, and D-galactose were dried for 24 h at 76° under reduced pressure in an Abderhalden drying pistol. Three different mixed standard solutions were prepared, each containing 1.7 to 1.8 mg/10 ml of each of the above monosaccharides.

Aliquots of 300 μl were taken from each of the standards and placed in 6 × 50 mm glass culture tubes and concentrated to dryness in the bio-dryer.

Fifty microliters of H_2O and 50 μl of a 40 % (w/v) suspension of Dowex 50 X2 (H^+) resin in 0.02 N HCl was added to each tube; the tubes sealed with a silicone rubber septum. The samples were not hydrolyzed. The internal standard was added and the samples carried through the previously described procedure and the recovery of each sugar calculated for each experiment. The results indicate that the reproducibility of the method with known standards is 100 ± 3.8 %.

Reproducibility of method on a glycoprotein sample

Ten 300 μl aliquots of a solution of orosomucoid containing 3.273 mg/ml in 6 × 50 mm culture tubes were concentrated to dryness in the bio-dryer. The samples were hydrolyzed for 40 h in a steam bath using Dowex 50 X2 (H^+) resin and were carried through the standard procedure. The μmoles/mg of fucose, galactose, and

mannose were calculated for each sample. The results of these experiments indicate that the reproducibility of the method with a glycoprotein is within 3 % for each of the sugars.

Proportionality of response with amount of protein

In order to demonstrate that the neutral sugar values obtained is proportional to the glycoprotein added, 0.366, 0.733, and 1.466 mg of orosomucoid were subjected to the standard procedure. The relation of fucose, galactose, and mannose to the amount of orosomucoid added is shown in Fig. 5. These results show that the method gives linear relationship with different amounts of protein.

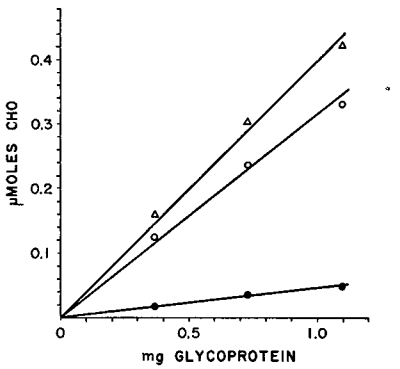


Fig. 5. Proportionality study on the glycoprotein orosomucoid showing the linear relationship between the amount of carbohydrates released and the amount of protein hydrolyzed (–●–) Fucose, (–○–) mannose and (–△–) galactose.

The method that has been described has been applied to a number of glycoproteins including albumin and aldolase which have no carbohydrate. These proteins yield no carbohydrate peaks in the gas chromatogram under the conditions that we have described.

A number of small unidentified peaks have been observed with several glycoproteins. One of these is between arabinose and xylose and another between 2-deoxyglucose and mannose. The nature of these peaks has not yet been established.

Studies similar to those of CROWELL *et al*[5] have been made varying the time, temperature, and concentration for the borohydride reduction steps. The conditions described in the procedure seem optimal for convenience and precision. Similarly, studies have been made of the conditions for acetylation in order to select the optimum for convenience and precision.

The present method is convenient, in that is employs a single column which does not bleed appreciably over the temperature range programmed. Freshly prepared columns give somewhat better resolution, but rapidly change to a steady performance. Columns have been used for more than 200 determinations without significant change in their characteristics.

The sensitivity of the method can be markedly increased by using balanced dual columns, but such sensitivity is not usually necessary.

The importance of the bio-dryer for the success of this procedure should be

emphasized, since with the small tubes used, other drying procedures are either too prolonged or lead to loss of material.

ACKNOWLEDGEMENT

The authors thank J. E. BLEECHER for his diligent efforts on the experimental work connected with this investigation.

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CHROM. 3392

CHROMATOGRAPHIC RESOLUTION OF A RACEMIC SUBSTANCE ON A MICROPOROUS RESIN

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SUMMARY

A microporous polymer of ethylvinylbenzene and divinylbenzene was chloromethylated and then aminated with (—)-α-phenethylamine to produce an optically active weak-base ion-exchange resin. Partial resolutions of mandelic acid were achieved with water and methanol as solvents but no resolution was observed with ethanol or chloroform. Some postulations are presented to explain the limited resolution that was observed and the structure of a more effective resolving resin is proposed.

INTRODUCTION

Considerable progress has been reported recently on the resolution of diastereoisomers by chromatographic methods. Racemic amino acids[1–5], alcohols[5–7], and amines[8,9] have been resolved by gas chromatography (GC) via volatile diastereoisomers on inactive substrates. Diastereoisomeric dipeptides have been separated by thin-layer chromatography[10] and esters of racemic *sec.*-butanol with active lactic or mandelic acid were resolved on Dowex-50[11]. Reports on the more difficult separation of enantiomers on active substrates have also appeared. Racemic amino acids have been resolved by GC on capillary[12] and packed[13] columns, and mandelic acid has been resolved partially on a variety of active substrates by liquid column chromatography (LC)[14–17]. Also, α-olefins have been resolved on optically active vinyl polymers[18], and coordination compounds of Ni(II) on cellulose by LC[19].

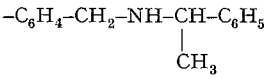
The GC separation of diastereoisomers or enantiomers is so much faster than LC, so that for the analytical determination of optical purity, GC is the method of choice. But for preparative purposes, LC of diastereoisomers or enantiomers is superior since much larger samples can be chromatographed at one time.

In LC, very long columns are usually avoided due to the enormous pressures necessary to force the liquid through the column. It was felt that a column of a microporous resin, as described by HOLLIS[20], would show a smaller pressure drop than a conventional ion-exchange resin, and hence permit LC with very long columns. Active (—)-α-phenethylamine was chosen to be incorporated into the polymer since the asymmetric carbon is adjacent to the exchanging site and the carbon is surrounded by fairly large groups to limit the number of conformations. A weak-base resin was felt to be superior to a strong-base resin prepared earlier[15] since closer approach to the

asymmetric carbon by the isomers of the racemic substance being chromatographed was shown to be possible using Fisher-Hirschfelder models.

EXPERIMENTAL

Synthesis of the optically active resin,



Chloromethylation. To a slurry of 8 g of Porapak Q*, 80–100 mesh, and 12 g of chloromethyl methylether, 12 ml of a 10 %, v/v solution of anhydrous stannic chloride in petroleum ether (b.p. 30–60°) was added and the mixture agitated well. The reaction mixture was kept at 25° for 48 h with occasional mixing. The chloromethylated resin was washed in a small column with 100 ml of each, dioxane, 45 % water 45 % dioxane 10 % 12 *M* HCl, 20 % water 70 % dioxane 10 % 12 *M* HCl, 95 % dioxane 5 % methanol, 50 % dioxane 50 % methanol, and with methanol until no further chloride appeared in the effluent. The resin contained 3.00 mmoles of chloride per gram of dry resin as determined by SCHÖNIGER's method[21].

Amination. The optically active amine, (—)-α-phenethylamine, was prepared by the procedure of THEILACKER AND WINKLER[22]. Six ml of the amine, $[\alpha]_D^{21} = -38.5°$ (neat, $d_4^{21} = 0.952$), was added to 3 g of the chloromethylated resin and the mixture kept at 2° for 48 h, then at 25° for 48 h and finally at 100° for 1 h. The recovered excess amine had $[\alpha]_D^{21} = -37.8°$ (neat). The aminated resin was slowly washed with 500 ml 1 *M* HCl, 500 ml 1 *M* NaOH, and water until no chloride appeared in the effluent. The weak-base and strong-base capacities were determined as described elsewhere[15]. The weak-base capacity was 2.26 mmoles per gram of dry, free-amine form resin. No strong-base capacity was found. The problem of extra cross linking to form quaternary nitrogens as reported previously[15] had been avoided.

Chromatography. In the experiments with chloroform, the optically active resin was slurried into the column with benzene. A glass wool plug was placed on top of the bed, and the benzene washed out with chloroform. In the other experiments, the resin was slurried into the column with the solvent used. The column was fitted with a reservoir above it, and an automatic fraction collector was used to collect 3 – 6 ml fractions. Frontal chromatography of racemic mandelic acid solutions was carried out. The solution was passed into the column until the optical activity of the fractions after the breakthrough of the mandelic acid was zero. The non-aqueous fractions were evaporated to dryness with a stream of air at 25° and the residue dissolved in 6.00 ml of water. In all cases, the mandelic acid content was determined by absorption at 257 mμ with a Beckman DB Spectrophotometer and the optical activity with a Perkin-Elmer 141 Polarimeter at the sodium D line. At this point the mandelic acid in the interstitial voids was washed out with the solvent and the exchanged mandelic acid was displaced with 0.1 *M* KOH and collected in one fraction. With the non-aqueous solvents, the fraction was evaporated, dissolved in water, acidified with 0.5 *M* HCl, and the mandelic acid content and optical activity determined. The same sample and quantity of resin in the free-amine form was used in all the experiments.

* A microporous polymer of ethylvinylbenzene and divinylbenzene. Available from Waters Associates, Inc., 61 Fountain Street, Framingham, Mass. 01701, U.S.A.

RESULTS AND DISCUSSION

The results of the frontal chromatography experiments are summarized in Table I.

TABLE I

DATA FOR EXPERIMENTS

Column height (I.D. = 2.5 mm) (mm)	*Concentration of feed solution (M)*	*Flow rate (cm/min)*	*Breakthrough volume (ml)*	*mmoles mandelic acid and optical purity in fraction at breakthrough*[e]	*Sum of measured α's, 1 dm cell*
1000	0.01[a]	0.60	150	0	0.000 0.000[f]
880	0.01[b]	2.0	138	0.0466 mmoles 5.7 % pure	−0.014 +0.011[f]
880	0.023[c]	2.2	40	0.0404 mmoles 3.9 % pure	+0.014 −0.011[f]
880	0.01[d]	1.0	5[g]	0	0.000 0.000[f]

[a] In $CHCl_3$.
[b] In H_2O containing 0.1 ml Zephiran per liter (as an antibacterial — a 17 % solution of alkyldimethylbenzylammonium chlorides, Winthrop Laboratories, 90 Park Ave., New York, N. Y., U.S.A.).
[c] In CH_3OH.
[d] In C_2H_5OH distilled from 0.01 *M* alcoholic KOH.
[e] Based on $[\alpha]_D^{20} = 150$ of mandelic acid[23].
[f] Of mandelic acid displaced from the column with 0.1 *M* KOH.
[g] Appeared after interstitial volume.

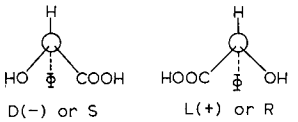
A 10 to 30 cm head was sufficient to produce the listed flow rates. Apparently the microporous structure of the beads was not affected during the synthesis. The optical activity of the fractions went to zero shortly after the breakthrough. With chloroform, water and methanol, the concentration of mandelic acid at the breakthrough went from near zero to the concentration of the feed solution in 10 ml or less. This indicates rapid reaction in the column.

No resolution was observed in chloroform perhaps due to a slow equilibration between the mandelate ions held on the resin and the mandelic acid in solution. A change in the shape of the solvated mandelic acid isomers in water and methanol and/or a change in the shape of the solvated resin group may account for the reversal in the sign of the rotation of the mandelic acid which appeared at the breakthrough.

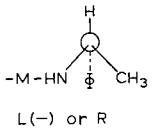
The unusual results with ethanol as the solvent are being investigated further. The experiments were performed in the order listed in Table I. Decomposition of the resin did not occur since the apparent capacity to mandelic acid in water was rechecked, and it agreed with the earlier experiments with water. A much longer column would have improved the resolutions observed. Considerable quantities of partly resolved mandelic acid could be prepared with columns of greater length and/or cross-sectional area.

If the difference in interaction between the two isomers and the active sorbent

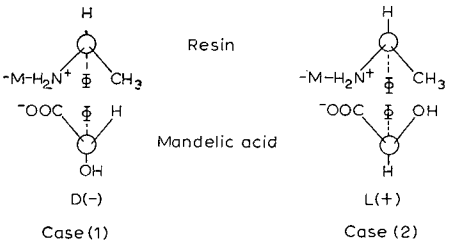
could be increased, the resolution would be improved. The absolute configurations of D(—)- and L(+)-mandelic acid are[24]:

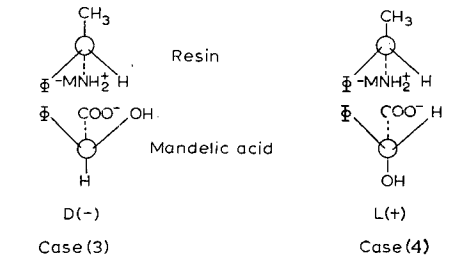


The absolute configuration about the asymmetric carbon in the resin is[25] (M refers to the resin matrix):



For resolution to occur, the resin group and the mandelic acid must contact each other at at least three points[26]. Assuming that the phenyl groups of the resin and acid are in contact, then the mandelate form of the resin can have four possible configurations:





In cases (1) and (2), any discrimination between the two mandelic acid isomers rests on a difference in the interaction between CH_3 and H, and CH_3 and OH. A large difference in interaction could hardly be expected since the difference in the van der Waals interactions is very small. In cases (3) and (4), a difference in the interaction must be due to the H and OH, and the H and H interactions. A large difference in the interactions here could also not be expected for the reason given.

If the methyl group on the active carbon of the resin were replaced with a

carboxylic acid group, a more discriminating resin may result since the difference in the interaction in cases (1) and (2) would then be due to H and COOH versus OH and COOH. Hydrogen bonding is possible in case (2). Cases (3) and (4) would be unchanged. A resin with this structure could be prepared by incorporating active α-phenylglycine into a polymer matrix. A resin of this type should be effective in resolving mandelic acid and perhaps racemic amino acids. The resin could be used as a weak-base or weak-acid ion-exchange resin by adjusting the pH of the feed solution appropriately. An investigation of this resin is being carried out.

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CHROM. 3401

DISC AND CELLULOSE ACETATE ELECTROPHORESIS OF HUMAN PLACENTAL PROTEINS

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(First received December 13th, 1967; modified January 19th, 1968)

SUMMARY

A protein-rich concentrate, PLSR, has been isolated from acetone-dried human term placenta by steps involving extraction with water, dialysis and treatment of the dialysis residue with 95 % ethanol and the composition analyzed by electrophoresis on cellulose acetate and polyacrylamide gel. The latter criterion showed the presence of two pre-albumin bands and which could be further enriched by subjecting PLSR to COHN fractionation procedures. Pre-albumins occurred in only trace amounts in concentrates isolated from late pregnancy and cord sera. As based on electrophoresis on cellulose acetate, splitting of the γ-globulin was observed with several of the fractions. Of a number of media tested, water was quite effective in the extraction of the acetone-dried powder although PLSR obtained by way of a borate buffer of pH 9.0 contained appreciable pre-albumin. Disc electrophoresis of human chorionic gonadotrophin applied as a solution containing 39,000 IU/ml revealed bands analogous to those of PLSR but in contrast to the latter, the zones were of low intensity.

INTRODUCTION

The composition of placental proteins has been studied by several investigators and the plasma proteins identified by means of fluorescent antibodies by BARDAWIL *et al.*[1] and isolated by others[2–4]. MUZHNAI[5] extracted fresh washed minced placenta with barbital buffer and on agar gel electrophoresis, noted the presence of a weak albumin, a strong β_2-globulin and a zone in the α-globulin when staining was carried out without recourse to an immune developer. However, by use of the latter, a total of 7 arcs appeared consisting of a weak pre-albumin (PA), an albumin, an α_1- and α_2-globulin and two in the γ-globulin region. In view of the availability of placenta, commercial schemes based on COHN fractionation procedures have been instituted for the isolation of plasma proteins, notably albumin and γ-globulin[6–8]. Although complete removal of blood proteins from placenta is virtually an impossibility, yet definite placental protein components have been described and tested. Thus, the placenta is reported to produce, among others, the purified protein, PPP[9,10], a shock-producing factor[11], a contractile protein related to or identical with actomyosin[12], a lactogen[13], a lipid-mobilizing entity[14] and a hormone simulating human

somatotrophin[15–17]. The latter has been extracted from a placental by-product, Fraction VII, in γ-globulin isolation[17]. A protein, TPP, is purported to occur in γ-globulin isolated by the COHN procedures from frozen placenta but only in minimal amounts in products from the fresh unfrozen tissue; it could not be detected in human blood[18].

In the present study, placenta was dried with acetone and an aqueous extract submitted to fractionation by COHN methods, emphasis being directed toward the concentration of PA and the relative distribution followed by electrophoresis on cellulose acetate and polyacrylamide gel. In this respect, DEUTSCH AND GOODLOE[19] using a modified Tiselius apparatus, noted the presence of a component which moved ahead of albumin on electrophoresis of blood plasma of a variety of mammalian and avian species and recently, GERSHBEIN AND SPENCER[20] demonstrated PA levels up to 7 % in mink serum. The occurrence of PA in cerebrospinal fluid is well known and the distribution of such components in this fluid under various pathological conditions continues to occupy the attention of many workers. Although it occurs in small quantity in human serum, PA has been isolated from this source and purified by SCHULTZE *et al.*[21] and GOT AND BOURRILLON[22]. PA plays a role in biological phenomena, especially in relation to its binding with thyroxine[23,24], in which a greater affinity is displayed than in the combination of the hormone with thyroxine-binding protein, TBP[25]. The latter has been shown to exist electrophoretically in the α_1- and α_2-globulin region[26]. TATA[27] observed the formation of a complex between PA and α_1- and α_2-globulin based on recovery experiments in which serum containing added PA was electrophoresed. He concluded that the thyroxine binding power of TBP was due mainly to the PA present in the α-globulin–PA complex. A high concentration of serum ^{131}I–trichloroacetic acid-soluble radioactivity has been shown to occur in the PA fraction[28].

EXPERIMENTAL

Cellulose acetate strips (S&S) originated from Serometrics and measured 12 × 2.5 cm with a thickness of 150–200 μ and porosity of 1–3 μ. For paper electrophoresis, an Owen buffer of pH 8.6 was employed (composition: 5.0 g sodium barbital, 1.9 g anhydrous sodium acetate, 0.38 g calcium lactate and 34.2 ml of 0.1 *N* HCl per liter[29]). The concentration of the latter was altered occassionally so as to enable the voltage across the system to be maintained at 160 V. The dye solution for staining the strips contained 1.0 g Buffalo Black 10B, 20 ml glacial acetic acid and 180 ml methanol. The latter dye in addition to Amido Schwarz and bromphenol blue applied to the polyacrylamide gels was obtained from the National Aniline Corporation. For disc electrophoresis, a kit was purchased from Canal Industrial Corporation and in later experiments, a Canalco Model 6 apparatus was used. The combination of gels produced a 7.5 % polyacrylamide gel of pH 9.4; a Tris buffer of 8.4 was employed in the disc electrophoresis. Human chorionic gonadotrophin (HCG) isolated from pregnancy urine and containing 1300 IU/mg was secured from Glogau and Company, Chicago.

Placental extracts

Drying of placenta. Human term placenta, refrigerated at 4°, was processed up to 24 h following delivery. The tissues were trimmed of cord and membranes, washed free of blood clots with portions of water (total: 1 l/placenta), cut into pieces and

blended with acetone in a Waring blender. The blend was transferred to an 18 l wide mouth jar and covered with excess solvent; 12 placentas were employed per bottle. The mixture was allowed to stand at 25° up to 7 days with frequent daily stirring, filtered under suction, washed with copious amounts of distilled acetone and the cake air-dried. Connective tissue fragments were removed and the product stored at 4°, a temperature used throughout in conjunction with aqueous solutions, unless otherwise stated. The weight of the placental parenchyme averaged 300 g and the dry powder amounted to 71 g or about 24 % based on the wet tissue.

Fractionation of acetone-dried placental powder. The dried powder was blended with water (10 ml/g), centrifuged for 10 min at 3000 r.p.m. and the supernatant portion passed through four thicknesses of cheesecloth and recentrifuged. The volume of the filtrate represented 60–70 % of the water employed, the difference being involved in the wetting of the powder. The fluid was shell-frozen and lyophilized, the dry solid being stored at 4°. In several runs where large volumes were handled, final filtration was affected in a Sharples centrifuge and the fluid preserved with merthiolate (25–50 mg/l) prior to freezing and concentration. The resulting product was blended with 95 % ethanol and the supernatant fluid decanted. Extraction with fresh portions was continued until the washings were colorless and generally, 50 ml alcohol was required per g powder over a period of 48 h. The air-dried solid was taken up in water (10 ml/g) and the mixture allowed to steep for 1 h at 4° after which time it was centrifuged in the cold for 20 min at 15,000 r.p.m. The supernatant liquid was dialyzed in Visking cellophane casing (size 36–100) against water for 48 h with frequent changes at 4°. The freeze-dried dialysis residue is designated PLSR as depicted in the flow diagram, Fig. 1.

PLSR types were also isolated by extraction of 100 g portions of one batch of acetone-dried powder with Sorensen phosphate buffer of pH 7.1, acetate buffer

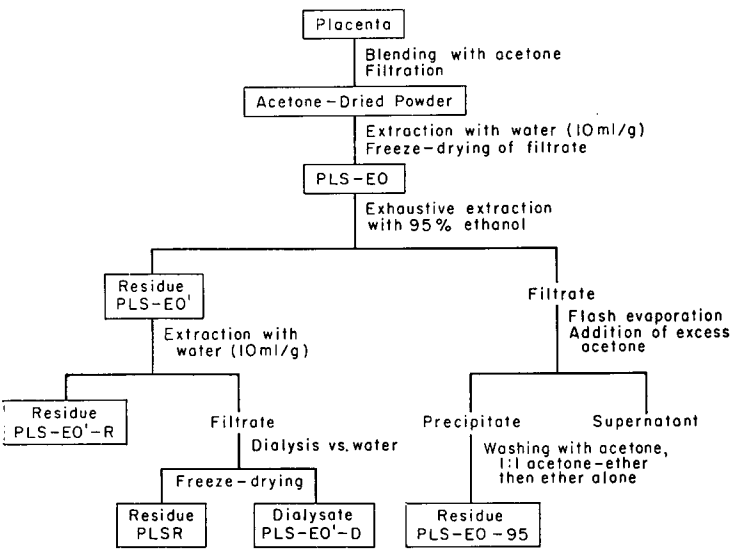


Fig. 1. Flow diagram depicting steps in the isolation of PLSR and allied concentrates from human placenta.

TABLE I

PLACENTAL CONCENTRATES FROM EXTRACTION OF THE ACETONE-DRIED TISSUE

Batch number[a]	*Acetone-dried tissue powder*	*Volume of filtrate*	*PLS-EO*[b]		*PLS-EO'*[b]	*Solid removed by alcohol*	*PLSR recovery*		
								Based on PLS-EO	*Based on dry powder*
	(*kg*)	(*l*)	(*g*)	(%)	(*g*)	(%)	(*g*)	(%)	(%)
1	2.3	15	108	4.7	76	30.0	14.0	13.0	0.61
2	12.0	72	560	4.7	433	22.0	106.0	18.9	0.88
3	8.0	58	314	3.9	231	31.7	60.0	19.1	0.75
4	10.0	70	498	5.0	436	30.5	70.6	14.2	0.71
5 (Saline)	0.100		5.12	5.1			0.33	6.4	
5 (Phosphate buffer; pH 7.1)	0.100		6.92	6.9			0.68	9.8	
5 (Acetate buffer; pH 3.5)	0.100		3.50	3.5			0.04	1.1	
5 (Borate buffer; pH 9.0)	0.100		3.93	3.9			0.68	9.8	

[a] Unless otherwise stated, water was employed in the extraction of the acetone-dried powder.
[b] For description, see Fig. 1.

(Walpole; pH 3.5), borate buffer (1.3 g boric acid and 8.4 ml 1 *N* NaOH per l; pH 9.0) and 0.90 % sodium chloride. The recoveries of fractions from 5 batches of acetone-dried powder, including the smaller runs with special buffer systems are presented in Table I. The subcutaneous injection of a saline solution containing 25 mg PLSR into the virgin rabbit did not affect the tubes or ovaries over a period of 48 h. The test for chorionic gonadotrophin was positive in both the rabbit and amphibian, *Xenopus laevis*, when the dosage was increased to 150 mg. The protein content of PLSR based on nitrogen (biuret) was 70.9, 76.5 and 78.1 % for batches 1–3, inclusive and ranged lower with the products extracted from the acetone-dried powder with buffers. Thus, the values were 44.9 and 58.5 % for PLSR isolated by means of the acetate and borate buffers, respectively.

Resolution of PLSR by COHN *procedures.* 'Method 6' of COHN *et al.*[30], originally designed for plasma protein fractionation by variations in alcohol concentration, ionic strength and pH at low temperatures was applied to 6 % solutions of PLSR. Adjustment was attempted to cope with the fact that the solution had a pH of 6.5–6.7, in contrast to plasma with a pH of 7.4–7.5. However, as too large a volume of buffer was required to raise the pH to the plasma value, this step was abandoned. A second COHN method[31] was also employed for the fractionation of PLSR. The resulting fractions were dissolved in water and freeze-dried. The final filtrate was flash-evaporated to remove alcohol, lyophilized, the solid redissolved in water, dialyzed against water (12 v/v solution) for 48 h and finally relyophilized (PLSR VI). The conditions and weight percentage yields of the various fractions derived from PLSR are shown in Table II. The weights of PLSR of batches 1–3 inclusive submitted to resolution by 'Method 6' were 16.0, 50.0 and 18.0 g, respectively, and of batch 4 employed in the second COHN method, amounted to 3.0 g. Unless otherwise indicated, the Roman numeral follows the conventional COHN fraction designation.

Human blood extracts

Cord serum. A pool of cord serum (49 ml) collected from 12 cases was treated with excess acetone and the resulting powder (2.87 g) blended with 50 ml water. The filtrate was freeze-dried and the solid (680 mg; recovery: 24 %) extracted with ethanol in an amount of 70 ml. The residue was taken up in 40 ml water and dialyzed against 800 ml water for 48 h. Processing of the residue yielded the PLSR-type concentrate in amounts of 378 mg or a recovery of 56 %.

Prenatal serum. Serum (70 ml) pooled from 10 women in the last trimester of pregnancy yielded 5.14 g powder on drying with acetone. The lyophilized aqueous extract (1.56 g; 30 % recovery) gave rise to 745 mg or a 48 % yield of the PLSR product.

Cellulose acetate electrophoresis

The cellulose acetate was soaked in Owen buffer, blotted to remove excess fluid and the 6–8 % test solution applied in amounts of 5–10 μl. The runs were carried out in a Shandon electrophoresis unit at 1.0–1.2 mA/strip and at 140–160 V; duration: 80–90 min. The strips were then dried at 90° for 15 min and stained with Buffalo Black 10B solution for 10 min, the excess dye being rinsed with methanol. The strips were dipped in 5 % acetic acid prior to drying between filter paper, cleared with mineral oil and scanned in a Varicord microdensitometer.

TABLE II

CONDITIONS AND RECOVERIES OF PLACENTAL FRACTIONS BY THE COHN PROCEDURES

Placental batch	COHN *fraction*	*pH at which fraction was removed*	*T* (°C)	*Alcohol concentration* (%)	*Recovery* (*g* %)
	'Method 6'[30]				
1	I	6.9	−2.5	8	2.0
	II + III	5.8	−5	25	6.7
	IV-1	5.1	−5	18	44.3
	IV-4	5.8	−5	40	2.4
	V	4.8	−6	40	8.4
	VI				22.0
2	I	6.6	−2.5	8	0.7
	II + III	6.5	−5	25	0.9
	IV-1	5.4	−5	18	42.6
	IV-4	6.0	−5	40	8.5
	V	4.8	−6	40	13.3
	VI				22.8
3	I	6.2	−2	8	1.3
	V	4.8	−6	40	3.7
	VI				15.3
	Later COHN *procedure*[31]				
4	I + III-3	—	0	—	2.7
	II	5.6	0	14	25.1
	III-0	6.4	0	15	17.0
	III-1,2	6.8	0	15	4.5
	IV-1	—	0	—	1.9
	IV-6 + 7	6.3	0	15	1.3
	V	5.5	0	15	1.0
	VI	5.8	0	18	24.8

Electrophoretic analyses for PLSR and subfractions isolated by 'Method 6' of the COHN group appear in Table III and tracings for PLSR as such and the derived fraction VI of batch 1 are shown in Fig. 2. The splitting of β-globulin did not occur consistently and accordingly, was calculated as one band. The splitting of γ-globulin as illustrated in Fig. 2 was noted in PLSR VI of batches 1–3 inclusive as well as in some of the other fractions and where splitting was absent, the values are expressed as total γ-globulin. PA was very distinct in PLSR VI of each batch in which it occurred at a high level. However, in most fractions, PA diffused into the albumin portion so that a line of demarcation was required. The unresolved protein, U.P., signifies an amount which did not migrate but remained at the site of application.

Disc electrophoresis[32]

With the pH of the buffer at 8.4 and at a constant current of 5 mA per sample gel, the corresponding voltage was 180–200. A solution of bromphenol blue was added to the buffer as tracking dye and in amount sufficient to give a slight tinge of blue in a 7.5 cm path. The sample gel was prepared by mixing 5 μl of the 6–8 % test solution into 0.25 ml of dilute upper gel and applying 0.15 ml. Electrophoresis was carried out for 30–40 min at which time the migration front was about 3.5 cm into the lower

TABLE III

WEIGHT PERCENTAGE DISTRIBUTION OF PLACENTAL COMPONENTS AS BASED ON PAPER ELECTROPHORESIS[a]

Batch	*Fraction*	*PA*	*Albumin*	*α-Globulin*	*β-Globulin*	*U.P.*[b]	*γ_1-Globulin*	*γ_2-Globulin*	*Total γ-globulin*
1	PLSR[c]	2.5	62.3	5.5	9.4	14.2			6.0
	PLSR I	1.8	20.6	12.1	14.9	44.6			6.0
	PLSR II + III	1.0	14.7	10.3	30.7	34.3			9.0
	PLSR IV-1	2.3	61.8	8.1	7.3	15.9			4.6
	PLSR IV-4	1.2	23.2	27.4	23.6	16.6			7.9
	PLSR V	1.0	71.1	6.5	7.0	8.5			5.9
	PLSR VI	10.2	28.1	6.7	24.3		16.1	14.6	30.7
2	PLSR	1.7	47.4	10.9	10.9	15.1	5.9	8.1	14.0
	PLSR I	1.5	48.6	20.6	22.3				7.0
	PLSR II + III	1.7	26.4	18.0	29.9	17.5			6.5
	PLSR IV-1	1.2	57.5	20.6	12.8		5.3	2.5	7.8
	PLSR IV-4	1.8	75.5	12.6	7.2	0.7			2.2
	PLSR V	1.8	86.0	8.1	2.0	1.5			0.6
	PLSR VI	11.1	3.9	14.5	19.8		33.8	16.9	50.7
3	PLSR	0.7	67.0	14.7	8.4	5.9			3.3
	PLSR I	2.2	57.3	17.0	16.3		5.3	1.9	7.2
	PLSR V	3.4	65.5	14.7	11.5				4.9
	PLSR VI	13.4	3.6	19.6	20.5		29.0	13.9	42.9

[a] Fractionation by 'Method 6' of COHN *et al.* [30].
[b] Unresolved protein or the portion remaining at the site of application.
[c] Tracing appears in Fig. 2.

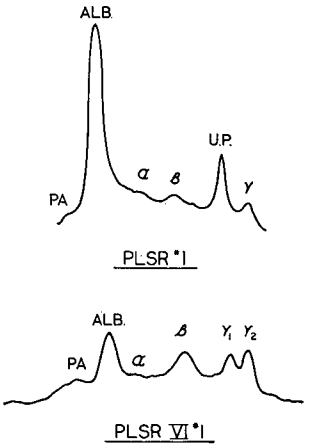


Fig. 2. Tracings of PLSR of placental batch 1 and fraction VI, the processed supernatant portion from the COHN procedure, obtained by cellulose acetate electrophoresis. U.P. denotes the unresolved protein at the site of application.

TABLE IV

WEIGHT PERCENTAGE DISTRIBUTION OF PLACENTAL FRACTIONS DETERMINED BY DISC ELECTROPHORESIS[a]

Batch	*Fraction*	*PA_1*	*PA_2*	*Albumin*	*Post albumin*	*β-Globulin*	*γ-Globulin region*
1	PLSR	3.4	4.1	58.0	14.2	13.6	6.7
	PLSR I	14.2	9.2	54.8	5.2	10.0	6.6
	PLSR II + III	4.3	2.8	36.2	22.7	20.0	14.0
	PLSR IV-1	12.4	10.0	50.8	14.8	8.0	4.0
	PLSR IV-4	4.0	1.6	46.4	11.9	21.2	14.9
	PLSR V	4.1	3.4	59.0	9.1	11.8	12.6
	PLSR VI	17.0	10.0	26.4	19.0	12.0	15.0
2[b]	PLSR	8.1	11.8	55.6	7.1	10.4	7.0
	PLSR I	14.3	7.8	49.2	7.0	8.7	13.0
	PLSR II + III	18.3	9.4	44.8	4.9	10.9	11.7
	PLSR IV-1	15.3	19.7	41.2	5.2	11.2	7.4
	PLSR IV-4	5.2	9.9	51.0	6.5	13.6	13.8
	PLSR V	4.7	6.1	62.9	8.0	13.9	4.4
	PLSR VI	11.3	32.5	2.2	5.3	31.2	17.5
3	PLSR	8.6	16.5	41.8	9.5	10.6	13.0
	PLSR I	14.7	8.4	60.0	3.9	7.9	5.1
	PLSR V	3.7	7.8	64.2	4.6	9.3	10.4
	PLSR VI	6.4	11.0	3.1	48.3	18.8	12.4
4	PLSR II	0.0	0.0	64.5	16.0	11.7	7.8
	PLSR III-0	0.0	0.0	61.3	14.7	17.5	6.5
	PLSR III-1,2	3.1	7.0	42.5	16.6	17.5	13.3
	PLSR V	51.6	16.8	7.9	5.5	13.3	4.9
5	PLSR[c]	20.0	10.6	26.3	15.2	15.2	12.6

[a] Subfractions were obtained by 'Method 6' of the COHN group[30] except for batch 4, which employed a later procedure[31].

[b] Tracings and electrophoretograms appear in Figs. 3 and 4.

[c] From extraction of the acetone-dried placental powder with borate buffer of pH 9.0

gel. The gel was stained with Amido Schwarz solution for 16 h and destaining was performed in 7.5 % acetic acid. The destained gel was preserved in 7.5 % acetic acid and an integrated tracing obtained in a Canalco Model E microdensitometer.

Disc electrophoretic data are presented in Table IV for PLSR and COHN subfractions and in which the β-globulin is calculated as a single peak. Although visual observation indicated two sharp bands lying close together, the β-globulin appeared as one peak on scanning. The region between the β-globulin and the spacer gel constituted the slow moving globulins and is denoted as the γ-globulin area, whereas, the region between the albumin and β-globulin is labeled the 'post albumin'. Tracings for COHN fractions from PLSR of batch 2 depicting the relevant areas and electrophoretograms for two of these samples appear in Figs. 3 and 4, respectively. Some of the sub-

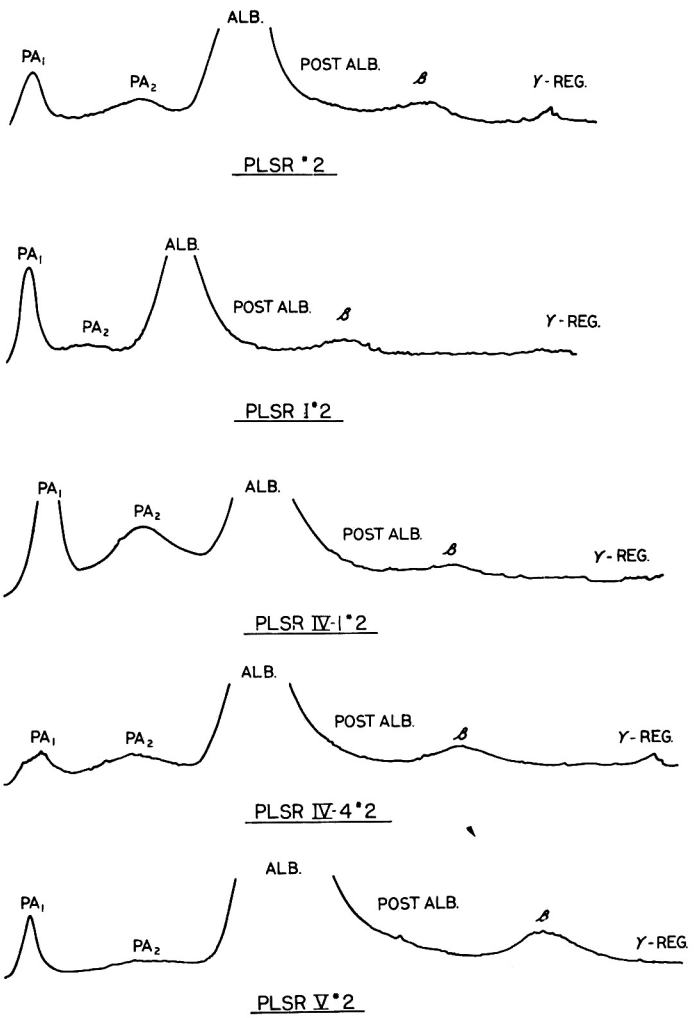


Fig. 3. Disc electrophoretic tracings of PLSR of batch 2 and derived COHN fractions showing off-the-paper scan of albumin and one PA_1 band.

fractions from batch 4 isolated by way of the second COHN method[31] displayed a few measurable peaks. Thus, fractions I + III-3, IV-6 + 7 and VI presented two faint bands in the β-globulin region and in common with fraction IV-1, only one faint PA_1 band. Although PLSR prepared by extraction of the dried placental powder with borate buffer contained a high level of PA (30.6 %; Table IV), the analogous products obtained by use of saline and phosphate and acetate buffers did not present measurable or organized patterns. The acetate-extracted PLSR displayed faint PA_1, PA_2 and albumin and two sharp bands in the β-globulin region; each of the bands of PLSR isolated by way of the phosphate buffer was faint at concentrations of protein comparable to those employed with the conventional concentrate.

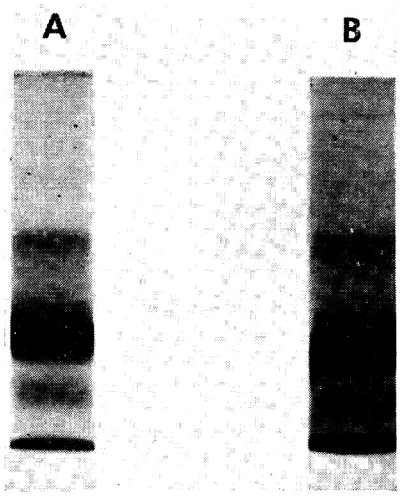


Fig. 4. Polyacrylamide gel electrophoretograms of (A) PLSR and (B) PLSR IV-1 derived from batch 2 and for which tracings and analyses are presented in Fig. 3 and Table IV, respectively. Increasing electrophoretic mobility is toward the bottom as represented.

PLSR-type products isolated from prenatal and cord sera showed disc electrophoretic patterns similar to an analogous concentrate from a large adult male serum pool. PA was observed as faint bands, two occurring in cord and pregnancy PLSR and up to three, in the male serum product but these could not be recorded by the microdensitometer. On disc electrophoresing a solution of HCG containing 39,000 IU/ml, 7 bands were obtained, each of which was far lower in intensity as compared to those of placental PLSR.

DISCUSSION

The present report advances information on various protein concentrates from washed human term placenta which was converted to a powder of good storage properties on drying with acetone. The powder was extracted with water and the resulting freeze-dried filtrate treated with 95 % ethanol, whereby a total of about 30 % of the solids was removed (Table I). When an aqueous solution was dialyzed against water, the nondialyzable dried product, PLSR. was obtained with a recovery

of just under 1 % as based on the acetone-dried powder. Similar PLSR products were isolated from late pregnancy, cord and adult male sera but only 4 % by weight was extracted by ethanol. The present treatment brings about the dissociation of lipoproteins and other components. Thus, the yellowish solid obtained on addition of excess acetone to the alcoholic extract from PLS-EO (Fig. 1) whitened on washing with ether and the solubility became less in water but increased in chloroform.

Such preliminary processing yielded a suitable product toward the concentration of PA, a component which was essentially absent in placental protein concentrates reported in the past, except possibly for the study of MUZHNAI[5]. PLSR lent itself to further fractionation by COHN procedures for plasma proteins, 'Method 6'[30] and a later one designed for smaller amounts of material[31]. The basic difference between the two is that the latter procedure deals with the extraction of fractions from precipitates, whereas, the first one involves the removal of subfractions by precipitation. However, it must be emphasized that the pH of PLSR solutions (6.5-6.7) was not raised to that of plasma, a step which would have required relatively large amounts of buffer and no attempt was made to employ alkali in order to avoid artifacts. As based on paper electrophoresis, with few exceptions, the resulting PLSR subfractions possessed the same type of components as in the case of serum as such, but varied in their respective distribution from fraction to fraction. PLSR and allied products are not simply mixtures of blood proteins but are unique in containing a variety of protein factors and hormones as reviewed above.

In relation to paper electrophoresis, 'Method 6' gave rise to fractions containing a PA peak, the latter being especially prominent in PLSR VI, the supernatant remaining after removal of fractions I–V, inclusive. Disc electrophoresis revealed two distinct PA bands, one of which occurred in the tracking dye region. That PA_1 is not tracking dye is evidenced by the disappearance of the band from human serum but not from PLSR and derived COHN fractions when many samples were electrophoresed simultaneously in a larger apparatus. No significant level of PA occurred in PLSR-type concentrates derived from late pregnancy and cord sera but the two faint bands observed in the gels might be related to those of placenta. COHN subfractions from serum PLSR were likewise low in PA. It is quite probable that by the enrichment procedures, liberation of PA might have resulted from the dissociation of PA-globulin complexes, not unlike those described for TBP by TATA[27]. Although the presence of calcium lactate in the Owen buffer enhances the resolution of β-globulin into two components, the splitting was not observed with all fractions. However, splitting of γ-globulin was frequent (Table III).

By disc electrophoretic criteria, PA was further concentrated in PLSR IV-1 and occurred in even greater amount in PLSR VI. The highest percentage was encountered in PLSR V of batch 4 fractionated by way of the later COHN procedure[31]. On comparing the PA levels by paper and disc electrophoresis, it is noted that the values by the latter approach are much higher; extensive shouldering of the peak was evident with cellulose acetate. In this respect, as disc electrophoretic criteria have much better resolving power, such PA data are probably much closer to the true values. The splitting of γ-globulin in PLSR VI and other fractions observed with cellulose acetate could not be followed too closely with the gels due to the fact that γ-globulin was not as pronounced as such areas as the albumin region. The extraction of the acetone-dried powder with the other aqueous media led to PLSR products of lower PA content

or in which the patterns were not as consistent in make-up as compared to the usual concentrate, except for the borate-extracted material in which a similar or even higher level resulted (Table IV).

The presence of chorionic gonadotrophin in PLSR was demonstrated by bioassay, dosages of 150 mg but not 25 mg, being effective on subcutaneous injection into the virgin rabbit doe. CAWLEY *et al.*[33] applied polyacrylamide gel electrophoresis to a commercial HCG preparation from human pregnancy urine and reported the presence of 7 bands consisting of two PA, one albumin, one post albumin, two in the transferrin region and one in the slow moving γ-globulin or slow α_2-globulin; the relative concentration or zonal intensity of each was not given. Immunodiffusion electrophoresis revealed that the transferrin region was the center of hormonal activity. In corroboration of such findings, a similar distribution obtained with a sample of HCG applied at a level of 39,000 IU/ml and which paralleled the PLSR gel bands but the latter were far more intense. Presumably, the presence of gonadotrophin contributes to the observed gel patterns of the placental products. A profitable approach to this problem might be a model investigation involving the concentration of human pregnancy urine, isolation of the protein portion and extensive disc electrophoretic analysis of components, experiments of which are not included in the current study. In this connection, according to a recent report[34], some differences were noted between amniotic fluid and maternal and cord sera by disc electrophoresis but no PA component was described. In yet another account[35], fluids obtained from women by amniocentesis were concentrated, dialyzed and submitted to gel electrophoresis. A constant feature of the fluids was the presence of β-globulin and four PA bands to the virtual exclusion of γ-globulin.

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CHROM. 3374

ZUR IDENTIFIZIERUNG VON POLYSACCHARIDEN

NACHWEIS DER ZUCKERBAUSTEINE DURCH DÜNNSCHICHT-CHROMATOGRAPHIE UND INFRAROTSPEKTROSKOPIE

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(Eingegangen den 8. Januar 1968)

SUMMARY

Identification of polysaccharides. Detection of sugar components by thin-layer chromatography and infrared spectroscopy

A method for the identification of sugars and uronic acids as components of polysaccharides of various origin was developed. The sample (*e.g.* pure substances, mucopolysaccharides of muscle or additives to food) was hydrolyzed by a cation exchange resin. The resulting mixture of compounds was separated on cellulose thin layer plates. Isolated bands of sugars were pressed into KBr disks after elution and identified by their infrared spectra. The separated uronic acids were converted into their cinchonic salts, whose infrared spectra allowed a differentiation of D-glucuronic, D-galacturonic and D-mannuronic acid.

EINLEITUNG

Der Nachweis endogener und zugesetzter Polysaccharide (Quellstoffe) in biologischem Material und Lebensmitteln ist infolge eines grossen Überschusses an störenden anderen Stoffen erschwert. Da die Quellstoffe infolge ihrer geringen Konzentration von 0.5 bis 1.0 % als solche kaum identifiziert werden können[1,2], haben GRAU UND SCHWEIGER[3,4] ein Verfahren entwickelt, bei dem ein Säurehydrolysat dieser Substanzen auf Celluloseschichten getrennt wird. Bei der sauren Hydrolyse können allerdings gewisse Schwierigkeiten auftreten:

(1) Es kommt zu Reaktionen der freiwerdenden Zucker mit den aus Proteinen in Freiheit gesetzten Aminosäuren. Die dabei gebildeten braun gefärbten Produkte wirken sich störend auf weitere Identifizierungsreaktionen aus.

(2) Uronsäuren können während der Hydrolyse abgebaut werden[5,6].

(3) Die Hydrolyseprodukte vereinigen sich in manchen Fällen sekundär zu Disacchariden[7].

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Um diese Schwierigkeiten zu vermeiden, wandten wir die Hydrolyse an Ionenaustauschern nach HAAB, ANASTASSIADIS UND COMMON[5,6] an. In der vorliegenden Arbeit wird gezeigt, dass es möglich ist, Quellstoffe in reiner Form und als Zusatz, sowie Polysaccharide des Muskelgewebes mit Hilfe einer Kombination von Dünnschichtchromatographie und Infrarotspektroskopie zu identifizieren.

MATERIAL UND METHODEN

Die Ausrüstung zur Dünnschichtchromatographie stammte von der Firma Desaga, Heidelberg. Zur Aufnahme der Infrarotspektren mit Hilfe der KBr-Presstechnik wurde ein Gerät der Firma Perkin-Elmer, Überlingen "Infracord 137" und ein Gerät der Firma Beckmann, München "IR 10" verwendet.

Alle Reagentien waren p.A. und stammten von der Firma Merck, Darmstadt. Als Trägermaterial für die Dünnschichtchromatographie wurde das Pulver MN 300 HR der Firma Macherey und Nagel, Düren verwendet. Als Kaliumbromid wurde KBr für Spektroskopie benutzt, Pectinase stammte von der Firma Serva, Heidelberg und Cinchonin (rein) von der Firma Schuchardt, München. Der Enzymtest auf Galactose stammte von der Firma Worthington-Biochemical Corp. Freehold/N.J., U.S.A.

1. Aufarbeitung der Probe und Chromatographie des Hydrolysates

Die Probe wird nach einer evtl. notwendigen Entfettung mit Petroläther und Entzuckerung mit 50 %igem Äthanol (vergl. 3) mit deionisiertem Wasser kurz aufgekocht. Man zentrifugiert die ausgefallenen Proteine und andere unlösliche Stoffe ab und versetzt den Überstand, der die gelösten Polysaccharide enthält mit 1 vol 96 % Äthanol. Die Polysaccharide werden nach einigen Stunden abzentrifugiert. Das Material wird in 5 bis 10 ml deionisiertem Wasser aufgenommen und mit 1 bis 3 g Kationenaustauscher Merck I in der H^+-Form pro 20 bis 50 mg Polysaccharid bei 105° im Trockenschrank unter mehrfachem Umschütteln 12 Std. stehen gelassen. Nach dem Abfiltrieren und Auswaschen des Austauschers kann man durch Filtration über eine Säule mit Kieselguhr–Kohle im Verhältnis 1:1 die Huminstoffe entfernen. Das klare, helle Filtrat wird auf eine Celluloseschicht vom 0.5 mm Dicke, die einmal mit dem Laufmittel vorgewaschen wurde, in Form eines Striches aufgetragen. Als Laufmittel wurden verwendet: (1) Isopropanol–Pyridin–Eisessig–Wasser (8:8:1:4) und (2) Äthylacetat–Pyridin–Wasser (2:1:2). Die Plattenränder werden nach dem Lauf zur Lokalisierung der Banden in einer Breite von 0.5 bis 1.0 cm mit Anisidin-Phthalat[8] angefärbt und etwa 20 bis 30 min auf 50° erwärmt. Die Banden werden abgeschabt und das Pulver wird mit Äthanol–Wasser (1:1) eluiert. Das Eluat wird nach Filtration durch einen Glasfiltertiegel auf ein kleines Volumen eingeengt. Bei Anfärbung auf Uronsäuren mit Naphthoresorcin in 2 %iger Trichloressigsäure[9,10] muss mindestens 30 min lang auf 105° erhitzt werden. Die Uronsäuren treten dann als blaue Banden nach einigen Stunden hervor.

Beim Nachweis von Pectin versetzt man 5 g der nicht vorbehandelten Probe mit 100 mg Pectinase und einigen ml deionisiertem Wasser, lässt 15 min bei 30° und pH 5.5 stehen[11], zentrifugiert den Rückstand ab und lässt die Lösung durch einen Kationenaustauscher Merck I in der H^+-Form laufen. Das Filtrat wird auf die Celluloseschicht aufgetragen.

2. Darstellung der Cinchonin-Derivate und Infrarotspektroskopie

Zur Darstellung der Cinchoninsalze der Uronsäuren wurden 10 ml des alkoholischen Eluates (5 bis 10 mg Substanz aus der Celluloseschicht) mit 5 bis 10 mg Cinchonin versetzt und über Nacht stehen gelassen[12]. Bei den Lactonen muss vorher der Ring bei pH 8.0 (30 min) geöffnet werden; das Reagenz wird nach Ansäuern mit Essigsäure zugegeben[13]. Durch Reiben mit einem Glasstab fällt das farblose Salz aus. Nach dem Einengen wird das überschüssige Reagenz mit 1.0 ml Chloroform entfernt. Der Rückstand wird in Wasser gelöst und eingeengt.

Man überführt 0.5 bis 1.0 ml des eingeengten Eluates oder des gelösten Cinchonin-Derivates in kleine Glasschälchen, in die man vorher etwa 200 bis 220 mg KBr eingewogen hat. Die Schalen werden in einen Exsiccator gesetzt und über Nacht unter Vakuum stehen gelassen. Der trockene Rückstand wird herausgekratzt, im Achatmörser gut verrieben und nach dem Wägen zu einer durchsichtigen Scheibe gepresst. Die Einwaage an Substanz soll 1.0 bis 1.5 mg nicht überschreiten, andernfalls muss mit KBr "verdünnt" werden.

ERGEBNISSE UND DISKUSSION

(1) Bei der Hydrolyse von pflanzlichen Polysacchariden können die Pentosen Arabinose, Xylose, Ribose, die Hexosen Galactose, Glucose, Mannose, die Methylpentose Rhamnose und die Uronsäuren Galacturonsäure, Glucuronsäure, Mannuronsäure, Guluronsäure in verschiedenen Konzentrationen frei werden. Dazu kommen bei tierischen Polysacchariden Iduronsäure (aus dem Chondroitinsulfat B) und Glucuronsäure.

Bei mineralsaurer Hydrolyse entstehen aus den Monosen und Uronsäuren durch Sekundärreaktionen Oligomere, vor allem Dimere in geringer Konzentration[7]. Diese Beobachtung konnte durch die vorliegenden Ergebnisse bestätigt werden. Die Laufwerte dieser Produkte bei der Chromatographie liegen in gleicher Höhe wie die von natürlich vorkommenden Disacchariden und Uronsäuren (vergl. Tabelle I); eine Interferenz lässt sich nicht vermeiden. Neu gebildete Oligosaccharide konnten durch eine zweite saure Hydrolyse in einfache Zucker gespalten werden.

(2) Eine Anzahl von pflanzlichen Polysacchariden (Agar, Johannisbrotkern-

TABELLE I

R_G-WERTE EINIGER ZUCKER

Bezogen auf R_F-Wert Glucose = 1.0 nach zweimaliger Chromatographie im System 2 (Äthylacetat–Pyridin–Wasser, 2 : 1 : 2, beide Phasen).

Substanz	R_G	*Nachweisgrenze mit Aminoanilin* (*μg*)	*Nachweisgrenze mit Anisidin-Phthalat* (*μg*)
Raffinose	0.42	1–2	
Lactose	0.53	0.5	
Trehalose	0.62	5	
Maltose	0.71	0.5	
Saccharose	0.84	0.5	
Glucuronsäure	0.50	—	0.5
Galacturonsäure	0.44	—	0.5

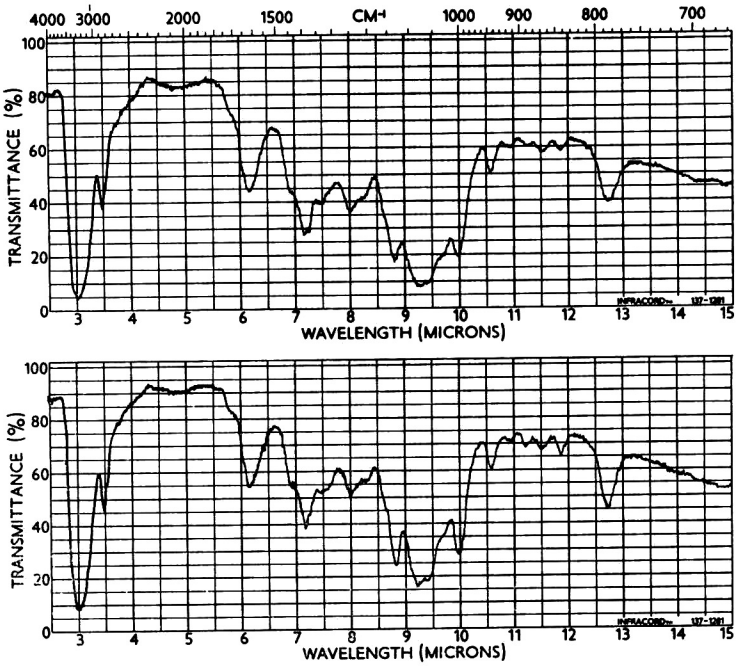


Fig. 1. Infrarotspektrum von Arabinose. Oben: Reinsubstanz nach Chromatographie; unten: aus Lebensmittel als 1%iger Zusatz isoliert (Austauscherhydrolyse).

mehl) wurde nach der Austauscher-Hydrolyse analysiert. Als Beispiel eines neutralen Zuckers wurde das Verhalten der Arabinose genommen. Sie wurde aus der Celluloseschicht nach der Chromatographie eluiert. Das Infrarotspektrum wurde aufgenommen; es stimmte mit dem von TIPSON UND ISBELL[14] angegebenen überein (Fig. 1). Ebenso lässt sich Xylose aus pflanzlichen Polysacchariden nachweisen. Ribose kann als *p*-Phenylhydrazon durch das Infrarotspektrum eindeutig nachgewiesen werden[15]. Dagegen war das Spektrum anderer Zucker wie z.B. der Galactose weniger gut zur Identifizierung geeignet, da es erst im Bereich über 15 μ ausgeprägte Banden aufweist. Dieser Zucker konnte schneller und sicherer mit einem enzymatischen Test, z.B. mit Galactostat, auf den Celluloseschichten nachgewiesen werden.

Das Verhalten von Polymeren, welche Uronsäuren enthalten wurde eingehend untersucht.

Fig. 2 zeigt die Auftrennung eines schwefelsauren Hydrolysates von Pectin auf einer Celluloseschicht. In der mit (a) bezeichneten Zone befindet sich die Galacturonsäure zusammen mit sekundär gebildeten Produkten und Resten von Polygalacturonsäure. Die Fig. 3 bringt zum Vergleich die Auftrennung eines Hydrolysates, das durch enzymatische Hydrolyse oder durch Hydrolyse am Ionenaustauscher aus Pectin gewonnen wurde. Hierbei waren die Bedingungen des Nachweises bewusst ungünstig gewählt, da das Pectin zu einer Fleischkonserve zugesetzt war, also neben einem grossen Überschuss an Proteinen und Fett vorlag. Dennoch ist die Galacturonsäure als Hauptbestandteil des Hydrolysates (b in Fig. 3) deutlich zu erkennen. Sie konnte auch als Cinchonin-Derivat durch das Infrarotspektrum nachgewiesen werden (Fig. 4).

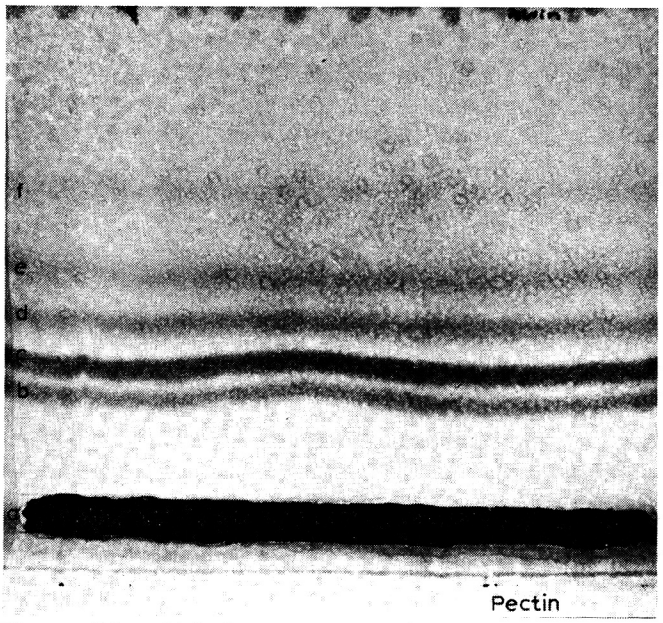


Fig. 2. Dünnschichtchromatogramm eines Pectinhydrolysates nach Hydrolyse mit 5%iger Schwefelsäure auf Cellulosepulver MN 300 HR, System 2. Anfärbung mit Anisidin-Phthalat. Banden von unten nach oben: a = Uronsäure bzw. abgeschabte Oligosaccharide; b = Galactose; c = Glucose; d = Arabinose; e = Xylose; f = Rhamnose.

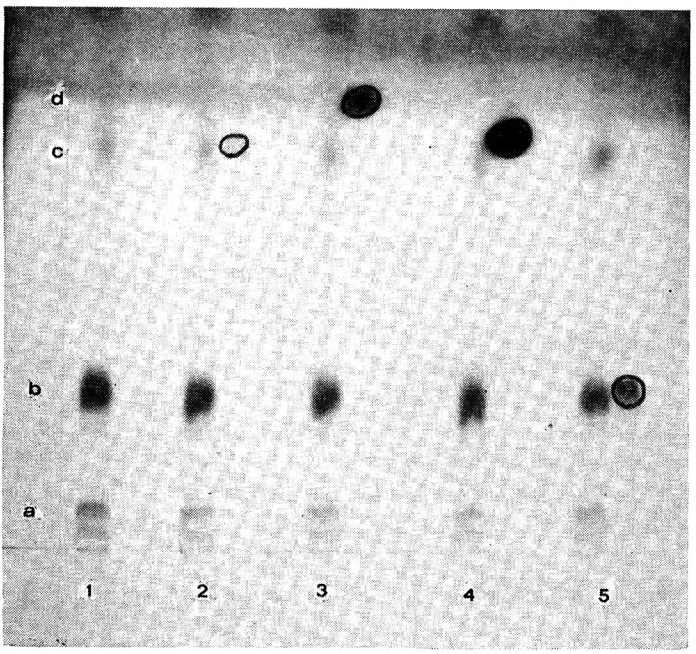


Fig. 3. Dünnschichtchromatogramm eines Pectinhydrolysates nach Hydrolyse mit Pectinase, System 1. Anfärbung mit Anisidin-Phthalat. a = Nicht gespaltener Anteil Polygalacturonsäure; b = Galacturonsäure; c = Glucose; d = Xylose. Referenzsubstanzen rechts vom Startpunkt zugegeben: 2 = Glucose; 3 = Xylose; 4 = Arabinose; 5 = Galacturonsäure.

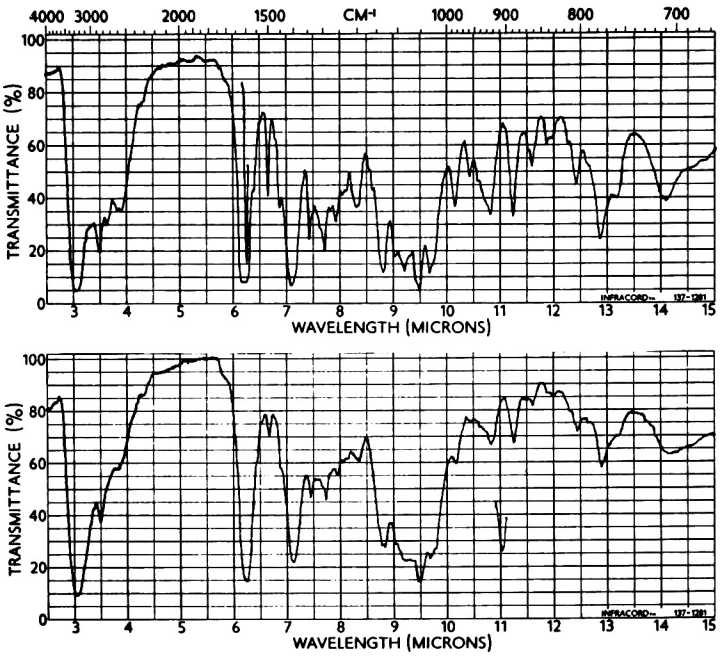


Fig. 4. Infrarotspektrum von Galacturonsäure-Cinchoninsalz. Oben: Reinsubstanz nach Chromatographie aus Cellulosepulver isoliert; unten: aus mit Pectin versetztem Material isoliert (Pectinase-Hydrolyse).

(3) Eine weitere Polyuronverbindung, die durch mineralsaure Hydrolyse weitgehend zerstört wird, ist das Alginat, welches vor allem aus verschiedenen Meeresalgen isoliert wird. Alginat enthält nach FISCHER UND DÖRFEL[16] neben Mannuronsäure grössere Mengen Guluronsäure; dies wurde durch HAUG *et al.* bestätigt[13,17,18]. Unsere Vorversuche mit Reinsubstanzen zeigten, dass die Guluronsäure im System 1 (vergl. Methoden) zu etwa gleichen Teilen als Lacton und als freie Säure läuft. Die Mannuronsäure dagegen liegt unter den Bedingungen der Hydrolyse und der Chromatographie überwiegend als Lacton vor, das sich gut von anderen Substanzen des Hydrolysates abtrennen lässt (Substanz (f) in Fig. 5). Das Mannuronsäure-Lacton ist nach Hydrolyse von Alginaten mit Ionenaustauscher im Dünnschichtchromatogramm sichtbar (Fig. 5). Es konnte aus dem Chromatogramm eluiert werden; nach Ringöffnung

TABELLE II

R_G-WERTE* VON URONSÄUREN UND TYPISCHEN I.R.-BANDEN IHRER CINCHONINSALZE**

Substanz	*Freie Säure*	*Lacton*	*Wellenlänge (μ)*
Galacturonsäure	0.40		9.50–10.80–14.20
Glucuronsäure	0.46		8.60–10.55–13.50–14.80
Guluronsäure	0.53	1.50	——
Mannuronsäure	0.56	1.28	7.15–12.05–13.25

* Einmalige Chromatographie im System 1.
** Wellenlänge in Micron.

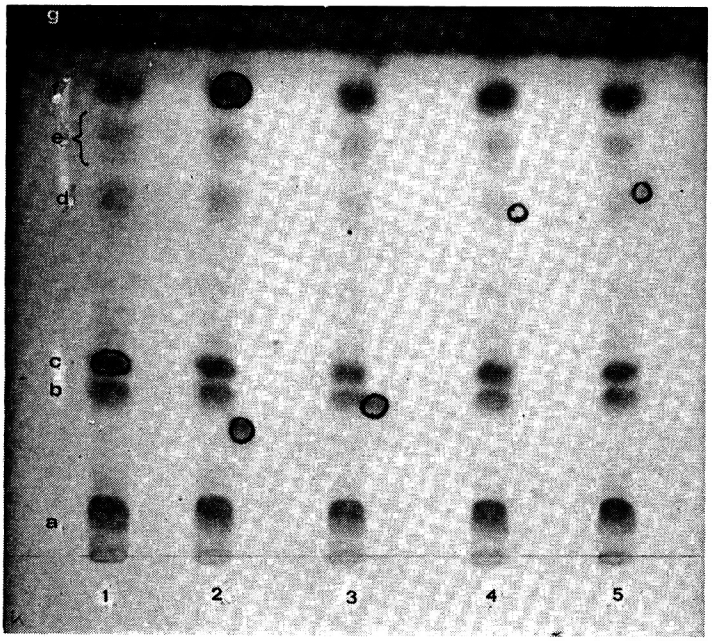


Fig. 5. Dünnschichtchromatogramm eines Alginat-Hydrolysates mit Ionenaustauscher (auf Cellulosepulver MN 300 HR nach zweimaligem Lauf). a = Polyuronsäuren; b = fraglich (Glucuronsäure); c = Guluronsäure; d = Glucose; e = Pentosen; f = Mannuronsäurelacton; g = Guluronsäurelacton. Zusätze zum Hydrolysat: 1 = Guluronsäure; 2 = Galacturonsäure (unten); 2 = Mannuronsäure (oben); 3 = Glucuronsäure; 4 = Glucose; 5 = Arabinose. Zusätze 2 (unten) 3–5 rechts neben der Auftragstelle.

wurde es in das Cinchonin-Derivat übergeführt. Das Infrarotspektrum des Derivates ist in Fig. 6 wiedergegeben. Es ist wichtig, dass sich dieses Spektrum eindeutig von den Spektren der Cinchoninsalze von Galacturonsäure (Fig. 4) und Glucuronsäure (Fig. 7) unterscheidet. Aus diesem Grund wurde die Mannuronsäure und nicht die Guluronsäure zur Identifizierung von Alginaten herangezogen. In der Tabelle II sind nochmals die R_G-Werte im System 1 und die typischen IR-Absorptionsbanden der Cinchoninsalze der verschiedenen Uronsäuren zusammengefasst.

Nach den angeführten Ergebnissen ist es möglich, die in Hydrolysaten von pflanzlichen Polysacchariden enthaltenen Hexuronsäuren durch ihre Infrarotspektren zu unterscheiden.

DANK

Wir sind Herrn Dr. ARNE HAUG vom Norwegischen Institut für Seetangforschung in Trondheim für die freundliche Überlassung von Reinsubstanzen von Mannuronsäure und Guluronsäure zu Dank verpflichtet. Ferner danken wir Herrn Prof. Dr. E. FAHR, Organisch-Chemisches Institut der Universität Würzburg, für die Anfertigung der Infrarotspektren der Cinchoninsalze der Mannuronsäure, Dr. H. ISBELL vom National Bureau of Standards in Washington für die freundliche Überlassung des Infrarotspektrums der Mannuronsäure und Frl. A. BADERSCHNEIDER für ihre wertvolle technische Mitarbeit.

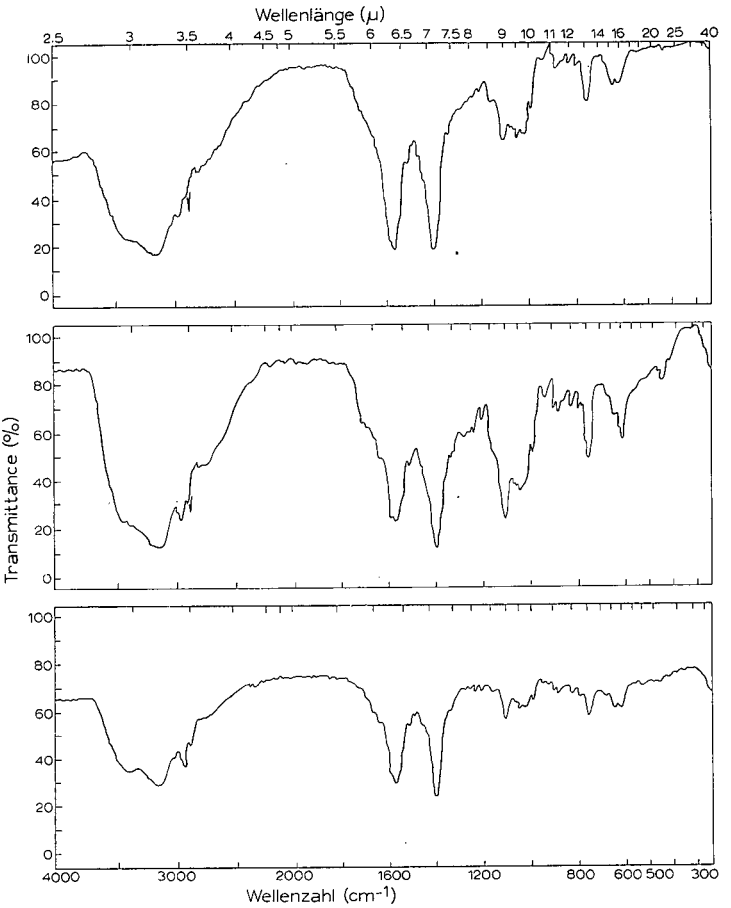


Fig. 6. Infrarotspektren von Cinchoninsalzen der Mannuronsäure nach Dünnschichtchromatographie. Oben: Reinsubstanz; Mitte: aus Alginatprobe 1 (Substanz von Fig. 5); unten: aus Alginatprobe 2.

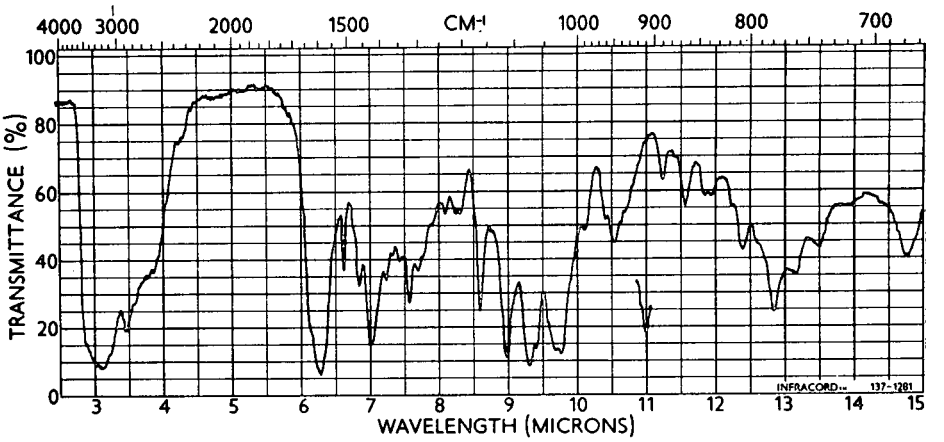


Fig. 7. Infrarotspektrum von Cinchoninsalz der Glucuronsäure nach Dünnschichtchromatographie.

ZUSAMMENFASSUNG

Es wird eine Methode angegeben mit der Zuckerbausteine und vor allem Uronsäuren aus pflanzlichen Quellstoffen in Reinsubstanz als auch aus biologischem Material sowie Lebensmitteln mit Hilfe von Ionenaustauschern schonend isoliert werden können. Sie werden mit Hilfe der Dünnschichtchromatographie an Celluloseschichten aufgetrennt und durch Infrarotspektren identifiziert.

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CHROM. 3367

QUANTITATIVE ESTIMATION OF MONOHYDROXY SATURATED STEROIDAL SAPOGENINS IN PLANT MATERIALS BY DENSITOMETRIC THIN-LAYER CHROMATOGRAPHY

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(First received December 19th, 1967; modified January 17th, 1968)

SUMMARY

A thin-layer chromatographic technique using Silica Gel G as the adsorbent and six-fold development with *n*-hexane–ethyl acetate (12:1, v/v) has been devised for the separation and analysis of monohydroxy, saturated sapogenins in *Asparagus*, *Smilax* and *Yucca* species. It was shown that a linear relationship exists between the logarithm of the weight of sapogenin applied and the square root of the absorbance of the spot produced. Quantitative evaluation was achieved by using a photoelectric densitometer coupled to an integrating logarithmic recorder, which computed the absorbance of the spot. The experimental error was found to be approximately 7 %. Smilagenin and sarsasapogenin were found to be insufficiently separated by the described method and were estimated together. Similarly, tigogenin and neo-tigogenin were not separated and were estimated together. Equal spot intensities were produced by equal weights of sarsasapogenin, smilagenin, neo-tigogenin and tigogenin. The results obtained using the densitometric assay procedure showed close correlation with results obtained by an infra-red spectroscopic method.

Steroidal sapogenins are of economic importance as precursors of many of the medicinally useful steroids. They occur in plants as glycosides and are of somewhat restricted distribution. Economically, steroidal sapogenins are isolated mainly from species in the genera *Dioscorea*, *Agave* and *Yucca*, although they are to be found in several other genera, including *Smilax*, *Asparagus*, *Strophanthus*, *Digitalis* and *Trigonella*. The individual sapogenins of primary industrial value are diosgenin (Δ^5-25α-spirosten-3β-ol) from *Dioscorea* species, hecogenin (5α,25α-spirostan-3β-ol-12-one) from *Agave* species and sarsasapogenin (5β,25β-spirostan-3β-ol) from *Yucca* species. In addition to the genus *Yucca*, sarsasapogenin is found in *Asparagus officinalis* L[1] and *Smilax* species[2], the latter including the commercially used sarsasaparillas.

After hydrolysis of the naturally occurring saponins and extraction of the liberated sapogenins with a hydrocarbon solvent, the sapogenins are usually assayed by gravimetric[3,4] or infra-red techniques[5,6]. The gravimetric methods are applicable

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to plant materials with high sapogenin yields, but are inaccurate when the proportion of contaminants extracted is high. Infra-red spectroscopic methods are more suitable, as they are based on the spirostan structure of the sapogenin and are thus specific for steroidal sapogenins. It has been found, however, that non-sapogenin compounds extracted by the hydrocarbon solvent may interfere with the infra-red assay method and cause high results. To overcome these disadvantages it was felt that a convenient analytical technique, suitable for small quantities of plant material, was required. Densitometric thin-layer chromatography has been studied and its use for the estimation of diosgenin in *Dioscorea* species reported[7]. Extension of the method for sarsasapogenin and other monohydroxy, saturated sapogenins is reported in this paper. This method was developed for future studies on the genus *Yucca*, in particular *Y. glauca* Nutt., a plant found growing in the U.S.A. from Iowa and South Dakota to Montana, south to Missouri, Texas and Arizona[8]. From the leaves of *Y. glauca*, sarsasapogenin has been isolated[9,10], but in addition to this sapogenin they contain smilagenin (5β,25α-spirostan-3β-ol), neo-tigogenin (5α,25β-spirostan-3β-ol) and tigogenin (5α,25α-spirostan-3β-ol)[11]. As a result, this paper reports the development of an assay procedure for the estimation of monohydroxy, saturated sapogenins.

EXPERIMENTAL

Glass plates, 15 × 20 cm, were spread with a layer of Silica Gel G (Merck) to a thickness of 250 μ, according to the method of STAHL[12] and air-dried before use. Known quantities of sarsasapogenin, smilagenin, neo-tigogenin and tigogenin were applied to the plates by micropipettes and developed with *n*-hexane–ethyl acetate (12:1, v/v), using the super-saturated method of STAHL[13]. After the solvent had advanced 15 cm from the starting line, the plates were removed from the tank, dried and re-developed five more times with the same solvent system. Following development, the plates were dried and sprayed uniformly with a solution of antimony trichloride in concentrated hydrochloric acid (3:1, w/w)[14]. The sapogenins were visualised by heating the plate at 100° in a circulating air-stream until the spots were a blue-black colour. This normally took 30 min.

The plates were allowed to cool before the absorbances of the spots were estimated by a Vitatron densitometer* using a slit width of 20 mm by 2 mm and either a tungsten lamp or mercury lamp source. The values so obtained were calculated by an automatic integrator. Spots were scanned in the direction opposite to that of solvent flow and erratic readings from extraneous light were eliminated by the use of a light-tight box.

Preparation of the standards

Pure sarsasapogenin, smilagenin, neo-tigogenin and tigogenin, dissolved in chloroform, were applied to thin-layer plates in concentrations ranging from 18 μg to 80 μg. After development, the spots produced were scanned and a linear relationship between weight applied and absorbance was established for all four sapogenins within the range tested.

Preparation of plant material

The sapogenins were extracted from plant material of known weight and

* Fison's Scientific Apparatus Ltd., Loughborough, Leic., Great Britain.

moisture content by the method of BLUNDEN, HARDMAN AND WENSLEY[10]. This involved maceration of the fresh material, incubation of the distintegrated material, acid hydrolysis of the saponins by refluxing with 2 *N* hydrochloric acid for 2 h, and extraction of the sapogenins from the dry, acid-insoluble residue with light petroleum (40–60°). Dried, powdered plant material was incubated first with water before being treated by the above procedure. The petroleum extract was evaporated to dryness, re-dissolved in chloroform and made up to a known volume. These extracts were applied to thin-layer plates along with standard sapogenin solutions. In the case of *A. officinalis* and *Smilax* species, only a standard solution of sarsasapogenin was necessary, but with *Y. glauca*, standard solutions of sarsasapogenin and tigogenin were applied. The applied spots of the plant and standard solutions were alternated on the plate to minimize the effects of variation in thickness and opacity of the adsorbent layer. The sarsasapogenin and tigogenin contents were calculated relative to the standard solution, and the resultant value obtained from the mean of six plates, each containing two spots of the plant extract.

To determine the variation in spot intensity produced by equal weights of sarsasapogenin, smilagenin, neo-tigogenin and tigogenin, 20 μg and 60 μg of each compound were applied to thin-layer plates and the spot intensities produced were compared.

The extent of breakdown during extraction of each of the four sapogenins, sarsasapogenin, smilagenin, neo-tigogenin and tigogenin was determined by refluxing 20 mg samples of each compound with 50 ml of 2 *N* hydrochloric acid for 2 h and subsequently extracting them with chloroform. These solutions were assayed and compared with appropriate standards of untreated compounds.

Samples of *A. officinalis*, *S. aristolochaefolia*, *S. regellii*, and *Y. glauca* were assayed by the thin-layer chromatographic and infra-red spectroscopic methods for purposes of comparison. The infra-red spectroscopic determinations of *Asparagus* and *Smilax* samples were performed by dissolving the sapogenin extracts in chloroform and assaying them using a Hilger H 800 spectrophotometer. The total sapogenin concentration was estimated from the intensity of the band near 980 cm^{-1} and the

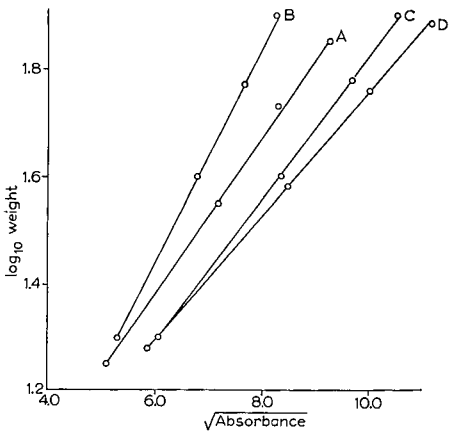


Fig. 1. Relationship between spot absorbance and weight of sarsasapogenin, smilagenin, neo-tigogenin and tigogenin. A = Sarsasapogenin; B = smilagenin; C = neo-tigogenin; D = tigogenin.

TABLE I

RELATIONSHIP BETWEEN WEIGHTS OF SARSASAPOGENIN, SMILAGENIN, NEO-TIGOGENIN AND TIGOGENIN APPLIED AND THE ABSORBANCIES OF THE SPOTS PRODUCED, EXPRESSED AS A READING FROM THE AUTOMATIC INTEGRATOR

Sapogenin	*Wt. (μg)*	*Log_{10} wt.*	*Integrator[a] reading*	*$\sqrt{Integrator\ reading}$*
Sarsasapogenin	17.9	1.25	26	5.1
	35.8	1.55	52	7.2
	53.7	1.73	69	8.3
	71.6	1.85	86	9.3
Smilagenin	19.7	1.29	28	5.3
	39.4	1.60	46	6.8
	59.1	1.77	59	7.7
	78.8	1.90	69	8.3
Neo-tigogenin	20.0	1.30	38	6.2
	40.0	1.60	70	8.4
	60.0	1.78	94	9.7
	80.0	1.90	111	10.5
Tigogenin	19.0	1.28	34	5.8
	38.0	1.58	72	8.5
	57.0	1.76	100	10.0
	76.0	1.88	126	11.2

[a] Mean result from six plates.

TABLE II

QUANTITATIVE RESULTS FOR SARSASAPOGENIN, SMILAGENIN, NEO-TIGOGENIN AND TIGOGENIN RECOVERIES

Sapogenin	*Wt. applied (μg)*	*Recovered[a]*		*No. of estimations*
		μg	%	
Sarsasapogenin	17.9	17.4 ± 1.0	97.0 ± 5.5	40
	35.8	35.5 ± 2.5	99.2 ± 7.0	40
	53.7	53.3 ± 2.4	103.0 ± 4.3	40
Smilagenin	19.7	19.6 ± 1.3	99.7 ± 6.6	40
	39.4	39.2 ± 2.6	99.6 ± 6.7	40
	59.1	58.2 ± 4.1	98.7 ± 7.0	40
Neo-tigogenin	20.0	20.1 ± 1.4	100.3 ± 6.8	40
	40.0	40.5 ± 2.0	101.3 ± 4.9	40
	60.0	60.2 ± 3.7	100.3 ± 6.1	40
Tigogenin	19.0	19.1 ± 1.1	100.3 ± 5.8	40
	38.0	38.2 ± 2.3	100.3 ± 5.9	40
	57.0	58.1 ± 4.7	101.9 ± 8.1	40

[a] Recovered percentage is expressed as the mean of all determinations ± one standard deviation.

major components, if 25β-epimers, are estimated from the intensity of the band near 915 cm^{-1}, or if 25α-epimers, from that of the band near 900 cm^{-1}. The minor component was obtained by difference. This technique is applicable only when the sample is predominantly of one configuration and there is not much contamination from non-sapogenin materials. For the assay of *Y. glauca* samples it was necessary to ignore the 980 cm^{-1} peak owing to the appreciable 25α-components and to use a ratio method, based on the ratio of the intensities of the 915 and 900 cm^{-1} peaks[15].

RESULTS AND DISCUSSION

PURDY AND TRUTER[16] found that there was a linear relationship between the logarithm of the weight of substance applied and the square root of the area of the spot produced. BLUNDEN, HARDMAN AND MORRISON[7] found that this linear relationship held for the logarithm of the weight of diosgenin applied and the absorbance of the spot produced. Four monohydroxy, saturated steroidal sapogenins, sarsasapogenin, smilagenin, neo-tigogenin and tigogenin have now been studied and a similar linear relationship found to apply, from approximately 18 μg to 80 μg. The limits of the linear relationship were not determined (Fig. 1 and Table I). These results showed that densitometric thin-layer chromatography provides a suitable method of assay for these sapogenins.

The percentage error in the method was calculated by applying approximately 20, 40 and 60 μg of each of the test sapogenins to thin-layer plates and estimating the variations observed in spot intensity. Ten chromatograms, each of four spots, were prepared in these determinations (Table II).

The solvent system used for the separation of diosgenin from the other constituents in *Dioscorea* extracts was *n*-hexane–ethyl acetate (4:1, v/v)[7]. Double development of each chromatogram was necessary to effect sufficient separation of the sterol and diosgenin spots to enable accurate densitometric estimations to be made. The same solvent system when applied to the plant extracts in the present study did not separate the sarsasapogenin from the sterol sufficiently for the quantitative estimation of the genin. A number of other solvent systems commonly used for sapogenins[17,18] were tried, but without success. Finally, six-fold development in *n*-hexane–ethyl acetate (12:1, v/v) proved satisfactory. When β-sitosterol was added to previously assayed samples of *A. officinalis*, *S. aristolochaefolia* and *Y. glauca* in quantities equal to the sarsasapogenin yield and the mixtures assayed again, results were obtained which were not significantly different from previously obtained figures, showing that the added sterol had been effectively separated from the sapogenin spots and did not interfere with the assay.

S. aristolochaefolia, *S. regellii* and *Y. glauca* extracts contained smilagenin in addition to sarsasapogenin. Six-fold development in the *n*-hexane–ethyl acetate solvent system did not effectively separate these two compounds, although smilagenin was clearly visible as a cap over the sarsasapogenin spot. As a result, smilagenin present was estimated along with sarsasapogenin. In addition to sarsasapogenin and smilagenin, *Y. glauca* contains tigogenin and neo-tigogenin. These latter two sapogenins were not separated from each other on the thin-layer chromatograms, but were well separated from the combined smilagenin/sarsasapogenin spot (Fig. 2). As a result, tigogenin and neo-tigogenin were also estimated together and calculated as

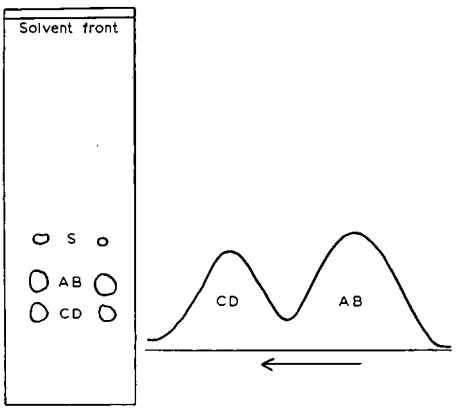


Fig. 2. Thin-layer chromatogram of *Y. glauca* extract and integrator tracing of the sapogenin spots. S = Sterol; AB = sarsasapogenin/smilagenin; CD = tigogenin/neo-tigogenin.

tigogenin. It was shown that equal weights of sarsasapogenin, smilagenin, neo-tigogenin and tigogenin give equal spot intensities with the antimony trichloride reagent. It was concluded, therefore, that no error was involved in estimating smilagenin with sarsasapogenin and neo-tigogenin with tigogenin.

To determine the sensitivity of the assay procedure, sarsasapogenin was added to previously assayed samples of *A. officinalis* rhizomes, *S. aristolochaefolia* roots and *Y. glauca* leaves. To each of the different extracts of known volume, sarsasapogenin was added equivalent to approximately 25 % and 50 % of the total sarsasapogenin yield of the extracts and the chromatographic assay carried out on the mixture to determine the recovery of the added sapogenin. Similar recovery tests were performed

TABLE III

RECOVERY OF ADDED SAPOGENINS TO ASSAYED SAMPLES OF PLANT EXTRACTS

Sapogenin added	*Amount added as % of quantity in extract*	*Recovery (%)*
Sarsasapogenin	25[a]	97.4[c]
	50[a]	99.7[c]
Smilagenin	25[a,b]	99.8[c]
	50[a,b]	100.2[c]
Tigogenin	25	107.3[d]
	50	96.0[d]
Neo-tigogenin	25	104.1[d]
	50	100.0[d]

[a] Amount in extract taken as the sum of sarsasapogenin and smilagenin yields.
[b] Recovery of added smilagenin calculated as sarsasapogenin.
[c] Mean of results from *S. aristolochaefolia*, *A. officinalis* and *Y. glauca*.
[d] Only applicable to *Y. glauca*.

TABLE IV

COMPARISON OF DENSITOMETRIC AND INFRA-RED ASSAY RESULTS

Species	*Morphological part*	*Infra-red results* (%)			*Densitometric results* (%)[a]		
		25β-Sapogenins	*25α-Sapogenins*	*Total*	*5β-Sapogenins*	*5α-Sapogenins*	*Total*
A. officinalis	Rhizome						
Sample 1		1.00	—	1.00	0.99	—	0.99
Sample 2		0.78	—	0.78	0.75	—	0.75
S. aristolochaefolia	Root	0.43	0.02	0.45	0.46	—	0.46
S. regellii	Root	0.05	0.16	0.21	0.19	—	0.19
Y. glauca	Leaf						
Sample 1		0.86	0.39	1.25	0.84	0.38	1.22
Sample 2		1.15	0.29	1.14	1.44	0.26	1.40
Sample 3		1.12	0.27	1.39	1.14	0.28	1.42

[a] Moisture-free basis.

on extracts of the same three species to determine the recovery of added smilagenin, calculated as sarsasapogenin. The recovery from an extract of *Y. glauca* of added neo-tigogenin and tigogenin was also carried out. In all cases, within the limits of experimental error, quantitative recoveries of the added sapogenins were obtained (Table III).

Close correlation was found between the assay results obtained using the infra-red and densitometric methods (Table IV). In the assay of diosgenin it was shown that the results obtained by the infra-red spectroscopic method were higher, as it estimated diosgenin and 25α-spirosta-3,5-diene together, whereas the two compounds were separated by thin-layer chromatography, resulting in the estimation of diosgenin only by the densitometric method. Sarsasapogenin, smilagenin, neo-tigogenin and tigogenin are saturated compounds and it was shown that refluxing with 2 *N* hydrochloric acid for 2 h did not produce any significant decomposition of the sapogenins, although solutions prepared from the acid-treated compounds were yellow in colour. The unsaturated compound, diosgenin, however, was found to decompose by between 5.1 and 7.3 %[7].

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CHROM. 3395

ISOTOPE SEPARATION FACTORS OF ^{14}C-AMINO ACIDS IN ION-EXCHANGE ON RESINS WITH CARBOXYLIC GROUPS

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SUMMARY

The overall isotope effect in ion exchange for ^{12}C–^{14}C amino acids with isoionic points > 7 was measured by an automated procedure. Isotope separation factors were then calculated by use of breakthrough curves at 25° and the height equivalent to a theoretical plate for the chromatographic column employed. They were found to be chiefly correlated with the pK_i of the basic groups which are concerned in exchange interactions.

The use of continuous flow radioactivity detectors in the study of ion-exchange processes involving labelled molecules is well-known in the literature[1]. More recently this technique has gained widespread acceptance, mainly because it does not interfere in any way with subsequent manipulation of the effluent, as in amino acid colorimetric analysis. A comprehensive study on this technique was published by PIEZ[2], who was also able to verify the isotope effect in amino acid analysis during the exchange on cationic resins with sulphonic groups.

In previous papers[3] by the present author, the exchange of labelled amino acids, with an isoionic point between 7.5 and 10.5, was studied on a buffered cationic resin with carboxylic groups. The elementary separation factor, *i.e.* the equilibrium constant K for the isotope exchange reaction

$$(A^+R^-)_{res} + (^*A^+)_{sol} \rightleftarrows (^*A^+R^-)_{res} + (A^+)_{sol} \quad (1)$$

where R^- is the anion of the resin macromolecule, A^+ and $^*A^+$ are the ^{12}C and ^{14}C amino acid cations, respectively (the subscripts refer to resin and solution phases), was calculated for 1-^{14}C-DL-lysine from the overall isotope effect using the breakthrough curve and the height equivalent to a theoretical plate for the chromatographic column employed. In the present report the procedure was automated, and a continuous flow radioactivity detector connected in series with a recording ultraviolet spectrophotometer, so that the analyzer could run unattended and breakthrough curves started at any time.

A schematic drawing of the equipment is given in Fig. 1. The apparatus is basically an automatic amino acid analyzer[4], with some modifications, in order to record breakthrough curves, instead of elution chromatograms. The cationic exchange

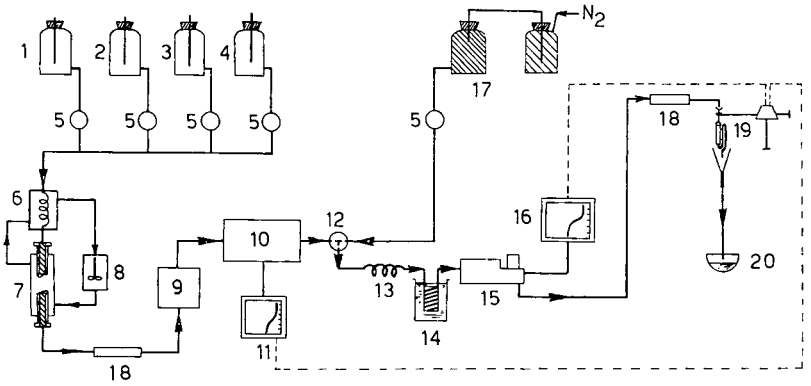


Fig. 1. 1 = Buffer solution reservoir; 2 = amino acid solution reservoir; 3 = water reservoir; 4 = acid (regenerating solution) reservoir; 5 = micro pump; 6 = preheater; 7 = 150-cm chromatographic column; 8 = thermostatic unit; 9 = pH-meter and recorder; 10 = radioactivity flow detector; 11 = radioactivity recorder; 12 = T-fitting; 13 = mixing coil; 14 = colorimetric reaction coils; 15 = spectrophotometer; 16 = absorbance recorder; 17 = ninhydrin solution reservoir; 18 = flow meters; 19 = siphon stand; 20 = waste.

resin was Amberlite CG 50 (BDH; Rohm and Haas) minus 400 mesh. The finest particles were isolated and fractionated by repeated sedimentation in water to obtain beads in a narrow range of size distribution (10–14 μm). The particle size was determined microscopically with the resins swollen in water, and the fractions carefully inspected before use. The 150 cm chromatographic column was made of precision bore borosilicate tubing, had an internal diameter of 0.35 cm and was flanged at the top and bottom so that the teflon fittings described by JONSSON AND SAMUELSON[5] could be attached. Buffers and solutions[3,6] were stored in Mariotte flasks and fed by means of micro pumps (Beckman, type 74608, Fullerton, Calif., U.S.A.) to the chromatographic column, after preheating in a jacketed coil 0.5 m long, made of teflon tube, with an inner diameter of 2 mm. The temperature was maintained constant at 25 $\pm$ 0.1° by the circulation of water from a thermostatic unit through the jackets of the preheater and the column. The bed height was 120 cm, when the resin was buffered at pH 6.7. All solutions were prepared with CO_2-free, triply distilled water. Each reservoir bottle was connected to a de-aerator in order to prevent the formation of gas bubbles. The resin was buffered at the pH of maximum capacity (pH_{max}) for each amino acid[3,6] and washed with CO_2-free water. A 10^{-3}–10^{-4} M amino acid solution (containing 10–30 μ C/l of labelled compound), the pH of which was carefully adjusted to pH_{max} by means of a small addition of $NaHCO_3$ or HCl solution, depending on the case, was then passed through the column. Change-over of operations was performed with the aid of solenoid-operated valves, which were actuated by a robot-timer (Industri AB Reflex, Stockholm, Sweden), not indicated in the figure. Pre-set times were obtained empirically during preliminary runs. The effluent from the column was continuously monitored for pH and subsequently passed into a flow detector (Packard Tri-Carb, Model 320, Lagrange, Ill., U.S.A.) for the radioassay of labelled amino acids[7]; the apparatus was equipped with a strip chart recorder (Packard, Model 380). The column effluent was then mixed in a commercial T-fitting with the ninhydrin reagent stored at a slight overpressure[8] under nitrogen in a dark Mariotte flask and delivered at a constant rate by a micro pump.

The solution passed through teflon capillary tubing (30 cm long) to achieve mixing. The colour was then developed in a teflon reaction coil about 20 m long and with an inner diameter of 1 mm, kept in a thermostated bath at 100°. Absorbance was read at 570 nm in a spectrophotometer (Spectronic, type 600E, Bausch and Lomb, Rochester, N.Y., U.S.A.), the output of which was recorded (Speedomax type G potentiometer recorder, Leeds and Northrup, Philadelphia, Pa., U.S.A.). In order to check the flow rate (0.150 cm^3/min per gram of dry resin) at regular intervals, flow meters were inserted in the line. To record the flow rate of the effluent, event markers, as described by LINDQVIST[9] and LARSEN[8], were connected to the absorbance and radioactivity recorders. The event markers were actuated by a mercury switch in the syphon stand of a fraction collector (Komby Rak, Terzano, Milan, Italy).

The equilibrium constant K of reaction (1) at 25° for labelled amino acids (I)–(IV) (see Table I) was calculated from the overall isotope effect φ for the breakthrough curve by means of the following equations[3]:

$$K = 1 + [(\varphi - 1)H_{EZ}/H_T N_{EZ}] \tag{2}$$

where H_{EZ} and H_T are the height of the exchange zone and the total height of the chromatographic column, respectively, and N_{EZ} is the number of effective plates of the exchange zone;

$$\varphi = (1/NA_{s_0}) \int_0^N n\mathrm{d}A_s \tag{3}$$

where N is the overall exchange capacity (gmoles) of the buffered resin in the column towards the amino acid at the experimental pH value, A_{s_0} the specific activity of the ^{14}C amino acid solution at the exhaustion point, A_s the specific activity, and n the overall gmoles of the amino acid at any point of the breakthrough curve. The integral of eqn. (3), which was evaluated graphically, is an obvious extension of the previously employed relation[3] for the evaluation of φ.

Results are summarized in Table I. ^{14}C-amino acids (I), (II) and (IV) were commercially available compounds (Radiochemical Centre, Amersham, Bucks, Great Britain), while (III) was synthesized[3]. ^{12}C-Amino acids were all analytically pure reagents (BDH, Poole, Great Britain), critically examined for purity[10].

TABLE I

ISOTOPE SEPARATION FACTORS IN ION EXCHANGE OF ^{12}C–^{14}C AMINO ACIDS

No.	*Amino acid*	pH_{max}	$(K-1) \times 10^{5}$[a] *at 25° (eqn. 2)*	pK_i'[b]
I	L-(+)-histidine 2-^{14}C-imidazole ring	4.5	4.5 ± 0.5 (4)[c]	5.97 (pK_2')
II	1-^{14}C-DL-lysine	6.7	7.7 ± 0.6 (5)	10.28 (pK_3')
III	6-^{14}C-DL-lysine	6.7	5.9 ± 0.5 (6)	8.90 (pK_2')
IV	L-(+)-arginine, guanidine, ^{14}C	7.7	8.6 ± 0.7 (6)	13.2 (pK_3')

[a] Average value, with standard deviation, measured on 4–6 replicate chromatograms.
[b] K_i' is the acid dissociation constant.
[c] The figure in parentheses is the number of determinations.

As was previously stated[3], the isotope effect was only significant if the labelled C-atom was adjacent to the basic function or if it constituted the carboxylic group itself. The present results are in agreement with preliminary measurements[3], substantiating a correlation between isotope separation factors and pK_1' of basic groups which are concerned in exchange interactions, as is shown in Table I (K_1' = acid dissociation constants): the isotope effect increases with increasing basicity of these groups. The secondary isotope effects under study and their variation with radiocarbon position are probably caused to a large extent by an activation energy effect on the rate of chromatographic displacement, the frequency factor effect being either small or constant. Changes in non-bonding interactions and tunnelling effects must be unimportant, since no bond rupture is involved, except in interactions which concern quaternary basic groups and active sites of the resin molecule[6]. The only effects remaining which might influence the activation energy are anharmonicity, inductive interactions and hyperconjugative interactions. It is difficult to assess the relative importance of these factors, because the quantitative use of small secondary isotope effects is not a reliable tool for ascertaining mechanisms. From the correlation between isotope separation factors and pK_1' of the basic functions, which are adjacent to the centre of isotopic substitution, a marked dependence on the slightly different dissociation constants of these functions in the labelled and unlabelled amino acids studied could be argued. The source of this difference may arise from considerations of zero-point vibration for the anharmonic system, as well as from inductive effect or hyperconjugative release of electrons from the isotopically substituted C–C or C–H bonds. Inductive and hyperconjugative influence on secondary deuterium isotope effects has been thoroughly discussed[11] but C–C hyperconjugation may also and probably does have a significant role and should not be neglected[12]. In the labelled molecules of Table I, particularly, the net effect is an "increased" basicity of the nitrogen atom, which enhances affinity towards the resin macromolecule. Similar conclusions were recently reached in the examination of the primary isotope effects shown by amines on cation exchange resins, the main factor affecting constants of isotope exchange reactions being the ratio of the ionization constants for the two pure isotopic forms of the amines. These factors, rather than the somewhat greater electronegativity of ^{14}C, as was postulated by PIEZ AND EAGLE[14], can adequately account for all the isotope effects observed and for the increase in retention volume of radiocarbon-labelled amino acids, during ion-exchange chromatography[3,15], which is usually maximal when the carboxyl group is labelled. Vanishing secondary isotope effects, which were observed in some instances[15], could be considered from this point of view as a result of a fortuitous cancellation between opposing and apparently counterbalanced effects.

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CHROM. 3402

SYNERGISM IN THE REVERSED-PHASE PARTITION CHROMATOGRAPHY OF AMERICIUM, CERIUM AND LANTHANUM

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SUMMARY

Retention of the ions of $^{241}Am^{3+}$, Ce^{3+} and La^{3+} in reversed-phase chromatography is considerably higher on paper treated with a mixture of HTTA and TOP than if each extractant is used separately. The value for log $(1/R_F-1)$ for all three ions reaches a maximum at a fixed composition of components in the mixture. Thus a synergistic effect occurs not only in the liquid–liquid extraction process but also in the reversed-phase partition chromatography.

The composition of the metallic species formed in the organic phase is determined by correlating log $(1/R_F-1)$ with the amount of HTTA and TOP fixed on the paper and with the concentration of H^+ ions in the mobile phase at constant ionic strength. The results imply that species of composition $M(TTA)_3(TOP)_2$ are formed, which is in agreement with the data obtained by solvent extraction on the same system.

Reversed-phase partition chromatography with various organic solvents as the stationary phase has proved to be a very useful method of investigating the behaviour and separation of metallic ions. Previous results obtained by this method suggest an analogy with solvent extraction processes[1–7].

Taking into account the similarity of these two processes, it would be interesting to know whether the synergistic effect, so often found in solvent extraction[8–10], also occurs in chromatographic processes.

Our previous investigation[11] on the behaviour of rare earths on papers impregnated with tri-*n*-octylphosphate (TOP) showed a very low affinity towards this solvent and consequently a low retention on the stationary phase. In the present paper the behaviour of Am(III), Ce(III) and La(III) on papers treated with a mixture of thenoyltrifluoroacetone (HTTA) and TOP was examined. A considerable increase in the retention, *i.e.* decrease in the R_F value, appears for all three tervalent ions. This behaviour closely follows the synergistic effect found in solvent extraction[9,10]. The composition of the complexes and the optimal conditions for mutual separation of the ions were determined from the results obtained.

EXPERIMENTAL

Impregnation of the chromatographic paper with a mixture of a TOP (Fluka, Buchs, Switzerland) and HTTA (BDH, London) solution in carbon tetrachloride was carried out as described previously[6,7]. An aqueous hydrochloric acid solution of selected concentration was used as the mobile phase.

Investigations were carried out with chloride solutions of La^{3+}, Ce^{3+} and $^{241}Am^{3+}$. In the case of La^{3+} and Ce^{3+}, 0.010 ml aliquots containing about $5 \cdot 10^{-7}$ equivalents of each ion were spotted on the paper. To check the R_F values, the radioisotope cerium-144 (Ce^{3+}) was used in some runs. The R_F values of $^{241}Am^{3+}$ were determined radiometrically with a γ-scintillation NaI (Tl) counter.

The experiments were carried out on Whatman No. 1 paper strips, 2 × 35 cm, in glass cylinders 14 × 40 cm, by ascending development. The chromatograms were developed for 4–5 h at room temperature.

RESULTS AND DISCUSSION

The chromatographic papers were impregnated with solvents having different molar ratios of HTTA to TOP, but the total concentration of the two extractants was kept constant at 0.2 *M*. Table I gives the R_F values of La^{3+}, Ce^{3+} and Am^{3+}, while Fig. 1 shows log $(1/R_F - 1)$ *versus* the molar concentrations of HTTA and TOP in the mixture. La^{3+} and Ce^{3+} were run with 0.01 *M* HCl and Am^{3+} with 0.05 *M* HCl at ionic strength μ = 0.1 Na(H)Cl. The R_F values were determined relative to the acid front. When impregnated papers are treated with dilute mineral acids as the mobile phase, it is possible that acid is absorbed by the paper. Therefore it is convenient to

TABLE I

R_F VALUES FOR Am^{3+}, La^{3+} AND Ce^{3+}

Paper impregnated with a mixture of HTTA and TOP. The mobile phase for La^{3+} and Ce^{3+} is 0.01 *M* HCl and for Am^{3+} 0.05 *M* HCl; μ = Na(H)Cl.

Mixture of organic extractants		R_F		
		Am^{3+}	La^{3+}	Ce^{3+}
TOP	0.20 *M*	0.99	0.97	0.96
HTTA TOP	0.02 *M* 0.18 *M*	0.98	0.95	0.81
HTTA TOP	0.05 *M* 0.15 *M*	0.66	0.55	0.12
HTTA TOP	0.10 *M* 0.10 *M*	0.14	0.14	0.03
HTTA TOP	0.15 *M* 0.05 *M*	0.16	0.21	0.05
HTTA TOP	0.18 *M* 0.02 *M*	0.28	0.39	0.15
HTTA	0.02 *M*	0.98	0.97	0.97

define the acid front as the distance between the starting line and the line where the acid no longer appears on the paper. The acid free zone is immediately behind the solvent front[12].

From the results presented it is seen that the R_F values for all three ions are nearly equal to unity when the paper is impregnated only with 0.2 *M* HTTA or 0.2 *M* TOP. A mixture of these extractants causes a considerable increase in the retention of all three ions. A maximum value for log $(1/R_F - 1)$ is obtained at a certain composition of the mixture.

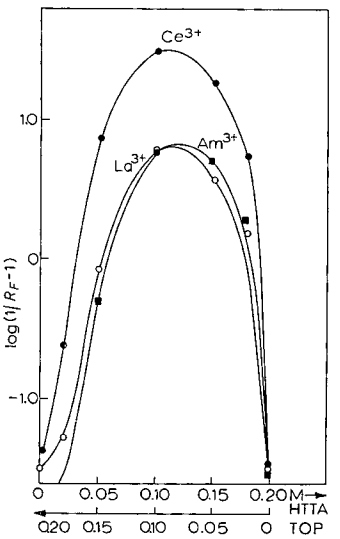


Fig. 1. Plot of log $(1/R_F - 1)$ for Am^{3+}, La^{3+} and Ce^{3+} as functions of molar concentrations of HTTA and TOP in the mixture.

To determine the composition of the complexes we used a relation which allows us to correlate reversed-phase partition chromatography with solvent extraction. If the aqueous and organic phases in both processes are identical the following equation is valid[1,13]

$$\log (1/R_F - 1) = \log E_a^0 + \log k \qquad (1)$$

where E_a^0 is the extraction coefficient, and k is a constant depending on the chromatographic experimental conditions.

The dependence of the R_F values for La^{3+}, Ce^{3+} and Am^{3+} on the H^+ ion concentration and on the amount of HTTA and TOP was examined. In these experiments two variables out of three were always kept constant. Fig. 2 shows the plot of log $(1/R_F - 1)$ against log $[H^+]$. The chromatographic paper strips were impregnated with a mixture of 0.15 *M* HTTA + 0.05 *M* TOP.

The behaviour of Am^{3+}, La^{3+} and Ce^{3+} has been investigated as a function of the amount of HTTA at constant TOP concentration in the organic phase and at constant H^+ ion concentration in the mobile phase. The amount of HTTA, mg/cm^2, is determined by weighing the paper before and after impregnation. It has been

proved that the weight increases obtained by treating the paper with a mixture of HTTA and TOP are additive compared with the values obtained by impregnation only with HTTA or with TOP. On the graphs, the HTTA and TOP concentrations are expressed in millimoles.

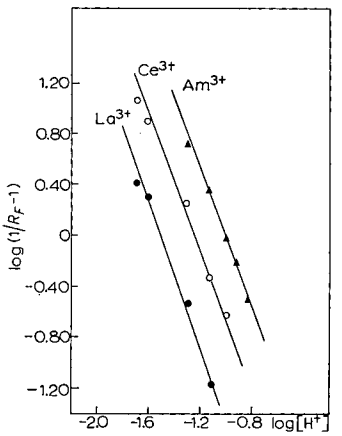
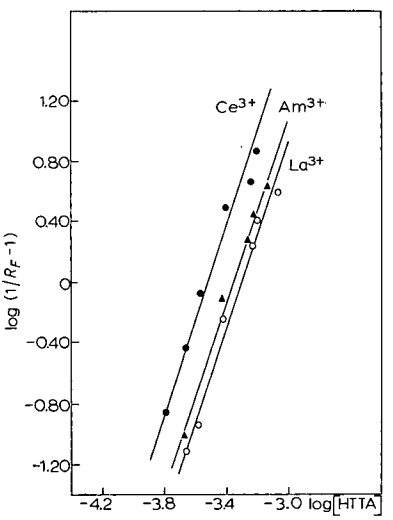


Fig. 2. Plot of log $(1/R_F - 1)$ *vs.* log $[H^+]$ for La^{3+}, Ce^{3+} and Am^{3+}. The slope of the straight lines is −3.

Fig. 3 shows log $(1/R_F - 1)$ *versus* log [HTTA] for Am^{3+}, La^{3+} and Ce^{3+}. The TOP concentration in the impregnation mixture was always 0.05 *M*. The eluent for La^{3+} and Ce^{3+} was 0.01 *M* HCl and for Am^{3+} 0.05 *M* HCl at constant ionic strength, $\mu = 0.1$ Na(H)Cl.

The dependence of log $(1/R_F - 1)$ on log [TOP] for La^{3+}, Ce^{3+} and Am^{3+} was investigated in the same way (Fig. 4). The HTTA concentration in the mixture with



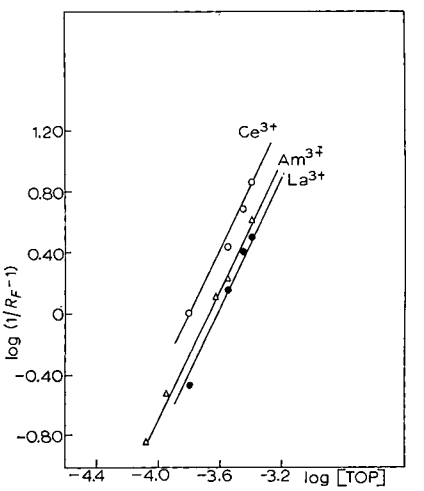


Fig. 3. Plot of log $(1/R_F - 1)$ *vs.* log (HTTA] in a mixture with TOP for Am^{3+}, La^{3+} and Ce^{3+}. The slope of the straight lines is 3.

Fig. 4. Plot of log $(1/R_F - 1)$ *vs.* log [TOP] in a mixture with HTTA for Am^{3+}, La^{3+} and Ce^{3+}. The slope of the straight lines is 2.

TOP was 0.15 *M*. The concentration of hydrochloric acid for La^{3+}, Ce^{3+} and Am^{3+} was the same as in the preceding graph.

If synergistic effects[10,14] occur in the extraction of metallic ions with a mixture of two organic extractants (in the present case, one is HTTA which forms chelates with metallic ions, and the other is a ternary organophosphate, TOP) the over-all reaction may be represented in the following way:

$$M^{n+} + n\text{HTTA} + m\text{S} \rightleftharpoons \text{M(TTA)}_n\text{S}_m + n\text{H}^+ \qquad (2)$$

where S is the organic electron donor molecule.

Taking into account the valency of the ions, one obtains:

$$M^{3+} + 3\text{HTTA} + 2\text{TOP} \rightleftharpoons \text{M(TTA)}_3\text{(TOP)}_2 + 3\text{H}^+ \qquad (3)$$

The equilibrium constant of this reaction is:

$$K_e = \frac{[\text{M(TTA)}_3\text{(TOP)}_2]_{org}[\text{H}^+]^3}{[\text{M}^{3+}]\,[\text{HTTA}]^3_{org}[\text{TOP}]^2_{org}} \times f(\gamma) \qquad (4)$$

where $f(\gamma)$ is the ratio of the activity coefficients of the components. For certain experimental conditions it is assumed that $f(\gamma)$ is constant. The extraction coefficient of the metallic species is then:

$$E^0_a = \frac{[\text{M(TTA)}_3\text{(TOP)}_2]_{org}}{[\text{M}^{3+}]} \qquad (5)$$

combining these with eqn. (1), one obtains:

$$\log (1/R_F - 1) = 3 \log [\text{HTTA}] + 2 \log [\text{TOP}] - 3 \log [\text{H}^+] + \text{const.} \qquad (6)$$

The term "const." includes K_e, $f(\gamma)$ and k. Taking into account the slopes of the straight lines in Figs. 2, 3, and 4, the extracted complexes fixed on the impregnated paper are in the form of $\text{M(TTA)}_3 \cdot \text{(TOP)}_2$. The results are in accordance with the data for the extraction of some tervalent lanthanides and actinides with a mixture of HTTA and ternary organophosphate[10]. The synergistic effect noted is in agreement with the assumption that an increased solubility of metallic chelates in the organic phase is caused by the formation of an adduct molecule with the ternary organophosphate[15,16].

The behaviour of other tervalent lanthanides (Pr, Nd, Sm, Eu) was also investigated on paper impregnated with the aforementioned mixture of extractants. The R_F values obtained have shown that the retention of these ions increases with the increase of their atomic number or with the decrease of their crystalline ionic radius.

The separation factor $(1/R_F - 1)_{z-1}/(1/R_F - 1)_z$ for ${}_{58}\text{Ce}-{}_{57}\text{La}$ can be calculated from Fig. 2. The mean value for the separation factor for this pair of lanthanides with HCl in the concentration range of 0.01–0.075 *M* ($\mu = 0.1$) is 4.5. When compared with their separation factor obtained on paper treated with acid esters of phosphoric acid[1,6] this value is more than twice as high.

Conditions for separation of Am^{3+}, Ce^{3+} and La^{3+} can be chosen from Fig. 2. The best separation is obtained with 0.05 and 0.075 *M* HCl.

ACKNOWLEDGEMENTS

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Notes

CHROM. 3431

Analytical chemical studies on steroids

Part XIX. Gas chromatographic separation of isomeric 16- and 17-oxosteroids

In the previous publications of this series, the authors reported the gas chromatography of C-13 and C-14 epimeric androstanes[1-3]. The occurrence of 16-oxosteroids as the natural products prompted us to explore the gas chromatographic separation of the usual 17-oxo compounds and their corresponding 16-isomers. The present paper deals with the gas chromatographic behavior of five pairs of isomeric oxosteroids and their O-trimethylsilyl oxime derivatives.

Experimental

Materials. Almost all the samples were prepared by the methods described in the preceding papers[1-3] and the others by known procedures.

Preparation of derivatives. To a solution of the sample (about 1 mg) in pyridine (0.5 ml) was added hydroxylamine hydrochloride (about 1.5 mg), and the resulting solution was heated at 70–80° for 1 h. This solution was further treated with hexamethyldisilazane (0.2 ml) and trimethylchlorosilane (0.1 ml) in the manner described by SWEELY *et al.*[4] After evaporation of the solvent the residue was extracted with *n*-hexane (0.5 ml), centrifuged and the supernatant was used for gas chromatography.

Gas chromatography. The apparatus used for this work was a Shimadzu Model GC-1C gas chromatograph equipped with a hydrogen flame ionization detector and a U-shaped stainless steel column (1.875 m × 3 mm I.D.). The column was packed with either 1.5 % SE-30 on a support of Chromosorb W (60–80 mesh) or 2 % OV-17 on Shimalite W (60–80 mesh). The temperatures of column, detector and injection chamber were kept at 220°, 280° and 250°, respectively. Nitrogen was used as carrier gas at a flow rate of 60 (for SE-30) or 80 ml/min (for OV-17).

Results and discussion

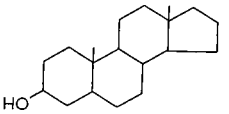
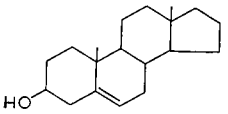
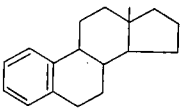
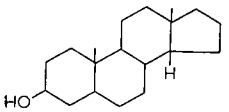
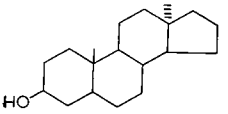
Gas chromatography of five pairs of isomeric 16- and 17-oxosteroids was carried out with 1.5 % SE-30 and 2 % OV-17. The relative retention times observed, using cholestane as a reference compound, are given in Table I.

When the oxosteroid itself was applied, satisfactory separation of isomeric 16- and 17-oxosteroids could not be attained on any kind of stationary phase. It is generally believed that one of the chief advantages of a non-selective phase lies in its effectiveness with different molecular shapes. Therefore, transformation of these oxosteroids into suitable derivatives was undertaken to accentuate the existing positional difference. In gas chromatographic work it is desirable that the derivative should be formed quantitatively and, in addition, the retention time of the product should be different from that of the starting material. Some pertinent carbonyl reagents, such as

TABLE I

RELATIVE RETENTION TIMES OF ISOMERIC 16- AND 17-OXOSTEROIDS

Conditions: stainless steel column (1.875 m × 3 mm I.D.); 1.5 % SE-30 on Chromosorb W (60–80 mesh) or 2 % OV-17 on Shimalite W (60–80 mesh); N_2 flow rate, 60 or 80 ml/min; column temp., 220°; detector temp., 280°; injection chamber temp., 250°.

Compound structure	*Position of keto group*	*Free*[a]		*NOTMS derivative*[b]	
		1.5 % SE-30	*2 % OV-17*	*1.5 % SE-30*	*2 % OV-17*
 (I)	16	0.42	0.90	0.86	0.95
	17	0.42	0.91	0.82	0.80
 (II)	16	0.40	0.90	0.85	0.96
	17	0.39	0.89	0.79	0.80
 (III)	16	0.21	0.43	0.36	0.59
	17	0.22	0.43	0.34	0.49
 (IV)	16	0.43	0.91	0.70	0.75
	17	0.41	0.85	0.81	0.79
 (V)	16	0.40	0.79	0.68	0.73
	17	0.39	0.68	0.58	0.53
Cholestane		1.00 (13.2 min)	1.00 (10.8 min)	1.00 (13.2 min)	1.00 (10.8 min)

[a] Sample without any pretreatment.
[b] Trimethylsilylated product of the oxime.

dimethylhydrazine and methoxyamine, have already been proposed for this purpose[5,6]. However, these techniques were still found to be unsatisfactory for the complete separation of these isomers, and hence introduction of the more accentuated substituent to the carbonyl group appeared to be necessary in order to increase the separation factors. Considering the steric interaction with the bulky reagent, the two-step method was devised for the preparation of the derivatives. Thin-layer chromatographic inspection demonstrated that formation of the oxime with hydroxylamine proceeded readily under the above-mentioned conditions. The procedure of SWEELEY *et al.* being then employed, subsequent O-trimethylsilylation of the oxime was also accomplished with relative ease.

The isomeric O-trimethylsilyl oxime derivatives thus prepared gave a single peak exhibiting different retention times on either SE-30 or OV-17. The complete

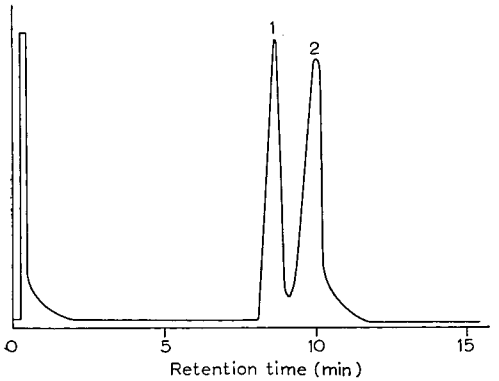


Fig. 1. Gas chromatogram of the O-trimethylsilyl oxime derivatives. 1 = 3β-Hydroxyandrostan-17-one; 2 = 3β-hydroxyandrostan-16-one. Column: 2% OV-17 (on Chromosorb W).

separation of the isomers was obtained with the latter of these two stationary liquids. A typical gas chromatogram is illustrated in Fig. 1. It is of particular interest that in almost all cases the 17-oxosteroid was eluted before the corresponding 16-isomer with only one exception, the 14β-series. This technique appears to offer a means of magnifying the existing slight difference in an arrangement of the functional groups on the steroid nucleus.

In addition the steroid numbers[7] were determined of the usual C/D-*trans* steroids having ring D ketone and their O-trimethylsilyl oxime derivatives. As shown in Table II, steroid number contributions (ΔSN) for the O-trimethylsilyl oxime of 16- and 17-ketones were found to be 2.1 and 1.9, respectively. The constancy of the values observed here may be useful in distinguishing the position of the ketone.

TABLE II

STEROID NUMBER CONTRIBUTION FOR O-TRIMETHYLSILYL OXIME

Compound No.	*Position of keto group*	*SN*[a]		*Δ SN*[a]
		Parent compound	*NOTMS derivative*	
I	16	24.3[b]	26.4	2.1
	17	24.3[b]	26.2	1.9
II	16	24.3[b]	26.4	2.1
	17	24.3[b]	26.2	1.9
III	16	21.1[c]	23.2	2.1
	17	21.1[c]	23.0	1.9

[a] *SN* = Steroid number (retention time: cholestane 13.2 min, androstane 1.6 min); ΔSN = steroid number contribution for O-trimethylsilyl oxime.

[b] Trimethylsilylated product.

[c] Sample without any pretreatment.

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CHROM. 3417

Gas chromatographic separation of pyridine homologues, chloroanilines and toluidines

During the course of studies on the separation of isomers by solvent extraction, GLC techniques have been used for quantitative analysis of the isomers present in the solvent phases. Several liquid phases have been studied and it has been found that glycerol, diglycerol and polyphenyl ether (5 ring-OS-124) give good resolution of pyridine bases, *o*- and *p*-chloroanilines and *o*- and *p*-toluidines.

Glycerol and diglycerol have previously been used[1,2] for the analysis of pyridine bases but have not been reported for *o*- and *p*-chloroanilines or *o*- and *p*-toluidines. Of these two stationary phases, diglycerol gave very good results. It was found with glycerol that, whilst it gave good resolution, its efficiency decreased with time for the chloroanilines and the toluidines. The resolution obtained for *o*- and *p*-chloroanilines and for *o*- and *p*-toluidines is given in Table I.

It is believed that the use of polyphenyl ether for the separation of pyridine bases, *o*- and *p*-chloroaniline and *o*- and *p*-toluidines has not previously been reported.

Experimental

Instrumentation. The Pye "Series 104" Dual Flame Ionisation, Temperature Programmed Chromatograph Model 24 was used.

TABLE I

RETENTION TIMES AND CONDITIONS

Conditions		*Compound*	*Retention time (min)*
Stationary phase and column length	*Temperature (°C)*		
20% polyphenyl ether; 9 ft.	110	Pyridine	14.5
		α-Picoline	17.3
		2,6-Lutidine	20.5
		β-Picoline	30.5
		γ-Picoline	33.7
		2,5-Lutidine	35.0
		2,4-Lutidine	40.5
	135	*o*-Toluidine	70.0
		p-Toluidine	75.0
	178	*o*-Chloroaniline	25.0
		p-Chloroaniline	30.0
12% diglycerol; 5 ft.	90	*o*-Toluidine	3.0
		p-Toluidine	4.0
	115	*o*-Chloroaniline	1.5
		p-Chloroaniline	2.5
20% glycerol; 5 ft.	90	*o*-Toluidine	3.0
		p-Toluidine	4.0
	115	*o*-Chloroaniline	1.5
		p-Chloroaniline	2.5

Solid support preparation. Chromosorb P of 80–100 mesh was treated with alcoholic sodium hydroxide (6% NaOH on dried Chromosorb P), the alcohol subsequently being evaporated.

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CHROM 3418

Separation of proteins of "Elapidae" snake venoms on polyacrylamide gel

The disc electrophoresis technique, first developed for acid proteins by ORNSTEIN AND DAVIS[1] was modified later for basic proteins by REISFELD *et al.*[2]. Slight modifications of the original method have been proposed[3–9]; the further extension and numerous applications of the method are summarized by Canalco[10] and more recently by HANSL[11] and REICH *et al.*[12].

A few publications on the separation of acid proteins from snake venoms have been presented by RUSSELL *et al.*[13,14] but none have been published up to now on the electrophoretic behaviour of "Elapidae" snake venoms in disc electrophoresis.

Materials and methods

Our study was made on eight different Asiatic and African "Elapidae" snake venoms: *Naja naja atra* (kindly supplied by Prof. C. C. YANG, Kaoshiung Medical College, Formosa), *Naja Tripudians*, *Bungarus Caeruleus* (kindly supplied by Prof. P. BOQUET, Garches, France), *Naja Melanoleuca*, *Dendraspis Augustiseps*, *Naja naja* (kindly supplied by the Thaïland Red Cross), *Naja nivea* and *Naja naja Haje*.

All these venoms were obtained in a freeze dried state and were dissolved at concentrations of 5–10 % in an appropriate buffer solution.

All chemicals and reagents were of pure analytical grade and were used without further purification. Acrylamide and N,N'-methylenebisacrylamide were purchased from Fluka A. G. β-Alanine, glycine and TEMED* were supplied by Serva (Heidelberg).

A first run was made according to the method of ORNSTEIN AND DAVIS[1] in order to encapture the fractions moving toward the positive pole.

Operating conditions. Acrylamide gel: 7.5 % (ratio acrylamide:Bis, 1:37); 20 V/cm of tube; time of migration: 150 min.

A second run following the method of REISFELD[2] was made for the basic proteins.

Operating conditions. Acrylamide gel: 15% (ratio acrylamide:Bis, 1:150); 20 V/cm of tube; time of migration: 150 min.

Both experiments followed the original methods with some slight modifications: small pore gel and spacer gel were prepared as usual; large pore gel preparation was omitted and the sample was placed at the top of the spacer gel in a 5% sucrose solution.

Results and discussion

Table I shows under A the number of fractions obtained by the above mentioned method and under B the number of fractions obtained by agar gel electrophoresis using a phosphate buffer pH 7.0 (ionic strength = 0.1).

Fig. 1 represents the separations obtained from the eight snake venoms. Proteins were colored with Amidoblack 10 B. Runs for basic and acid proteins are presented on the same drawing. The numbering of the tubes corresponds to that of Table I.

Fig. 2 shows the densitometric scanning of the fractions of *Naja naja atra* venom (see Fig. 1 tube No. 1). Scanning was made on a Vitatron densitometer using a slot especially adapted for polyacrylamide gel scanning purposes.

* TEMED = N,N,N',N'-Tetramethylethylenediamine.

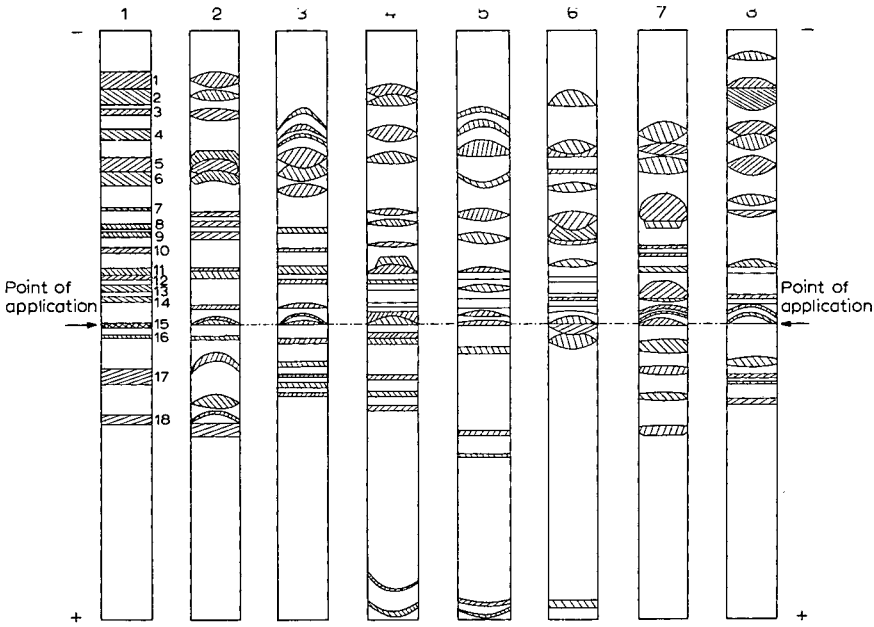


Fig. 1. Electrophoretic separations of eight "Elapidae" venoms.

TABLE I

No.	*Kind of venom*	*Number of fraction*	
		A	*B*
1	*Naja naja atra*	18	7
2	*Naja naja* (Thaïland)	19	4
3	*Naja nivea*	18	5
4	*Naja melanoleuca*	23	6
5	*Naja naja haje*	19	3
6	*Bungarus Caeruleus*	19	6
7	*Naja Tripudians*	17	5
8	*Dendraspis Augustiseps*	18	4

Disc electrophoresis is a very adequate separation tool for proteins in general and has many advantages over the other techniques commonly used in zone electrophoresis. As can be seen from Table I, the number of fractions obtained is great; moreover the reproducibility of the method is fairly good, resolution is excellent and the separation is accomplished in quite a short time.

Refrigeration during the separation was not necessary, the diffusion of the different fractions being small. The interpretation of the identity of the different fractions obtained and the measure of their enzymic activity are now being studied in our laboratory.

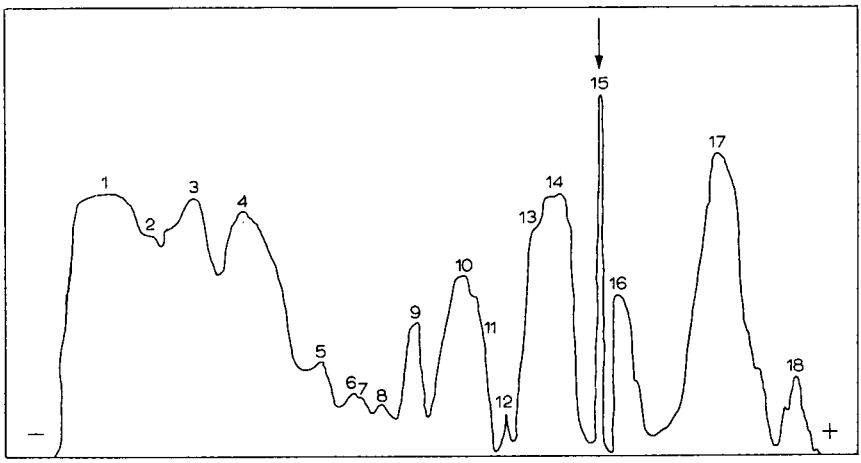


Fig. 2. Densitometric scanning of *Naja naja atra* venom (Vitatron densitometer). The point of application is indicated by the arrow.

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CHROM. 3455

Separation on a Sephadex column of a complex mixture of azo dyes used as fluorometric chelating agents

Separation of two very similar and heavily substituted azo compounds has been effected on a column of Sephadex G-10. The compounds involved were derived from *o,o'*-dihydroxyazobenzene by the introduction of one and of two methyleneiminodiacetic acid groups. *o,o'*-Dihydroxyazobenzene is used for the fluorometric determination of magnesium[1] and the methyleneiminodiacetic acid group, $-CH_2-N(CH_2COOH)_2$, which is half of the ethylenediaminetetraacetic acid molecule, has been introduced into acid–base indicators to convert them to metallochromic indicators; see, for example, the work of SCHWARZENBACH *et al.*[2] and of DIEHL *et al.*[3,4]. In the present work the methyleneiminodiacetic acid group was introduced into the *o,o'*-dihydroxyazobenzene molecule, thus producing a compound with two dissimilar chelating groups. The introduction of the methyleneiminodiacetic acid group was effected by the Mannich condensation of *o,o'*-dihydroxyazobenzene, formaldehyde and iminodiacetic acid using dimethyl sulfoxide as solvent. The reaction mixture was worked up by a process which removed the unreacted starting materials, including *o,o'*-dihydroxyazobenzene (H_2Dhab). The product obtained was a mixture composed of *o,o'*-dihydroxyazobenzene bearing one and two methyleneiminodiacetic acid groups (H_4Idhab and H_6Iidhab, respectively), more highly substituted products, and polymerized material. The dried crude product was soluble in basic solutions, basic organic solvents, and in concentrated acid solutions. Decomposition occurred in the latter solutions so that it was necessary to find some chromatographic system operable in basic solutions. No separations were obtained on alumina or silica gel. Separation proved possible, however, on Sephadex.

The use of dextran gels in aqueous systems is described in literature prepared by FLODIN[5] for Pharmacia, and more specifically the adsorption of aromatic compounds, especially phenols, on Sephadex was described by GELOTTE[6]. That aromatic acids are adsorbed on Sephadex columns had been demonstrated by PORATH[7]. That for adsorption to occur the aromatic molecule must be small enough to penetrate the matrix of the gel particle was shown by WOOF[8]. Further insight into the adsorption phenomena on Sephadex is found in the review article by JANSON[9].

On the basis of this information and the information furnished by the manufacturer[3], Sephadex G-10 was chosen as the column packing material. Sephadex G-10 has an exclusion limit of 700 molecular weight units. This means that molecules smaller than 700 molecular weight units will penetrate the matrix of the gel and molecules greater than 700 molecular weight units will be excluded and be eluted from the column. The molecular weight calculated for H_4Idhab is 359 and for H_6Iidhab 504. Both molecules thus can penetrate the matrix and the separation achieved must have resulted from selective elution.

Materials and methods

Column preparation and operation. Sephadex G-10 was obtained from Pharmacia Inc., Newark, N. J. Sixty-eight grams of Sephadex were allowed to equilibrate with 0.001 *N* sodium hydroxide according to the instructions supplied by the manufac-

turer. A column 80 cm long and 1.6 cm in diameter with a bed volume of 145 ml was poured and used throughout the study.

A sample of the crude product weighing 0.4 g was dissolved in a minimum amount of sodium hydroxide and the solution was adjusted to pH 10.5. This solution was loaded onto the column and eluted with 0.001 *N* sodium hydroxide. The flow rate of the column was adjusted to 10.5 ml/cm²/h by means of a pinch clamp on the outlet of the column.

Separation of components. All of the components of the crude product were observed to be intensely colored, and upon elution down the column with 0.001 *N* sodium hydroxide four distinct, differently colored bands became apparent. The first band appeared brown, the second yellow, the third red, and the fourth a light red-brown. These four components were collected in separate beakers and the elution volumes recorded. The separations of band two and three were very good. When these purified components were rechromatographed separately, they were found to be essentially free of material from the adjacent bands. The material in each beaker was precipitated by the addition of 1 *N* hydrochloric acid. The precipitate was filtered, washed, dried and weighed. The weight percent of each component was calculated on the basis of the weight of crude material added to the column. This relative concentration was then plotted versus the volume of effluent.

Results and discussion

The elution curve is shown in Fig. 1. The first band (I) appeared to contain high molecular weight compounds with molecular weights above 700 molecular weight units. This band was eluted in the void volume (V_0) of the column. Woof[8] has shown that the molecular sieving properties of the gel permit separation of lower molecular weight phenols from phenol polymers because the polymers cannot penetrate the gel matrix.

The second band (II) was shown to contain a yellow compound identified as 1-[3'-(N,N-bis(carboxymethyl)aminomethyl)-2'-hydroxy-1'-benzeneazo]-2-hydroxybenzene (H_4Idhab) with a molecular weight of 359. The third band (III) was identified

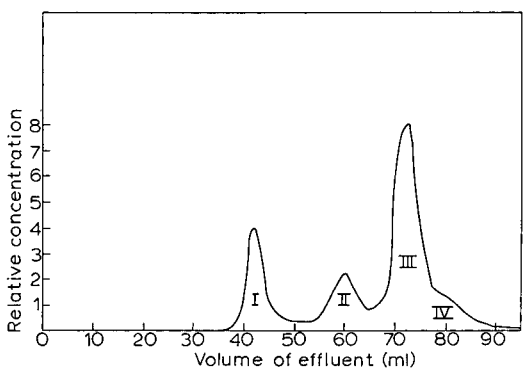


Fig. 1. Separation of products from the Mannich condensation of *o,o'*-dihydroxyazobenzene formaldehyde and iminodiacetic acid by elution from Sephadex G-10 with 0.001 *N* sodium hydroxide. I = High molecular weight material (brown); II = *o,o'*-Dihydroxyazobenzene-methyleneiminodiacetic acid (yellow, mol. wt. 359). III = *o,o'*-Dihydroxyazobenzene-di(methyleneiminodiacetic acid), (red, mol. wt. 504). IV = Unknown (red-brown).

as 1-[3'-(N,N-bis(carboxymethyl)aminomethyl)-2'-hydroxy-1'-benzeneazo]-3-[N,N-bis-(carboxymethyl)aminomethyl]-2-hydroxybenzene (H_6Iidhab), a red compound a molecular weight of 504. The fourth band (IV) was strongly adsorbed to the gel matrix and only part of it could be eluted. This band on elution formed a broad band with considerable tailing and the gel column remained a light red-brown color. This color was never completely removed from the column material. Insufficient amounts of the material in band IV were recovered to characterize the material.

The partition coefficient K_d was calculated for band II and band III using the equation $K_d = (V_e - V_0)/V_i$ where V_e is the elution volume of the component, V_0 is the void volume which is found from the elution volume of the substance that does not enter the gel matrix, and V_i is the inner volume. V_i can be calculated from the equation $V_i = g \cdot W_r$ where g is the dry weight of the gel in the column and W_r is the water regain in grams per gram of dry gel. V_i and V_o for this column were found to be 65 and 42, respectively. The K_d value for H_4Idhab was calculated to be 0.28 and for H_6Iidhab 0.52.

The reason that adsorption phenomena have been presumed to be the predominant action in effecting this separation even though the K_d values are less than one, is that band II contains a compound with a lower molecular weight than band III. GELOTTE[6] has shown that elution of aromatic substances with ammonium hydroxide pH 10.6 gave K_d values that were considerably less than when eluted with sodium chloride. Also, WOOF AND PIERCE[10] have shown that elution of phenolic acids with ammonium hydroxide have a trend of K_d values which they attribute to adsorption phenomena. The order of elution of H_4Idhab and H_6Iidhab with sodium hydroxide at pH 10.0 seems to be explainable when adsorption phenomena are considered.

The use of Sephadex to purify azo dyes and azo compounds modified to act as indicators in metal chelate titrations may help clear up some of the chemistry involved with these dyes. The use of these indicators in direct colorimetric or fluorometric analysis of metals should also profit from a close scrutiny of the purity of the compounds.

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CHROM 3450

Preparative separation technique for macromolecules by filtration through solid agar gel

Solid agar gel is not routinely used for preparative separation of macromolecules because of the slowness of diffusion and resistance to flow in this medium. However, under certain circumstances this medium presents advantages which offset its drawbacks. This paper reports an instance of successful separation on solid agar gel at a stage of purification where conventional methods failed. Nondiffusable material is trapped in the solid gel while the diffusables emerge as a mixture. Thus the method differs from fractionations on granulated agar and agarose, which do not necessarily prevent passage of nondiffusable fractions, but afford resolution of the filtrate[1,2].

The dialyzed purified extract of the coelenterate *Rhodactis howesii* contains an antigenic toxin, presumably a protein, which is part of a protein–polysaccharide complex[3,4]. This material clogged columns of DEAE and CM-cellulose and of Aminex and thus defied chromatographic fractionation; it emerged from Sephadex G-75 columns as a gelatinous mass in distilled water, or in a single frontal peak in 0.005 *M* NaCl. In the electrical field the complex moved as a block on acetate strips and did not penetrate into 5 % acrylamide gel.

In contrast, on immunodiffusion and immunoelectrophoresis on agar gel it yielded several precipitation lines showing that some degree of resolution occurs in this medium[3]. Furthermore, during the electrophoresis a fraction accumulated in the agar adjacent to the anodic edge of the antigen well causing thickening of this area of the agar sheet and deformation of this portion of the well edge. It was assumed that this fraction had mucoid properties which restricted its mobility in the medium, and had a high negative charge, and that these features, combined in the electrical field, caused compression of the agar gel. While this fraction is being trapped in the agar gel, it separates from other material with which it had been complexed, and which is freed to diffuse. Since this or a similar process occurs also on Ouchterlony plates without induced electrical field, it was inferred that properties of the agar gel play a role in this separation. Thus, if the mucoid fraction were responsible for the recalcitrant behavior of the original complex on chromatographic resins, a preliminary step of separating it on agar gel should make the other components available for conventional fractionation.

Based on these assumptions an apparatus which combined diffusion and filtration was designed for vertical migration of macromolecules through a horizontal agar gel plate; the gel was supported by a sieve; simultaneous movement of excess buffer through the gel enhanced the rate of marcromolecular migration. The eluate was subjected to chromatography on DEAE cellulose. The experimental results supported the assumptions.

Materials and methods

The extracts of *R. howesii* were dialyzed and purified as previously described[4]. Proteins were estimated by the optical density of the absorption peaks between 270 and 280 mμ[4]. Toxic activity was tested by bioassay and estimated in units[3]. Agar solutions of 0.6 % and 0.25 % of Difco Special Agar-Noble were freshly prepared in

0.01 *M* sodium phosphate buffered saline with 0.002 *M* sodium citrate at a final pH of 7.3.

A chamber with a filtering agar bottom was prepared as follows: A sieve of 2 mm mesh nylon fabric, reinforced by an 8 mm mesh rigid plastic grid, was attached with epoxy glue to the end of a 26 mm I.D. glass tubing. After the glue had hardened the apparatus was carefully cleaned, rinsed with alcohol, and air dried. The sieve was closed by a plastic film wrapping which was tightly secured to the outer glass wall by a rubber band. Into the chamber thus formed 2 ml of hot 0.6 % agar solution was poured and was left to congeal at room temperature. An agar gel sheet resulted with seals to both the inner glass wall and the substance of the sieve. The gel was overlaid with 2 ml of the 0.25 % agar solution. The plastic film was then removed and the device placed in a cold room at 4° where it was allowed to reach temperature equilibrium. The apparatus was positioned in a 50 ml Griffin beaker containing 2 ml of buffer so that the sieve–gel face contacted the surface of the buffer. This provision stabilized the seals.

The mechanical function of the 0.25 % agar is to fortify the seal between the 0.6 % agar gel and the glass wall. This function is apparently secured even when part of the 0.25 % agar becomes floating during the later operation. The device can take at least 3 cm water pressure. The sieve has enough rigidity to prevent critical deformation of the 0.6 % agar plate under this stress. However, the working load was always kept below 2 cm. The 0.6 % agar gel plate is about 3.7 mm thick. With a solution standing 2 cm high, the initial flow rate is about 0.25 ml/h. Eventually the chamber empties completely. For optimum flow rates during the filtration the fluid level in the beaker is adjusted at intervals. The moving water volume of the 0.6 % agar gel plate is about 2 ml as estimated from the passage of bromophenol blue.

Results and conclusions

Fifteen milliliters of *R. howesii* extract was filtered through this device and the filter washed with 4 ml of buffer. In the effluent, 85 % of the proteins and 75 % of the toxic activity were recovered. The subsequent fractionation of this effluent on a DEAE cellulose column yielded good resolution. One of the emerging fractions contained the toxic activity. The details will be reported elsewhere. This result indicates that the separation on agar gel was useful as a preliminary step in fractionating this material. It is suggested that this method may have a broader field of application. It may be tried on otherwise recalcitrant material whose heterogeneity can be revealed by diffusion on agar gel.

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CHROM. 3413

Dünnschichtchromatographische *in situ*-Analysen. Erreichbare Genauigkeiten in der Routine

Im Zusammenhang mit der in immer stärkerem Masse versuchten Anwendung der direkten Auswertung von Dünnschichtchromatogrammen scheint die Frage nach den im Routinebetrieb erzielbaren Genauigkeiten bzw. verwandter statistischer Grössen sehr aktuell zu werden. Wenn auch in einer Reihe von Veröffentlichungen[1–5] hierüber bereits gewisse Aussagen gemacht worden sind, so dürfte eine systematische Behandlung dieses Problemes doch sehr aufschlussreich sein.

Einige allgemeine Hinweise zur Beurteilung von Analysenverfahren übertragen auf die in situ-Auswertung

Einleitend sei auf die Differenzierung der Begriffe Richtigkeit und Genauigkeit hingewiesen. Während die Richtigkeit eines Ergebnisses die Ausschaltung etwa vorhanderer systematischer Fehler voraussetzt, werden durch die Angaben der Genauigkeit im wesentlichen die zufälligen Fehler charakterisiert. Entsprechend dem vorgegebenen Thema setzt die vorliegende Arbeit eine Kenntnis bzw. Berücksichtigung der bei *in situ*-Analysen möglichen, in der Literatur[5,6] bereits ausführlich behandelten systematischen Fehler bzw. Abweichungen voraus, sie verlangt also Richtigkeit der mit dem angewandten Analysenverfahren erhaltenen Ergebnisse.

An zufälligen Fehlerquellen dürften aufgrund von aus jahrelangen Direktmessungen gewonnenen Erkenntnissen neben einem beim Auftragen der Lösungen unvermeidlichen Pipettierfehler, einer gewissen Ungleichmässigkeit der Sorptionsschicht, deren Auswirkungen sich allerdings durch eine geeignete Messtechnik reduzieren lassen, die mit einem erforderlichen Aktivieren bzw. Besprühen in Zusammenhang stehenden Abweichungen zu erwähnen sein.

Hinsichtlich der eigentlichen statistischen Beurteilung von Analysenverfahren seien die beiden sich prinzipiell anbietenden Möglichkeiten angeführt:

(a) eine statistische Beurteilung der Einzelwerte, ausgedrückt z.B. durch die Standardabweichung der Einzelwerte,

(b) eine aus der statistischen Einzelbeurteilung abgeleitete Beurteilung der Mittelwerte, ausgedrückt z.B. durch die zu erwartende mittlere Abweichung der Mittelwerte.

Welches der beiden Kriterien nun im einzelnen angewandt wird, hängt von der jeweils in der Routine gewählten Arbeitsweise, diese wiederum von einigen substanzeigenen Faktoren, z.B. sind die Voraussetzungen bei der Analyse von pharmazeutischen Zubereitungen verschieden von denen der pharmazeutischen Grundstoffe, aber auch von den Ansprüchen des Analytikers ab. Wird bei Routineanalysen die Angabe von Einzelwerten für ausreichend befunden, so sind als Mass für die Genauigkeit die Gesetze der Fehlerrechnung der Einzelergebnisse angebracht. Wird dagegen die Angabe von Mittelwerten vorgezogen, so bieten sich zwangsläufig die Fehlerkriterien der Mittelwerte an. Entsprechend den in der Dünnschichtchromatographie vorwiegend gebrauchten und bewährten Auftragsverfahren—es folgen in der Regel alternierend Vergleichsflecke und Probeflecke nur einer Charge der zu prüfenden Substanz auf einer Platte—würde das Rechnen mit der Standardabweichung der

Einzelwerte eine nicht volle Ausnutzung der gegebenen Möglichkeiten bedeuten. Diesem Faktor, dem im allgemeinen seither—wie sich nicht nur aus der Literatur ergibt—nur sehr wenig Bedeutung beigemessen wurde, gilt im folgenden die besondere Aufmerksamkeit.

Effektive Angaben über erreichbare Genauigkeiten bei Routineanalysen

An Hand einiger Beispiele, ausgewählt unter Berücksichtigung der verschiedenen möglichen *in situ*-Messverfahren, seien die Grenzen der direkten Analysen aufgezeigt.

Die spektralphotometrische Gehaltsbestimmung von Chlormadinonacetat bereitet wegen einer Reihe störender Verunreinigungen gewisse Schwierigkeiten. Eine sich dem um ein relativ einfaches Bestimmungsverfahren bemühten Analytiker stellende Frage könnte nun folgendermassen präzisiert werden: Liegt, eine zufriedenstellende Trennung auf der Platte vorausgesetzt, der hier in erweitertem Sinne zu interpretierende Begriff Genauigkeit der *in situ*-Analyse in einer für eine Gehaltsbestimmung tragbaren Grössenordnung? Zur Beantwortung dieser Frage sei die Fehlerrechnung der Ergebnisse von fünf Platten herangezogen. Auf diesen (Fertigplatten Kieselgel 254) waren unter messtechnisch optimalen Bedingungen, jeweils sechs Eichpunkte dazwischen liegend, sechs Proben aufgetragen. Die Fluoreszenzen wurden mit einer in der Literatur[6] bereits mehrfach beschriebenen Messanordnung, bestehend aus einem Spektralphotometer PMQ II (Fa. C. Zeiss, Oberkochen) und einem Chromatogrammzusatz (Eigenbau) bei $\lambda = 525$ nm mit einer den Flecken angepassten kreisförmigen Messflächenbegrenzung, gemessen. Zur Reduzierung eines durch eventuelle Inhomogenitäten der Schicht hervorgerufenen Messfehlers wurde jeder Fleck auf zwei der belegten Zone benachbarte Plattenblindstellen bezogen. Letztere wurden je nach dem stärksten Untergrundgefälle in Steigrichtung bzw. Auftragsrichtung gewählt. Tabelle I gibt Aufschluss über die Mittelwerte der Gehalte $\bar{x}$ jeder der fünf Platten bzw. deren nach den Formeln

$$s = \sqrt{\frac{\Sigma \Delta^2}{(n-1)}} \text{ und } s_{\bar{x}} = \sqrt{\frac{\Sigma \Delta^2}{n(n-1)}}$$

errechneten Fehlerschranken der Einzel- und Mittelwerte. Es sei hierzu bemerkt, dass trotz der relativ kleinen Anzahl von sechs Einzelmesswerten auf jeder Platte bewusst nicht mit der Student'schen t-Verteilung gerechnet wurde, da die Abhängigkeit der t-Werte von der Zahl der Freiheitsgrade für das 68 %ige Vertrauensintervall nur gering ist.

TABELLE I

Platte	*Gehalt* $\bar{x}$ *(rel. Einh.)*	s (%)	$s_{\bar{x}}$ (%)
1	47.9	3.5	1.6
2	50.6	5.5	2.5
3	49.6	2.2	1.0
4	49.8	6.1	2.7
5	48.4	13.0	5.7

Mittelwert $\bar{\bar{x}} = 49.3$ rel. Einheiten

Auffallend in dieser Zusammenstellung ist die relativ hohe Streuung der Werte von Platte 5. Möglicherweise wird diese durch einen sogenannten Ausreisser verursacht, dessen Anerkennung allerdings nach den statistischen Regeln, z. B. der Fischer'schen F-Verteilung erfolgen müsste.

Eine Mittelbildung der zu erwartenden relativen mittleren Abweichung der Mittelwerte $s_{\bar{x}}$ der fünf Platten, begründet sei diese mit dem Argument, dass die errechneten Schranken $s_{\bar{x}}$ aufgrund voneinander abweichender Arbeitsweisen nicht *a priori* als konstant betrachtet werden können, liefert einen Wert von $s_{\bar{x}} = 2.7\,\%$. Diese Grösse berechtigt nun (unter Zugrundelegung des Mittelwertes $\bar{\bar{x}}$) entsprechend ihrer Definition zu folgender Aussage: 68 % aller Mittelwerte $\bar{x}$ liegen innerhalb der Schranken $\bar{\bar{x}} \pm 2.7\,\%$. Die Schranken $s_{\bar{x}}$ der Mittelwerte resultieren, dies sei ausdrücklich betont, aus einer Rechnung, bei der die einzelnen Werte $\bar{x}$ selbst nicht in Erscheinung treten.

Die Kenntnis der einzelnen Mittelwerte $\bar{x}$ der fünf Platten im vorliegenden Fall bietet andererseits die Möglichkeit, den ermittelten Wert $s_{\bar{x}}$ zu überprüfen. Führt man die Rechnung durch Einsetzen der entsprechenden Grössen in den Ausdruck $\sqrt{(\Sigma \Delta^2)/(n-1)}$ durch, so erhält man für die mittlere Abweichung der Mittelwerte $s_{\bar{x}}$ einen Wert von 2.2 %.

Geht man nun noch einen Schritt weiter, indem man die Fehlerrechnung auf die zu erwartende mittlere Abweichung der Werte $\bar{\bar{x}}$ ausdehnt, so betragen für diesen Fall die Schranken für das 68 %ige Vertrauensintervall sogar nur $\pm$ 1 %. Unter diesem Gesichtspunkt errechnet sich als Endergebnis ein Gehalt der zu analysierenden Ware von 49.3 rel. Einheiten $\pm$ 1 %.

Eine weitere Analyse der gleichen Substanz und unter den gleichen Bedingungen bewies die gute Reproduzierbarkeit dieses Ergebnisses. Die Wiederholung lieferte im Gegensatz zu der Erstanalyse eine geringere Streuung der Einzelwerte innerhalb der Platten und daraus folgend einen kleineren Mittelwert der zu erwartenden mittleren Abweichung der Mittelwerte der einzelnen Platten von 1.2 % (Erstanalyse 2.7 %). Die Streuung der gemessenen Mittelwerte der einzelnen Platten überstieg allerdings diesen Wert. Als Endergebnis dieses zweiten Versuches ergab sich mit dem Mittelwert aus fünf Platten bzw. der zu erwartenden Abweichung dieser Mittelwerte ein Gehalt von 49.9 rel. Einheiten $\pm$ 1.7 %.

Nach einer solchen in die Details gehenden Fehlerbetrachtung einer *in situ*-Analyse seien von einigen weiteren Verbindungen, deren Analysen seit Jahren dünnschichtchromatographisch erfolgen, lediglich zusammenfassende Angaben über die Fehlerrechnung der Plattenauswertung gemacht.

So errechnet sich für die Gehaltsbestimmung der Verbindung 16-Methylen-Reichstein S-Acetat unter den gewählten Bedingungen—es wird, u.a. mit einer Leuchtstoffplatte gearbeitet—ein Mittelwert für die aus den Messungen von jeweils einer Platte zu erwartende mittlere Abweichung der Mittelwerte von $s_{\bar{x}} = 2.9\,\%$.

Wie einleitend bereits indirekt angedeutet, ist zu erwarten, dass sich ein gegebenenfalls notwendiger grösserer Aufwand bei dem eigentlichen chromatographischen Teil der Analyse in der Streuung der Einzelwerte niederschlagen wird. Dies sei mit dem folgenden Beispiel belegt: Zur Verstärkung der Fluoreszenz von Dehydroemetin ist nach der chromatographischen Trennung eine etwa zweistündige U.V.-Bestrahlung der Platte angebracht. Infolge einer z.B. aus apparativen Gründen örtlich nicht konstant gehaltenen Bestrahlungsstärke muss für die verschiedenen

Flecke mit abweichenden Graden der Aktivierung gerechnet werden. Tatsächlich liegt der aus der Routine sich ergebende Mittelwert der zu erwartenden mittleren Abweichung der Mittelwerte, letzterer wiederum errechnet aus den Einzelwerten der einzelnen Platten, bei $s_{\bar{x}} = 5.6\,\%$. Es wurde bereits erwähnt, dass sich dieser Wert und damit die Genauigkeit durch eine entsprechende Technik des Bestrahlens beträchtlich vermindern lassen dürfte.

Die Ermittlung des Gehaltes des als Verunreinigung in einem Schädlingsbekämpfungsmittel vorkommenden 1,4-Naphthochinon erfordert neben einer Vortrennung zur Sichtbarmachung der belegten Zonen ebenfalls eine weitere Nachbehandlung der Messplatte. Trotz dieser umfangreichen Vorbereitungen mit einem erhöhten Mass an möglichen zufälligen Fehlern ergab sich in der Routine eine zu erwartende mittlere Abweichung der Mittelwerte $s_{\bar{x}}$ in der Grössenordnung von 2.5 % bei kleineren Mengen der Verunreinigung und eine solche von etwa 1 % bei stärker verunreinigten Waren.

Mit diesen Angaben über Routineanalysen einer Substanz aus dem Bereich der Schädlingsbekämpfungsmittel seien die Betrachtungen über erreichbare Genauigkeiten bei *in situ*-Analysen abgegrenzt. Die Zusammenstellung der einzelnen Beispiele, dies gilt im besonderen für die beiden letzteren, wurde bewusst zo gewählt, dass gerade bezüglich der chromatographischen Vorbereitungen der Dünnschichtplatten die Arbeitsweisen verschiedener Laboratorien sich in den Ergebnissen ausdrücken.

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CHROM 3439

Thin-layer chromatography of vinyl monomers based on undecenoic acid

A variety of vinyl monomers derived from undecenoic acid, a pyrolysis product of castor oil, and its derivatives have been prepared in this laboratory[1]. We now report the separation by thin-layer chromatography (TLC) of six such vinyl monomers, each representative of one of the following groups: allyl ester ($R–CO–OCH_2CH=CH_2$), allyl ether ($ROCH_2CH=CH_2$), vinyl ester ($R–CO–OCH=CH_2$), vinyl ether ($ROCH=CH_2$), acrylic ester ($RCH_2–O–CO–CH=CH_2$), and methacrylic ester ($RCH_2–O–CO–C(CH_3)=CH_2$).

Materials and methods

Allyl undecenoate was prepared by direct esterification of the acid with allyl alcohol in the presence of catalytic amounts of *p*-toluenesulphonic acid[2]. Allyl undecenyl ether was obtained by condensation of the sodium alkoxide of undecenol with allyl bromide[1]. Vinyl undecenoate and vinyl undecenyl ether were prepared by the familiar vinyl ester interchange[3] and transetherification techniques[4], respectively. Undecenyl acrylate and undecenyl methacrylate were prepared by REHBERG's procedure[5], involving alcoholysis of methyl acrylate or methyl methacrylate in the presence of an acidic catalyst and a polymerisation inhibitor.

Using a thin-layer applicator (Desaga, Heidelberg), glass plates (20 × 10 cm) were coated as usual with a well-stirred suspension of Silica Gel G (E. Merck, Darmstadt; 30 g in 60 ml water) to give a layer approximately 27 μ in thickness. The plates were dried and preserved in a desiccator. For reversed-phase TLC, the dried, coated

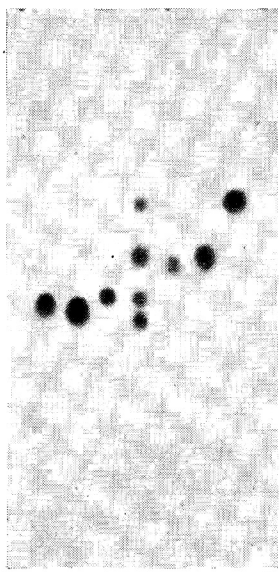


Fig. 1. Chromatogram showing the position of six vinyl monomers and a synthetic mixture of these on direct plate, the solvent system being *n*-hexane–ether (94:6, v/v); from left to right: allyl undecenoate, undecenyl acrylate, undecenyl methacrylate, synthetic mixture, vinyl undecenoate, allyl undecenyl ether and vinyl undecenyl ether.

plate was uniformly impregnated with silicone oil (Dow Corning, silicone fluid 200) by allowing a 5 % solution in diethyl ether to ascend the plate in a developing chamber. Argentated plates were obtained by uniformly spraying a 12% solution of silver nitrate in aqueous alcohol (50%) on the direct plate. The direct and reversed-phase thin-layer plates were charred with chromic acid while the argentated plates were charred with concentrated sulphuric acid.

Results and discussion

Fig. 1 shows the positions of the six monomers and that of a synthetic mixture of these on a direct thin-layer plate when developed in a *n*-hexane–ethyl ether (94:6, v/v) system. While the undecenyl vinyl ether and undecenyl methacrylate were resolved individually, allyl undecenoate and undecenyl acrylate, and the vinyl undecenoate and allyl undecenyl ether, resolved as pairs. The $R_F \times 100$ values for allyl undecenoate, undecenyl acrylate, undecenyl methacrylate, vinyl undecenoate, allyl undecenyl ether and vinyl undecenyl ether were 41, 41, 46, 52, 53 and 65, respectively.

Fig. 2 shows that clear resolution between vinyl undecenoate and allyl undecenyl ether could be achieved on the siliconised plate, the solvent system being acetonitrile–acetic acid–water (70:15:15, v/v/v). The $R_F \times 100$ values for allyl

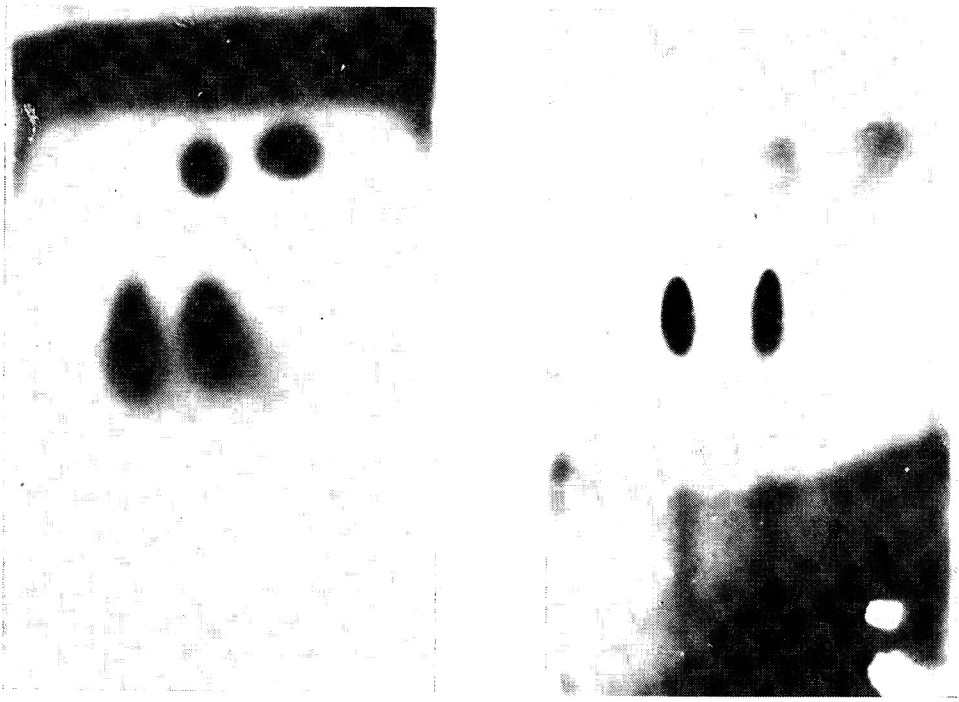


Fig. 2. Chromatogram showing the position of allyl undecenyl ether (left), vinyl undecenoate (right) and their synthetic mixture (centre) on a siliconised plate, the solvent system being acetonitrile–acetic acid–water (70:15:15, v/v/v).

Fig. 3. Chromatogram showing the position of allyl undecenoate (left), undecenyl acrylate (right) and their synthetic mixture (centre) on direct plate, the solvent system being *n*-hexane (dry)–ether (dry)–bromine (95:5:1.1, v/v/v).

undecenyl ether and vinyl undecenoate were 42 and 70, respectively. Allyl undecenoate and undecenyl acrylate moved together both on reversed-phase and on argentated thin-layer plates.

Since the double bond is in conjugation with the carbonyl group in the acrylic ester and not in the allyl ester, the procedure of KAUFMANN *et al*[6], of bromination on the plate was examined. Fig. 3 shows that a clear resolution between the allyl ester and the acrylic ester ($R_F \times 100$ values, 57 and 83, respectively) could be obtained on the direct thin-layer plate developed in *n*-hexane (dry)–ether (dry) (95:5) and containing 1.1 ml of bromine per 100 ml solvent.

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CHROM 3430.

Investigation of polymyxin E derivatives by thin-layer chromatography

In our communication entitled *Separation of polymyxin B, D, E and M by thin-layer chromatography*[1], we claimed that the solvent system acetone–water–acetic acid–2 *N* NH_4OH (15:5:1:2) proved to be convenient for the investigation on a Kieselgel G plate of antibiotics belonging to the polymyxin group. For the development of the spots, both ninhydrin spray and biological reactions, were used. One of our statements, however, needs correction. On the chromatogram of polymyxin E

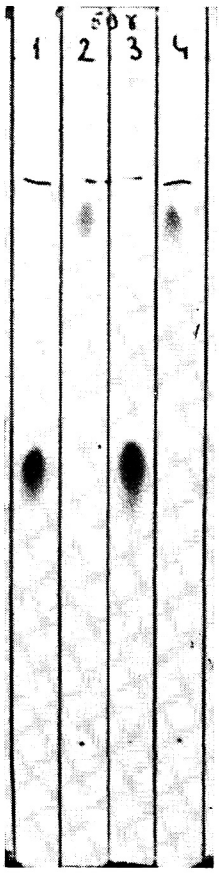




Fig. 1. Kieselgel G plate. Solvent system: acetone–water–acetic acid–2 *N* NH_4OH (15:5:1:2). Development: ninhydrin spray. 1 = Polymyxin B sulphate (Pfizer), 50 μg; 2 = The Colistin sample denoted as polymyxin E sulphate in our previous paper, 50 μg; 3 = Colistin sulphate (Laboratoire Roger Bellon), 50 μg; 4 = Colistin methanesulphonate (Laboratoire Roger Bellon), 50 μg.

Fig. 2. Kieselgel G plate. Solvent system: acetone–water–acetic acid–2 *N* NH_4OH (15:5:1:2). Development: *Bordetella bronchiseptica*. 1 = Polymyxin B sulphate (Pfizer), 20 μg; 2 = The Colistin sample denoted as polymyxin E sulphate in our previous paper, 20 μg; 3 = Colistin sulphate (Laboratoire Roger Bellon), 20 μg; 4 = Colistin methanesulphonate (Laboratoire Roger Bellon), 20 μg.

(Colistin; a product of the Laboratoire Roger Bellon), several biologically active spots were indicated, the R_F values of which deviated considerably from that of the chemically closely related polymyxin B sulphate. No reason for this phenomenon could be found. Recently, the Laboratoire Roger Bellon kindly supplied us with samples of their standard Colistin sulphate as well as of Colistin methanesulphonate, another of their preparations. The investigations mentioned above were carried out with both of these standards and the results compared with those obtained with our own substances. It became clear that the preparation of polymyxin E used originally by us was not the sulphate salt but the sulphomethyl-sodium derivative. This is shown by Figs. 1 and 2.

On the basis of the above investigation it can be stated that, in the system elaborated by us, the R_F values of Colistin sulphate and Colistin methanesulphonate differ from each other. The former gives a uniform spot, the R_F value of which is practically identical with that of polymyxin B. The multiple spot formation by the latter might be explained by formation of derivatives of varying degree of substitution in the mild acid medium of the solvent system.

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Detection of substances on thin layer chromatograms by the "hot-line" technique

We wish to describe an extremely simple and convenient method for detection on thin layer chromatograms which has the added advantage of relatively small percentage loss from a standard 20 × 20 cm chromatographic plate.

A nichrome wire of desired gauge is attached via two 5/8 in. washers and 1 in. springs to suitably insulated standard ringstand clamps. Current is applied to the washers via alligator clips. Our particular arrangement is shown in Fig. 1.

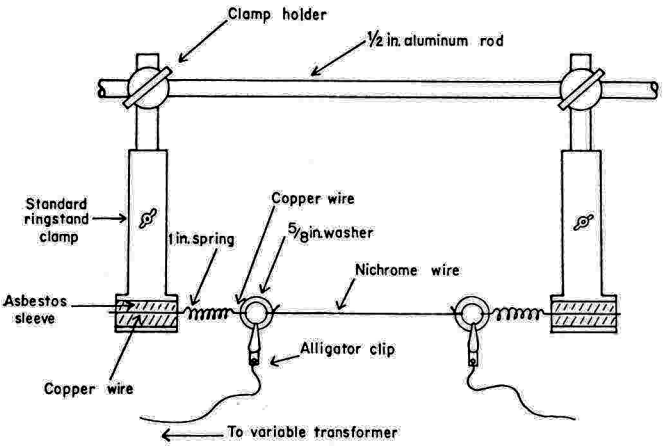


Fig. 1. "Hot-line" apparatus.

In the simplest techniques sufficient current is simply applied to the nichrome wire to cause it to glow red. The wire, of course, expands but is kept taut by the springs. The chromatographic plate is brought within a millimeter of the hot wire, very briefly, in order to char the organic material along a strip about 1- or 2-mm wide. If desired, the nichrome wire may touch the plate for an instant. We often used this latter technique as the wire was quite inexpensive and easily replaced, and this latter method minimized the loss of sample. If it is desirable to apply a reagent, *e.g.*, sulfuric acid, a technique which may be employed is to moisten a thin glass fiber pulled from a piece of capillary tubing. Reagents, of course, may be sprayed or spotted similar to the way the compound is applied to the plate. However, these methods would offer essentially no advantages over the usual procedures.

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Apparatus for impregnating thin-layer plates

Adsorbent layers impregnated with hydrophobic materials are commonly used to separate the members of relatively non-polar classes of compounds, such as glycerides and fatty acid esters. Suggested methods for impregnating include immersion of the plate in the impregnating solution[1,2], spraying[3], and allowing the plate to stand in the solution—as when developing the chromatogram[4,5]. The immersion method may present handling difficulties and requires great care to avoid disturbing loosely-bound adsorbent layers. Spraying may also disturb the adsorbent or result in uneven deposition of the impregnant. The development method obviates these difficulties but is more time-consuming and entails the possibility that due to frontal analysis the impregnated portion may not extent to the solvent front.

With the device here described (Fig. 1) an immersion method is used, but the impregnating solution is allowed to flow onto the plate, and off again, at a controlled rate. In this way the above-mentioned difficulties are avoided.

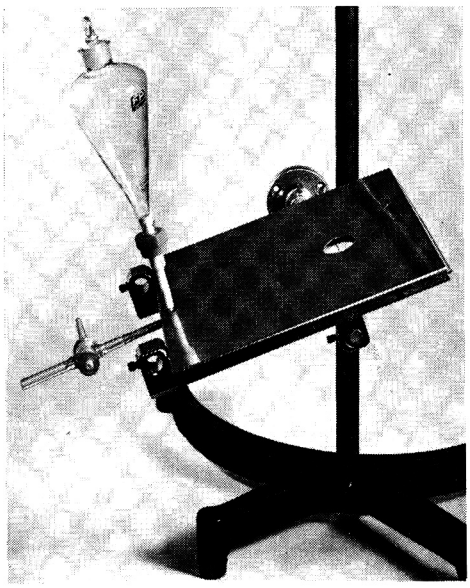


Fig. 1. Apparatus for impregnating thin-layer plates.

Method of operation

As shown in Fig. 1, the apparatus consists of an impregnating chamber resting on a Flexaframe* support which can be adjusted at various angles to the horizontal. The impregnating solution is placed in a separatory funnel connected to an inlet near the bottom of the chamber; the thin-layer plate rests against the ledge shown in Fig. 2, detail E. The funnel stopcock is opened and the solution allowed to flow into the chamber until it reaches the proper height as observed through the window

* Mention of trade or company names does not constitute endorsement by the Department of Agriculture over others not named.

(Fig. 2, D) in the top of the chamber. The funnel stopcock is then closed and the outlet stopcock opened.

The permissible flow-rate for the impregnating solution depends on the cohesiveness of the adsorbent layer and the angle of the chamber. With a 250 μ layer of silica gel containing 5 % $CaSO_4$ as binder, and with the chamber set at an angle of approxi-

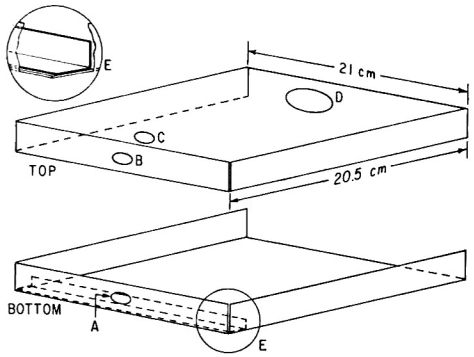


Fig. 2. Exploded view of impregnating chamber. Not drawn to scale; see text for complete dimensions.

mately eight degrees from the horizontal, 180 ml of solution is enough to impregnate the adsorbent for a distance of 15 cm in 5 min. Smaller amounts of solution may be used if more time is taken for the impregnation or if the chamber is set at a smaller angle. In the latter case, however, care must be taken to prevent the solution from flowing under the plate and out the open end of the chamber.

Construction

The chamber is made from two sheets of 20-gauge 18-8 stainless steel, as shown in Fig. 2. Each sheet is cut so as to have a 20.5 by 21 cm central portion surrounded on three sides by 1 cm wide tabs. Five millimeter diameter holes are punched out at A, B, and C, and a 25 mm diameter hole at D. Hole C, which is for the inlet tube, must be fairly close to the edge of the sheet so that the impregnating solution does not fall directly on the adsorbent; hole D has its center 4.5 cm from the opposite edge.

After the holes are made the tabs are bent in such a way that the top sheet can fit like a lid over the bottom. Fifteen millimeter lengths of 5 mm O.D. stainless steel tubing are then flanged at one end, fitted into holes B and C, and sealed in place with 50-50 solder (lead-tin) and stainless steel flux. An L-shaped strip of stainless steel with one arm 10 mm wide and the other 5 mm wide is spot-welded to the bottom sheet (see detail E). Top and bottom sheets are then put together and soldered along the joints.

A Fisher and Porter Company* 125 ml separatory funnel with Ultramax valve is fitted to the inlet tube. This funnel has an extension tube rigid enough to hold it erect without further support. A stopcock is fitted to the outlet. All plastic parts which come in contact with the impregnating solution should be Teflon.

* 18 Warminster Road, Warminster, Pa. 18974, U.S.A.

The chamber is supported on two parallel Flexaframe rods. These are connected by a third rod which extend through a clamp support on a stand and then through two Flexaframe feet arranged with their flat faces touching. The latter serve as a handle for adjusting the angle of the chamber.

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CHROM. 3403

Quantitative thin-layer chromatography of benzyloxycarbonyl dipeptide phenylhydrazides

Because in the recently-developed synthesis[1] of benzyloxycarbonyl peptide phenylhydrazides from benzyloxycarbonyl amino acid or dipeptide phenyldiimides and amino acid or dipeptide phenylhydrazides it was desirable to conduct many of the experiments with small quantities of reactants, a system of quantitative thin-layer chromatography was employed. Thin-layer separations of benzyloxycarbonyl amino acid and peptide esters have been effectively carried out on silica gel[2,3]. Using an optical densitometric method for quantification of glycerides, PRIVETT *et al.*[4–6] have found that an integrated function of the size and density of a charred spot on a thin-layer chromatogram is directly proportional to the carbon content, or weight of the sample, in the spot. The extension of such systems to the purification and quantification of four benzyloxycarbonyl dipeptide phenylhydrazides was undertaken. Optical densitometry of charred spots has been rather extensively applied to determinations of lipids and glycerides but does not appear to have been used before with peptide derivatives.

Experimental

Chromatoplates. Glass plates (16 × 20 cm) were coated with about a 0.25 mm layer of SilicAR TLC-7GF (12 g silica, 24 ml water) as described by LEIFIELD[7]. After drying at room temperature, they were activated by heating for at least 1 h at 110°. The plates so prepared were used within 30 min after removal from the oven. A 1–10 μl volume of solution containing 1–20 μg of product was spotted on the thin film 2.5 cm from the bottom edge of the plate in 0.5 μl drops in such a manner that the

surface of the film was undisturbed. The spots were dried during application in a gentle, unheated stream of nitrogen. For quantitative work, three spots each of two known amounts of the compound to be analyzed and three spots of a solution prepared from the peptide reaction mixture were applied in alternating fashion to the plate 1.5 cm apart.

Development. A Kontes covered glass tank was used which was lined inside with absorbent paper in contact with the developer. The chamber was allowed to reach saturation, the spotted plate was introduced into the chamber onto glass rods which kept the thin film from contact with the developer, and covered. After 15 min, the cover was slid back just enough from one corner to allow introduction of sufficient solvent to develop the chromatogram. The developer used and the development distance for each benzyloxycarbonyl dipeptide phenylhydrazide is defined in Table I. The R_F values obtained by development to a distance of 10.0 cm with a chloroform–methanol (95:5, v/v) developer are shown in the last column of Table I. R_F values were found to vary by $\pm$ 0.02 from one plate to another and development times by $\pm$ 1.5 min.

TABLE I

THIN-LAYER CHROMATOGRAPHIC ANALYSIS OF BENZYLOXYCARBONYL DIPEPTIDE PHENYLHYDRAZIDES

Dipeptide	*Development solution, chloroform–methanol* (v/v)	*Development distance* (*cm*)	*Development time* (*min*)	R_F	R_F *values with chloroform–methanol* (95:5)
Gly-Gly	85:15	10.0	22	0.44	0.21
Gly-L-Phe	91:9	15.0	43	0.58	0.53
L-Leu-L-Phe	95:5	10.0	22	0.69	0.69
L-Phe-L-Phe	95:5	10.0	22	0.67	0.67

Quantification. The developed chromatogram was dried at room temperature; then heated to 120° in an oven. The hot chromatogram was quickly and uniformly sprayed with an aqueous solution of 70 % sulfuric acid (v/v) saturated at room temperature with potassium dichromate. The plate was transferred to an oven at 120°; the temperature was raised to 200° and held constant 20 min, then decreased gradually to room temperature. As soon as the plate had cooled, measurements of optical density were made by scanning the chromatogram face up on a densitometer (Photovolt Corporation Model 52C and Gilford Model 220 absorbance indicator and optical density converter with a Heath Model EUW-20A recorder) with a specially designed carriage for holding 16 × 20 cm plates. The densitometer stage was covered with black construction paper and measurements were made in a darkened room. No filter was used. A 0.5 × 3.0 mm slit was employed. Three curves were recorded for each spot and the curves were cut out and weighed.

Results and discussion

Heating of the developed chromatogram prior to spraying was found essential to allow application of sufficient carbonizing reagent to insure complete carbonization

yet to eliminate the diffusive enlargement of the spots which occurred when the plates were sprayed cold.

For densitometric quantifications a variety of slit widths have been employed[4,5,8–11]. In the work under discussion, use of a slit width which was in most cases less than the diameter of the spot made it imperative that only good plates exhibiting symmetrical spots be used, and that the spots be carefully aligned between the slits.

It was found that standard curves were linear for plots of curve weight *vs.* weight of sample applied up to about 20 μg per spot. However good accuracy was not obtained using such standard curves as bases for quantitative determinations, and it was found necessary to use two known amounts of the compound to be analyzed as references. The method used was found accurate within about 8 %.

PRAVDA *et al.*[12] used thin-layer chromatography in separating benzyloxycarbonyl dipeptide methyl ester diastereomers sufficiently for identification, but not sufficiently for quantitative determinations. In the current research, an attempt was made to develop a quantitative thin-layer system for the benzyloxycarbonyl dipeptide phenylhydrazides for which diastereomers could be formed, but insufficient separation of the diastereomers was experienced during the use of several solvent systems.

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CHROM. 3414

A two-dimensional thin-layer chromatographic technique for the separation of admixtures of 5-bromouracil and DNA bases

The four principal DNA bases are readily resolved in single-dimension chromatography using solvents such as isopropanol–hydrochloric acid–water and butanol–acetic acid–water. When bromouracil is admixed with these bases complete separation is not achieved with these solvent systems since the bromouracil and thymine migrate together. It was found that bromouracil and thymine were readily separated using a phosphate buffer although with this solvent system the guanine/adenine and thymine/cytosine resolution was not satisfactory. The following technique describes a two-dimensional method developed for the separation of all five bases.

Ascending chromatography on Eastman cellulose chromatogram sheets (20 × 20 cm 6065 with fluorescent indicator) was carried out in tanks which were sealed with lids lined with "Parafilm". The bases were dissolved in $N/10$ hydrochloric acid (prepared from concentrated acid) and applied 2.0 cm from the lower edge of the sheet. The final diameter of the spot was 2 mm. The minimum quantity of each base required for adequate visualisation under U.V. light was 0.3 μg. However, for measurement of the absorption spectra of eluted spots between 0.5 and 1.5 μg amounts were required using a 1 cm path micro-cell in a Cary 15 spectrophotometer. Isopropanol–water–hydrochloric acid (65:18.4:16.6, v/v)[1] was used for development in one dimension and phosphate buffer[2] (123 ml 1 M disodium hydrogen phosphate, 74 ml 1.2 M ammonium sulphate, 3 ml isopropanol) in the other dimension. Two-way chromatography on a single sheet gave poor separations. Thus using the isopropanol solvent first, the phosphate solvent front migrated nearly twice as fast at one edge as at the other, while using the phosphate buffer first, dark streaks and a white deposit were formed on the sheet. For these reasons a technique was developed based on PUMPHREY'S method[3] of transfer to a second sheet.

The first dimension was carried out on a narrow strip, 3.5 × 20 cm; the mixture of bases was applied 2 cm from both the lower edge and one side edge and developed with isopropanol–hydrochloric acid–water. The solvent front migrated 14 cm in 6 h at 21°. After development the strip was completely dried in a current of cold air and the streaked area at the solvent front cut away. The sheet to be used for the second dimension was trimmed to the same length as the cut strip, and the sheet and strip were clamped along a 5 mm overlap with the thin layers face to face. Fig. 1 shows two



Fig. 1. Clamping system for holding the strip and sheet together and to maintain good contact between the cellulose layers.

5 mm wide teflon strips within a stainless steel frame which was closed by spring clips. This clamping device was used to hold the strip and sheet together and maintain the good contact necessary for complete transfer of material by the second solvent. The phosphate buffer solvent front travelled evenly under these conditions at a rate of 12 cm in 1 h. After development the strip and sheet were unclamped and separated from each other whilst still damp. The dried chromatogram was free from interfering streaks.

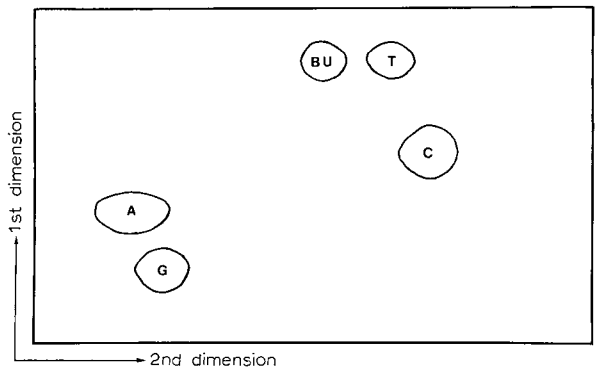


Fig. 2. Two-way chromatogram of DNA bases, showing separation with solvents (1) isopropanol–hydrochloric acid–water and (2) phosphate buffer. A = Adenine; G = guanine; C = cytosine; T = thymine; BU = bromouracil.

The separation achieved by this method is shown in Fig. 2. Thymine and bromouracil migrated together in the first solvent (R_F 0.84), separating from cytosine (R_F 0.55), adenine (R_F 0.39) and guanine (R_F 0.22). In the phosphate buffer the average R_F values were: adenine, 0.18; guanine, 0.23; thymine, 0.64; cytosine, 0.70; and bromouracil, 0.50.

I wish to thank Mr. H. HODT for the construction of the clamping system, Dr. A. B. ROBINS for helpful discussions and Dr. W. ANDERSON for useful criticism.

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CHROM 3451

An improved method for the separation and removal of organochlorine insecticides from thin-layer plates

In this laboratory, wildlife samples are analyzed for insecticide residues by gas–liquid chromatography (GLC). However, due to interfering compounds[1] and the inability of one gas chromatographic column to resolve all insecticides, the technique of eluting separated organochlorine insecticides from thin-layer plates before GLC analyses was investigated. Thin-layer scraping devices described by other workers[2–4] proved inadequate for the recovery of large quantities of compounds that were spotted along the entire origin line. This communication describes a collecting device and eluting solvent that quantitatively recovers insecticides from thin-layer plates.

Apparatus

The collecting device (Fig. 1) consists of: (1) a straight end adapter with the constricted end cut at an angle and a ⓢ 29/42 female connection; (2) an inner joint from a 20 mm I.D. chromatographic column with a Kimflow fritted disc, ⓢ 29/42 male connection, and lower end cut at an angle.

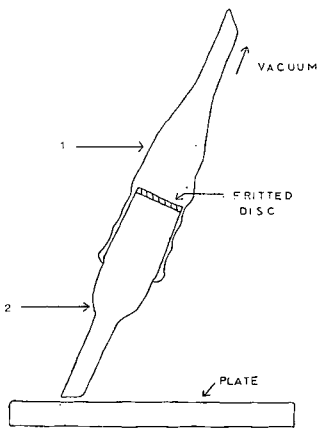


Fig. 1. Zone collecting device.

Experimental

The standard technique of ascending thin-layer chromatography (TLC)[5] was employed. MN Silica Gel GHR was layered on 20 × 20 cm glass plates, activated at 120° for 30 min and stored in a glass desiccator over anhydrous $CaSO_4$. One hundred microgram quantities each of the insecticides, dieldrin, *p,p'*-DDD, *p,p'*-DDT, *o,p'*-DDT, and *p,p'*-DDE and 50 μg quantities each of heptachlor epoxide and γ-BHC were spotted along the origin line. For zone identification, 5 μg of each insecticide were placed as one composite spot on the origin line at the far right end of the plate. Plates were placed in a lined tank and developed in a hexane–ethyl ether (98:2) mixture for a distance of 15 cm from the origin line. Developed plates were covered

except for the area above the far right spot, sprayed with silver nitrate solution[3] and exposed to germicidal U.V. light.

Before scraping, plates were divided into five horizontal zones using the color developed standards as a guide. These zones were outlined as shown in Table I.

TABLE I

SEPARATION AND RECOVERY OF PESTICIDES

Pesticides separated on silica gel thin-layer plates, removed in zones using new collecting device, eluted with hot benzene, and analyzed by GLC. Results are the mean of three determinations.

Insecticide	*Zone*	R_F	*Quantity spotted* (μg)	*Mean recovery* (%)
Dieldrin	1	0.21	100	103
Endrin	1	0.26	100	107
γ-BHC	1	0.29	50	100
Heptachlor epoxide	1	0.33	50	92
p,p'-DDD	2	0.47	100	102
p,p'-DDT	3	0.64	100	103
o,p'-DDT	3	0.71	100	93
p,p'-DDE	4	0.83	100	101
	5*	0.90–1.00	—	—

* Area above p,p'-DDE to 15 cm solvent front.

The outlined zones were removed, one at a time, with the zone collecting device. This was done by drawing the silica gel layer into the inner member of the device using a vacuum line connected to the small end of the straight end adapter. Elution of the insecticides was accomplished by disconnecting the vacuum line, inverting the device, placing it in a vertical holding clamp and eluting with 3 × 15 ml of hot benzene into a 50 ml glass stoppered centrifuge tube.

Analysis was performed on a gas chromatograph equipped with electron capture detector operated at 210° and 1×10^{-9} amp, 6 ft. glass column packed with 3 % OV-17 on 60/80 mesh Gas Chrom Q at 190° and a gas flow of N_2 at 100 ml/min.

Results and discussion

The quantitative recoveries obtained using this method are shown in Table I. The coefficient of variability for entire experiment was 7.2 %.

Other elution solvents and solvent mixtures investigated and found unsatisfactory were: *n*-hexane, petroleum ether 30–60 b.p., ethyl ether, ethyl acetate, and mixtures of petroleum ether–ethyl ether, 1:1 and 1:3. Attempts to visualize pesticides on developed thin-layer plates under U.V. light prior to zoning and removal from fluorescein impregnated plates resulted in poor recovery.

This procedure has been incorporated in our analysis procedure for determining organochlorine residues in biological materials and consequently gas chromatograms are less complicated and more interpretable. Repeated analyses of zone 5 of the thin-layer chromatoplates of field samples has revealed that most of the interfering com-

pounds present in wildlife samples are found in this area. The use of this method makes it possible to run analyses on one gas chromatographic column.

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CHROM. 3443

Ninhydrin reaction used for thin-layer chromatographic detection of bis(dimethylamino)fluorophosphine oxide (dimefox)

Dimefox is a valuable systemic insecticide and acaricide and because of its toxicity for mammals the problem of residue determination arises. In the absence of dimefox suitably labelled with radioactive phosphorus, these residues need to be determined by chemical methods based on the measurement of phosphorus[1], fluorine[2] or dimethylamine[3]. Although determination of dimethylamine should give quantitative results, it is not specific, as many plant substances decompose to give amines. Determination of fluorine is specific but insensitive. Total phosphorus determination is very sensitive and is used for similar compounds, but it is again non-specific.

Thin-layer chromatography, widely used for analysis of minute amounts of pesticides[4–7], was applied to the identification of dimefox residues and, in addition to having the advantage of simplicity, showed a reasonable degree of sensitivity.

Despite the increasing use of this technique for the separation, identification and determination of pesticides, most workers use non-specific spray reagents. Another investigator[8] has developed more specific reagents, but none of these was applicable to dimefox residue detection.

Dimefox, as an amine, reacts with ninhydrin and the reaction can be considered as a special case of the ninhydrin reaction with amino acids[9]. The interpretation is based on the mechanism of the Strecker degradation and explains the formation of diketohydrindylidene-diketohydrindamine and hydrindantin.

Experimental

Shake 10 g of silica gel and 10 g of Kieselguhr (E. Merck, Darmstadt) with 40 ml of distilled water for about 2 min and apply the resulting slurry to glass plates

(20 cm × 20 cm) at an applicator setting of 250 μ. Allow the plates to dry in air about 30 min and then heat in the oven for 30 min at 110°.

Spot 5 μl aliquots of standard solutions, containing 1–25 μg of dimefox, on to the chromatographic plate, 1.5 cm from one edge, keeping the spots as small and uniform as possible. Develop the plate with a mixture of petroleum spirit (b.p. 60–80°) and acetone (2:1, v/v) for about 45 min. Allow the plate to dry for 5 min, spray with a mixture of 2 % ninhydrin in acetone and concentrated hydrochloric acid (2:1, v/v) and heat in the oven for 30 min at 110°. Dimefox spots appear as red areas on a white background (R_F 0.58). Plot a graph relating the square root of the area of the spot and the logarithm of the weight of dimefox. The amount of dimefox in a sample spot may be determined from it.

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CHROM 3446

Die Verwendung von *Candida utilis* zur Lokalisierung stabiler Phosphatverbindungen auf Chromatogrammen

Kürzlich berichteten wir über einige empfindliche Nachweisreaktionen für papierchromatographisch getrennte Phosphatverbindungen[1]. Da der Nachweis über das freigesetzte Orthophosphat erfolgte (Hydrolyse bei hoher Azidität und Temperatur), lag die Empfindlichkeitsgrenze für besonders stabile Substanzen (z.B. AMP, 3-PGS) höher als für Orthophosphat und labile Verbindungen. Nachstehend wird ein enzymatisches Hydrolyseverfahren mitgeteilt, durch das sich diese Schwierigkeit weitgehend vermeiden lässt.

Mit Phosphomonoesterhydrolase[2–4] werden C–O–P-, nicht aber P–O–P-Verbindungen sicher gespalten. Bei einer Kombination Phosphomonoesterhydrolase/Polyphosphathydrolase kann eine gleichzeitige Hydrolyse beider Bindungen erwartet werden. Die Synthese dieser Enzyme unterliegt bei Mikroorganismen in Phosphatmangelmedien keiner Repression[5–7]. Auch die von uns verwendete *Candida utilis* zeigt unter diesen Bedingungen eine sehr hohe Polyphosphathydrolase- und Phosphomonoesterhydrolase-Aktivität. Zur Hydrolyse kann auf eine Isolierung dieser Enzyme weitgehend verzichtet werden; alle geprüften Phosphatverbindungen waren auf Papier- und Dünnschichtchromatogrammen mit gleich grosser Empfindlichkeit nachweisbar.

Material

Wir verwendeten *Candida utilis* (Stamm 653/3 von Biologycki Ustav ČSAV, Prag). Zur Anzucht von P-Mangelzellen überimpften wir vom Malzextraktagar auf Nähragar folgender Zusammensetzung[8]: 30 g Agar-Agar; 30 g Dextropur; 3 g Pepton; 1 g Asparagin; 1 g K_2SO_4; 0.5 g $MgSO_4 \cdot 7\ H_2O$; 0.1 g $CaCl_2$; 0.005 g $(NH_4)_2Fe(SO_4)_2$, Aqua dest. ad 1000 ml.

Nach 5-tägiger Bebrütung bei 26° wurden die Zellen mit Aqua dest. abgeschlämmt, durch Zentrifugieren und Resuspendieren mehrmals gewaschen und nach Ermittlung des Hämatokritwertes in 10%iger Suspension (V/V) im Kühlschrank aufbewahrt.

Ergebnisse

Die verwendete Hefe ist durch folgende Eigenschaften gekennzeichnet*:

(1) Keine P-Aufnahme (10^{-3} *M* KH_2PO_4 + 1 % Glucose): bei 50° nach 10 min.

(2) Tod (kein Wachstum auf Nähragar): bei 55° nach 10 min.

(3) Enzyminaktivierung (Suspension in Aqua dest.): Phosphomonoesterhydrolase bei 90° nach 10 min; Polyphosphathydrolase bei 70° nach 10 min.

(4) pH-Optimum (*M*/100 Zitrat/HCl-Puffer bei 30°): Phosphomonoesterhydrolase bei pH 2.9; Polyphosphathydrolase bei pH 3.1.

(5) Phosphatgehalt der Zellen: 0.8 mg P/g Frischsubstanz, davon 0.15 mg P als Ortho-P bzw. stark labiles P. Nach 10 min Erhitzen auf 62° und 8-stündiger Autolyse bei 20° ist der grösste Teil als Orthophosphat freigesetzt, Polyphosphathydrolase

* Eine ausführliche Mitteilung wird vorbereitet.

und Phosphomonoesterhydrolase bleiben weitgehend an die Zellstrukturen gebunden.

Unter Berücksichtigung dieser Daten wurde nach folgender Vorschrift gearbeitet:

Die 10 %ige Zellsuspension wird 10 min auf 62° erhitzt und nach 8 Std. Autolyse bei 20° zentrifugiert. Nach dem Resuspendieren des Sedimentes mit einer dem verworfenen Überstand entsprechenden Menge Aqua dest. werden 2 bis maximal 5 ml dieser Suspension mit 80 ml Aqua dest. und 0.5 ml $N/10$ Salzsäure versetzt und mit Aqua dest. auf 100 ml aufgefüllt. Mit dieser Suspension ist das Chromatogramm mässig feucht zu besprühen und nach 10 min Inkubation bei 30° im Trockenschrank bei 62° zu trocknen. Anschliessend erfolgt die Anfärbung nach Lit. 1. Dünnschichtchromatogramme können mit dem gleichen Farbreagenz besprüht werden.

Nach dieser enzymatischen Hydrolyse entspricht die Farbintensität der Flecke weitgehend den P-Mengen der Chromatogramme. Die mit der Zellsuspension aufgesprühten Orthophosphatmengen liegen unterhalb der Nachweisgrenze. Die Empfindlichkeitsgrenzen für Phosphomonoester sowie Pyro- bzw. Polyphosphate und Orthophosphat liegen in der gleichen Grössenordnung.

Bei der Untersuchung von Extrakten ist es angebracht, drei Chromatogramme (bzw. bei Keilstreifen nach MATTHIAS[9] jeweils ein Drittel des Chromatogrammes) vor dem Anfärben unterschiedlich zu behandlen:

(1) Ohne Hydrolyse: Nachweis von Orthophosphat und extrem labilen Phosphaten (z.B. Acetylphosphat, Carbamylphosphat).

(2) HCl/Ammoniummolybdat/Aceton-Hydrolyse nach Lit. 1: Nachweis von 1. und mässig labilen Phosphaten (z.B. FDP, ATP).

(3) Rohenzymgemisch-Hydrolyse: Nachweis von 1. und 2. sowie sehr stabilen Phosphaten (z.B. 3-PGS, AMP).

Auf diese Weise erhält man neben den R_F-Werten auch Informationen über die Hydrolyseeigenschaften der getrennten Phosphatverbindungen.

Eiweissfällende oder stark puffernde Laufmittelkomponenten (z.B. Trichloressigsäure, Ammoniumsulfat), die sich vom Chromatogramm nicht weitgehend entfernen lassen, sind ungeeignet. Die angefärbten Chromatogramme sind—zwischen Glasplatten gelegt—längere Zeit auswertbar.

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Herrn Prof. Dr. H. AUGSTEN möchte ich für helfende Diskussion herzlich danken.

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CHROM. 3434

Detection of 3-hydroxyflavanones on papergrams and thin-layer plates

A simple, specific detection technique for 3-hydroxyflavanones, based on PEW's[1] zinc–hydrochloric acid reduction, has been used for several years at the Forest Products Laboratory in locating these important flavonoids on papergrams[2].

Recently this technique has been modified to accommodate thin-layer silica gel plates. In the case of papergrams, or microcrystalline-cellulose thin-layer plates, zinc dust (technical-grade zinc powder) is lightly sprinkled or brushed evenly on the developed chromatogram and detection is achieved by spraying with 6 *N* hydrochloric acid. In the case of silica gel plates, the zinc dust can either be incorporated (2 % by weight) into the original silica gel mix before spreading or sprayed on the developed plate as a suspension in acetone. As before, a subsequent spraying with 6 *N* hydrochloric acid produces brilliant red-purple spots corresponding to the positions of the 3-hydroxyflavanones. If acidic-developing solvents are used —such as chloroform–methanol–acetic acid (7:1:1) which gives good separation between taxifolin (R_F 0.35) and aromadendrin (R_F 0.50)—the zinc must not be incorporated but sprayed on after development as a suspension.

The great advantage of this technique is the ability to differentiate between 3-hydroxyflavanones and other phenolics which have related R_F values and which give similar colour reactions with the commonly used phenolic detecting reagents, such as diazotized sulphanilic acid[3] and ferric-ferricyanide[4].

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CHROM 3447

A thin-layer chromatographic method for the determination of trace metals in cereal

The advantages of using thin-layer chromatography as opposed to paper chromatography for the separation of metal ions have been discussed before[1]. A shorter development time and sharper delineation of the spots are the most obvious advantages. It has also been shown in earlier papers that diffuse reflectance spectroscopy can lend itself well to quantitative determination of trace metals separated on thin-layer chromatograms.[1,2] Rubeanic acid and pyridine-2-aldehyde-2-quinolylhydrazone (PAQH) have been investigated extensively as chromatographic spraying reagents[1–5] for cobalt, copper and nickel. In this study a combination of these techniques has been adopted for the determination of traces of cobalt, copper and nickel in oats.

Experimental

Preparation of samples and standards. An improved ashing technique proposed by CONOLLY AND MAGUIRE[5] has been used. This involves ashing of a 20 g sample for 12 h at 550°. After cooling, 5 ml of dilute (1:1) hydrochloric acid are added and the mixture is evaporated on a steam bath and heated on a hot plate for 30 min. The hydrochloric acid treatment is repeated followed by the addition of 5 ml of hot dilute (1:1) hydrochloric acid. Silicates and other debris are centrifuged after the system has been heated in boiling water for 30 min. The residue is washed twice with 5 ml of hot (1:1) hydrochloric acid and the original supernatant liquid and washings are combined and evaporated to dryness. This residue is dissolved in 2 ml of (1:1) hydrochloric acid and kept for analysis.

Appropriate dilution series of synthetic copper, cobalt and nickel mixtures (1:1) in hydrochloric acid are prepared.

Doubly distilled water, reagent grade chloride salts and hydrochloric acid are used.

Chromatographic procedure. The chromatoplates were coated with a Desaga thin-layer chromatography applicator; the layer thickness was adjusted to 0.25 mm. After coating with Merck Silica Gel G, the plates were heated at 110° for 1 h and stored in a dry cabinet over silica gel adsorbent. The spots were applied with a 10 μl Hamilton microsyringe and then chromatographed in a preconditioned chromatography chamber. The solvent system used for chromatographic separation was 0.5 % concentrated hydrochloric acid in acetone.[1] Standards were developed simultaneously with the sample. The plates were then dried in air for 5 min and exposed to ammonia vapour for 10 min to neutralize. After 5 min drying at 110° in a convection oven the plates were sprayed with either a 0.1 % w/v rubeanic acid in ethanol solution or with a 0.03 % w/v ethanolic solution of PAQH until the plate appeared transparent. (For more details see Results and discussion.) Drying of the plates at 110° for 5 min is recommended for best results.[1,2]

Quantitative evaluation. The cells and technique used in preparing the separated components for quantitative analysis have been described previously.[6] Adsorbent from the same plate serves as a reference standard. The 30 mg or 20 mg samples removed from the plates for analysis of the spots are weighed to $\pm$ 0.1 mg on an Oertling auto-

matic balance and are ground in a small agate mortar for two periods of 1 min each to insure homogeneity of the sample. Samples that are not analyzed immediately are stored in a desiccator over silica gel. A Beckman D.U. spectrophotometer with a standard diffuse reflectance attachment is used for the photometric measurements. The wavelength settings are determined from spectra recorded previously.[1,2] For rubeanic acid complexes in this study they were 630 nm for nickel, 465 nm for cobalt and 580 nm for copper. For the PAQH complexes they were 508 nm for cobalt and 490 nm for nickel. For calibration curves the reflectance values R or the values of the Kubelka–Munk function $(1-R)^2/2R$ are plotted *versus* concentration.[1,2]

Results and discussion

Separation and detection. The separation on silica gel occurred in the order $Ni^{2+} < Co^{2+} < Cu^{2+}$. Complete resolution was achieved within 10–20 min. The separation time is about one-half the time required to separate the ions on cellulose and about one-tenth the time needed on alumina.[1]

The visual detection limits for the rubeanic acid complexes were found to be about 0.03 μg per spot (0.5 cm in diameter) for nickel and copper and 0.05 μg per spot for cobalt. The instrumental detection limits for 20 mg samples taken at an accuracy level of 50 % were 0.05 μg for nickel and copper and 0.1 μg for cobalt.

For sample spots applied in 5 μl amounts, no difficulty was encountered in detecting nickel and copper as rubeanic acid complexes. The cobalt however was present in such small amounts that either larger samples had to be applied on the chromatogram or a different spray reagent had to be used. Previous investigations had shown that with a 0.03 % PAQH solution as a spray reagent the detection limit for cobalt could be improved by a factor of ten (0.01 μg per spot at a 50 % accuracy level).[2] The plate was prepared in the same manner as for rubeanic acid. Colored zones for copper and iron (including iron impurities from the chromatographic adsorbent) did not interfere with the cobalt spot. Faint spots for large quantities of zinc and manganese, as well as the reagent background, faded completely upon heating the plate for 5 min at 110° after spraying. If nickel is present in very low concentrations, one can also use the PAQH complex for its detection. Spraying of the nickel spot with a 1.0 N sodium hydroxide solution is recommended; the detection limit is 0.01 μg per spot.[2]

Quantitative evaluation. Linear Kubelka–Munk plots extending up to concentrations of 10 μg per spot for rubeanic acid complexes and up to 1 μg per spot for PAQH complexes have already been reported.[1,2]

Four 20 g samples from the same batch of oats were prepared for analysis (see Preparation of samples and standards). Nickel and copper were determined as rubeanic acid complexes; cobalt as PAQH complex. The results are reported in Table I.

The recovery of metals from a sample of oats was tested by adding known amounts of the metal to the original sample (see Table II).

CONOLLY AND MAGUIRE[5] have reported fairly good agreement of data obtained by a similar chromatographic technique and a spectrographic technique. The main advantages of this method in comparison with a spectrographic method are better accuracy (between 2 and 5 % for samples in a medium concentration range[1]) and shorter time required for analysis. The two methods are comparable in sensitivity. From Table I it can be seen that metal concentrations of around 0.02 and 0.03 p.p.m.

TABLE I

ANALYSIS OF OATS*

Amount of metal found (p.p.m.)	*Sample No.*				*Average and standard deviation (p.p.m)*	*Relative standard deviation (%)*
	1	*2*	*3*	*4*		
Cobalt	0.03	0.02	0.02	0.03	$0.02_5 \pm 0.00_5$	$\pm$ 20
Copper	3.45	3.68	3.50	3.27	3.48 ± 0.17	$\pm$ 4.9
Nickel	0.96	1.05	0.90	0.99	$0.98 \pm 0.06_3$	$\pm$ 6.4

* The value for each sample is the mean obtained from four spots, developed simultaneously.

TABLE II

RECOVERY OF METALS FROM A SAMPLE OF OATS*

	Cobalt		*Copper*		*Nickel*	
Amount present in original samples (p.p.m.)	0.03	0.03	3.48	3.48	0.96	0.96
Amount added (p.p.m.)	0.05	0.10	3.0	6.0	1.0	3.0
Total amount found (p.p.m.)	0.07	0.15	6.69	9.98	2.08	4.12

* Mean of four results.

can be measured with an accuracy of $\pm$ 20 %. This method should also be applicable to other cereals and to a wide range of foodstuffs.

Acknowledgements

Miss CHARALYNE FREEMAN is to be thanked for technical assistance.

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Book Reviews

Countercurrent Separation Processes, by H. R. C. PRATT, Elsevier, Amsterdam, 1967, 537 pp., price Dfl. 95.—.

This book is concerned with the fundamental understanding of separation processes with the exception of mechanical separations such as filtration and settling. A general treatment applicable to all molecular separation processes is presented in a way that is readable and easy to follow. Consideration is given not only to the well documented processes of distillation, absorption and extraction but to less common ones such as gaseous and thermal diffusion.

A brief introductory chapter is devoted to a preliminary account of the principles and the thermodynamics of separation processes. The next two chapters present steady state cascade theory, chapter two the ideal cascade and chapter three the squared-off and square cascades, all the basic formulae for the steady state performance of columns being derived.

In his preface, Dr. PRATT refers to "the concept of the ideal tapered cascade, still largely unknown amongst chemical engineers", a view that is no doubt shared by many but not those engaged in isotopic separations. He explains that "steady state cascade theory" is concerned with the prediction of the stage requirements of a given separation. Thus chapters two and three derive generalised analytical solutions for binary systems from a combination of separation factor relationships and material balances in finite difference or differential form, techniques more familiar perhaps than the terminology "cascade theory".

The next six chapters then apply these principles to the various separation processes. The processes considered are divided into two groups on thermodynamic grounds, equilibrium processes such as distillation, extraction, ion exchange and gas centrifugation and non-equilibrium processes such as mass and thermal diffusion and molecular distillation. The majority, if not all, of the known separation processes, from the old established to the relatively new isotopic separations are considered and the stated aim of the book to present a general treatment applicable to all processes is achieved. Such a general text, although advanced in coverage, could not be expected to examine individual processes in the detail of a specialised text.

The two concluding chapters consider multicomponent separations and the unsteady state behaviour of cascades.

The book is well referenced, some four hundred references being given and a number of worked examples are included although they are not evenly distributed amongst the individual chapters. The approach is different and the book should be both useful and interesting not only to advanced students of chemical engineering, practising chemical engineers and plant designers, but to all workers, whatever their scale of operation, who are concerned with the separation of mixtures into their components.

A. J. BIDDLESTONE (Birmingham)

Technique of Organic Chemistry, Vol. XII. Thin-Layer Chromatography, by JUSTUS G. KIRCHNER, John Wiley & Sons, Inc., New York, 1967, xxi + 788 pp., price $ 21.95.

KIRCHNER's monograph on thin-layer chromatography (TLC) appears as a separate volume in WEISSBERGER's series *Technique of Organic Chemistry*. Following the familiar pattern of earlier monographs on TLC, the text is divided into two parts: the first (241 pages) is concerned with the technique of TLC, the second (489 pages) with its applications. Attached is a useful list of suppliers of equipment and materials mentioned in the text, and a sufficiently detailed subject index. There is no author index.

In the preface the author, one of the most prominent pioneers of thin-layer chromatography, states that it was not until he "dispensed with the idea of drop chromatography, employed by the earlier workers, and originated the use of uniformly coated glass strips and plates for development in a manner analogous to paper chromatography that the possibilities of the method were uncovered". This refers to his work published in the period 1950–1954. However, it was not until 1958 that the method exploded into its present popularity, when standardised adsorbents and equipment became commercially available, mainly due to the efforts of STAHL.

In the first part, the chapter on development of the chromatogram contains a good discussion of special development techniques. Included here is thin-layer electrophoresis, which could have deserved more attention. A separate chapter is devoted to reactions on plates, including oxidation, reduction, hydrolysis, preparation of derivatives, showing that the author has rightly realised the potential of this field. A table illustrates the use of such reactions in the identification of terpenes and other essential oil constituents.

The next chapter on the detection of colourless compounds contains a list of 204 spray reagents. For many reagents the reactions obtained are not described. The same applies to the detection limits of the reagents. For such details the literature references, given for each reagent, must be consulted. In addition, faced with the need to reveal a functional group, there is no easy way to find suitable reagents in the absence of a group-reagent cross-reference table. The use of radioactive methods in TLC has been dismissed on half a page (9 references).

This chapter is followed by a short (7 pages) but very useful discussion of factors affecting the reproducibility of R_F values in TLC. The next equally short chapter on preparative chromatography is concerned more with historical data (chromatobars) and the use of TLC for the selection of adsorbents for and the characterisation of effluents from column chromatography than with the state of the art of preparative-layer chromatography.

The first part closes with chapters on quantitative TLC and the combination of TLC with other techniques.

The second section of the book covers the applications of thin-layer chromatography. The material is divided into 21 chapters, containing a great number of reproduced chromatograms and tables of R_F values. Sometimes details are missing in the descriptions of procedures, thus making it necessary to consult the original paper. This should not be too difficult, because an outstanding feature of the monograph is the list of literature references, which in the reviewer's opinion represents

probably the most comprehensive coverage of literature on TLC published so far. It covers publications up to 1964 and partly 1965.

Compared with other monographs on TLC, KIRCHNER's book is certainly the most complete and up-to-date review of the subject available in English. Therefore it is of value to all users of the technique, particularly as a source of references.

F. C. A. KILLER (Abingdon)

Electronic Computers, by JOHN C. CLULEY, Contemporary Science Paperbacks No. 13, Oliver and Boyd, Edinburgh, 1967, 189 pp., price 7s.6d.

This book is divided into an introduction, one chapter on analogue computers, two on digital computers, one on languages and programming, one on digital computer applications and a short one on a discussion of future trends.

It is a little difficult to imagine for whom this book is written; it is both too detailed and not detailed enough. It requires at least a grounding in electronic notation and a good understanding of mathematical symbols. It whets the reader's appetite on Boolean algebra, for instance, and then leaves him without so much as a hint, much less a reference, as to where he can get further information. In fact the omission of even a sketchy bibliography is a serious fault in a book of this type.

The author gives a highly technical description of analogue computers and examples of their use and then somewhat cavalierly dismisses them as "generally restricted to engineering design and development"; although he has given a fascinating and tantalizingly brief glimpse of future pocket analogue computers which would be as "common as sliderules".

The chapter on digital computer fundamentals covers the necessary ground but shows a very confusing tendency to leap from binary to decimal and back in mid-sentence or mid-equation; *e.g.*, "0.0101 = 5/16" (this in a discussion on the location of the binary point, *not* on the conversion of binary to decimal or fractional notation).

The chapters on digital computer technology and computer programming and language would, I am sure, prove very heavy going for a general reader, again without reaching a text-book standard of complexity.

The author may fall between two stools in his more technical chapters, but his final two on "Digital computer applications" and "Future trends in computing" could hardly be bettered as a lucid account of the present uses and enormous future potential of digital computers. Although these two chapters occupy only 38 out of the 186 pages, they are probably worth the price of the book to the general reader. They will tell him clearly what computers are doing and will do, and the rest of the book will tell him how they do it, if he cares to do a fair amount of hard reading and thinking.

G. R. PRIMAVESI (Epsom)

News

Technological news

ABCOR, Inc., Cambridge, Massachusetts, pioneer in advanced chemical separation technology, particularly in the field of production-scale chromatography, and C. F. Boehringer & Söhne, Mannheim, Germany, a major factor in the pharmaceutical sector with world-wide operations, have reached an *agreement covering an exchange of know-how and licenses on patented equipment systems for high-capacity liquid chromatographic separation.*

This technology, covered by patents issued to Abcor and Boehringer, is essential to achieve economic production rates on large-scale chromatographic separation columns. It has already been employed in columns nearly 2 ft. in diameter (50 cm), producing tons of material per month.

In the U.S.A., this technology is presently available in gas chromatography equipment manufactured under license from Abcor, Inc., for the separation of a wide variety of chemicals, such as vitamin intermediates, essential oils, and hydrocarbon isomers. Henceforth Abcor will likewise be providing technology and equipment systems for liquid chromatographic separations.

By the use of these techniques, chromatographic separation—which has already become one of the most important analytical methods over the last ten years—can become economical for large-scale, commercial processing throughout the Chemical Industry.

Meetings

Fifth International Seminar on Gel Permeation Chromatography

Provisional arrangements for the Fifth International Seminar on Gel Permeation Chromatography have now been made.

The Seminar will take place from Sunday, May 19th to Wednesday, May 22nd, 1968 and the venue for this meeting will be the Mayfair Hotel, Berkeley Square, London W.1. The Seminar will formally convene at 9.00 a.m. Monday, May 20th and adjourn at mid-day on Wednesday, May 22nd. Mr. John Moore of the Dow Chemical Company, Freeport, Tex., who was concerned with the initial development of GPC, will be attending the Seminar.

COURSE ON THE SEPARATION OF MACROMOLECULES AND PARTICLES

EMBO Summer School, Institute of Biochemistry, Uppsala, Sweden, September 9th–14th, 1968.

Organizers: Professors A. TISELIUS AND J. PORATH AND Dr. S. HJERTÉN

The course will consist of lectures, demonstrations and practical exercises in biochemical separation methods, and will be conducted as were the two previous Summer Schools held at the Institute. The course is open to all European researchers including Scandinavians. A good speaking knowledge of English is required. The registration fee will be £ 25.

The number of participants that can be accepted will have to be limited to about 30. Preference will be given to candidates who have some experience in separation techniques. The closing date for applications is May 15th.

A limited number of fellowships have been made available by EMBO to be distributed upon application to the organizers of the course.

Further information and application forms can be obtained from: Dr. STELLAN HJERTÉN, Institute of Biochemistry, University of Uppsala, Uppsala, Sweden.

SECOND EUROPEAN SYMPOSIUM ON FLUORINE CHEMISTRY 1968

The Academy of Sciences at Göttingen in cooperation with the Institute of Inorganic Chemistry of the University at Göttingen prepared the 2nd European Symposium on Fluorine Chemistry from August 28th–31st, 1968. The Gesellschaft Deutscher Chemiker has taken over the organisation of the Symposium.

Only discussion lectures and no plenary lectures will be presented at the Symposium. Chairman of the scientific committee is Prof. O. GLEMSER, president of the Academy of Sciences at Göttingen. Further material for participation in the Symposium and for submission of discussion papers (only until May 15th, 1968) may be asked for from Dr. W. FRITSCHE, Gesellschaft Deutscher Chemiker, 6000 Frankfurt (M), Postfach 9075, Germany.

MEETING ON MASS SPECTROSCOPY

A meeting organized by the Mass Spectroscopy Group will be held at University College, London, on 16th–18th September, 1968. The meeting will be open to all and will cover all aspects of mass spectrometry, including its chemical and physical applications. The proceedings of the meeting will not be published, but abstracts will be circulated in advance.

Those interested are invited to submit abstracts of short papers on current work before 31st May, 1968. Further information may be obtained from the Secretary, Mr. R. M. ELLIOTT, GEC-AEI Electronics Ltd., Scientific Apparatus Division, Barton Dock Road, Urmston, Manchester, Great Britain.

FIFTH INTERNATIONAL SYMPOSIUM ON ADVANCES IN CHROMATOGRAPHY

The Fifth International Symposium on Advances in Chromatography will be held at Caesars Palace in Las Vegas, Nevada, U.S.A., January 20th–23rd, 1969. The format of the meeting will be similar to the previous symposia, which were held in Houston and New York. The subject matter will include gas chromatography, liquid chromatography and critical state chromatography.

Participation in the symposium will be on the basis of invited papers as well as contributions from the United States and abroad. Those who wish to present papers are requested to submit an abstract of about 500 words by September 15th, 1968. Complete manuscripts of accepted papers will be required by November 1st, 1968.

Abstracts of papers and inquiries concerning the conference should be directed to: Prof. A. ZLATKIS, Department of Chemistry, University of Houston, Houston, Texas 77004, U.S.A.

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