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Chromatographic Methods for the Separation and Identification of Porphyrins

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Reproducible results by chromatographic methods can only be ensured by strict adherence to experimental conditions. Although successful separations of porphyrin esters have been reported by previous workers using columns of alumina (Prunty, 1946; McSwiney, Nicholas & Prunty, 1950; Lederer & Tixier, 1947) or of calcium carbonate (Grinstein, Schwartz & Watson, 1945; Watson, Schwartz & Hawkinson, 1945; Gray & Holt, 1948), the adsorptive power of the commercial materials employed has not been strictly defined and, moreover, little if any reference has been made to the purification or standard of purity of the solvents employed. Repetition of their results, therefore, has often proved difficult.

Filter-paper chromatography has been applied to the porphyrins by Nicholas & Rimington (1949) and in the lutidine-water system used is capable of separating them into groups corresponding to the number of carboxyl functions in the molecule. It can thus be used as an analytical procedure, but appears to be not so well adapted for the preparation of material in quantities suitable for chemical characterization. For this purpose, adsorption chromatography of porphyrin esters upon solid columns is preferable.

Before attempting the isolation of certain hitherto unrecognized porphyrins, which partition chromatography has shown to be present in pathological materials (Nicholas & Rimington, 1951), we deemed it essential to study carefully the chromatographic behaviour of known porphyrin esters in precisely defined systems and to extend, if possible, the range of adsorbents available. This we have done, and our results are presented below. Paper chromatography has proved a very effective means of ascertaining the homogeneity of fractions, and therefore the sharpness of the separations obtained.

METHODS

Adsorbents

The commercial materials used were:

Al_2O_3 , 'Aluminium oxide for chromatographic analysis standardized according to Brockmann', grade I (Savory and Moore Ltd.).

MgO , heavy magnesium oxide (British Drug Houses Ltd.).

MgCO_3 , heavy magnesium carbonate (British Drug Houses Ltd.).

CaCO_3 , calcium carbonate, 'Analar' (British Drug Houses Ltd.).

From these materials, adsorbents of graded adsorptive power were prepared as described below, and all preparations were finally standardized according to the technique of Brockmann & Schodder (1941), whose system of classification for aluminas we have extended to all our materials. Brockmann & Schodder's classification is summarized by Williams (1946).

Graded aluminium oxide. The Al_2O_3 supplied by Savory and Moore (see above) is termed by the manufacturers 'Grade I Brockmann', but we have found that samples can vary from grade I to grade II. The adsorptive power appears to depend upon the amount of water present. Samples can be made more active by heating followed by cooling in a confined atmosphere. Conversely they may be deactivated by the addition of water. Grade II Al_2O_3 can be prepared from any other grade by suspending in water, filtering, drying at 120° and then cooling in a desiccator. Grade IV Al_2O_3 was similarly prepared by wetting followed by drying thin layers in air at 20° .

Graded magnesium oxide. The adsorptive power of the commercial material is rather variable. The grades used in our study were prepared as were the aluminium oxides, the conditions of drying being as follows: grade II, dried at 120° and cooled in a confined space; grade III, dried at 37° in thin layers in a constant temperature room; grade IV, dried in thin layers in air at 20° .

Graded magnesium carbonate. Heavy MgCO_3 as supplied by British Drug Houses was found to be grade III. It behaves chromatographically in all respects as does MgO of similar grade.

Graded calcium carbonate. Samples of CaCO_3 from several different sources, when standardized, all conformed to grade V. They were improved, however, by a preliminary heating to 120° followed by cooling in a desiccator before use.

Adsorption columns

For most experiments, glass tubes were used of size 1.5×20 cm., tapering sharply at one end to about 3 mm. diameter, and provided with a light plug of cotton wool to support the adsorbent. They were filled to a height of 10 cm. with adsorbent in the following manner. The lower end was closed by a stopper and the glass tube almost filled with the solvent in which the adsorbent column was to be packed. The adsorbent was then sprinkled into the solvent until the tube was half filled. The stopper was removed and the column allowed to drain under gravity or, if necessary, gentle suction. In this way the adsorbent particles became completely wetted and produced the most evenly packed columns. This method was used for all adsorbents except CaCO_3 . With this adsorbent the best results are obtained by first making a slurry with the solid and the solvent and then pouring it into the glass tube. Alternatively, the adsorbent may be packed tightly into the column in small portions at a time, using a tamping rod, and then wetted with the solvent. Although CaCO_3 is a very useful adsorbent for porphyrins, it is the most tedious to use owing to this difficulty in packing.

Solvents

The following solvents have been used, details of purification, where necessary, being given below.

Benzene. 'Analar' (British Drug Houses Ltd.). Suitable for use without further treatment; drying and redistilling did not affect its chromatographic behaviour.

Chloroform. The B.P. grade, containing ethanol, possesses a considerably greater eluting power than does the pure solvent. For use it was washed five times with 2 vol. of water and dried over CaCl_2 (see Vogel, 1948). It is then unstable and must be kept in the dark.

Light petroleum. The commercial product, b.p. $40-60^\circ$, was redistilled within these limits.

Methanol. The solvent was dried by the magnesium and iodine method according to Vogel (1948).

Application of porphyrin esters and development of chromatograms

Porphyrin esters (approx. 0.3 mg. of each) were applied to the columns as a concentrated solution in the first-named solvent of each system studied (see Fig. 1), the adsorbent having been packed suspended in this solvent. Development was accomplished by the mixture. Further details are included under the Results section. It may be noted here that in each case the last-named solvent in a system is that possessing the greatest eluting power. All chromatograms were run at room temperature, $19-22^\circ$.

Examination of fractions

Absorption spectra of fractions eluted from the columns were measured on the Beck-Hartridge reversion spectro-scope.

Melting points were observed upon an electrically heated micro-melting point apparatus (Gallenkamp).

Partition chromatography was carried out as described by Nicholas & Rimington (1949, 1951) at a controlled tempera-

ture, $21 \pm 1^\circ$. Porphyrin esters, eluted from the columns, were hydrolysed by evaporating the volatile solvent under reduced pressure and leaving the residue in contact with approx. 7N-HCl at room temperature for 36 hr. Excess acid was removed in a vacuum desiccator containing KOH, the porphyrin hydrochloride dissolved in 2N- NH_4OH and applied to the paper (Whatman, 'no. 1 for Chromatography').

RESULTS

A schematic survey of our results is presented in Fig. 1. It was not possible to separate satisfactorily the I and III series isomers of either uroporphyrin or coproporphyrin with any combination of solvents and adsorbent tried. In general, porphyrin esters are eluted in an order corresponding inversely with the number of carboxyl groups present in the parent porphyrin. The uroporphyrins are thus the most firmly retained. From magnesium oxide and magnesium carbonate, however, the order is exactly reversed, the uroporphyrins being the first to leave the column. The dicarboxylic porphyrin esters all behave very similarly under the solvent conditions which separate them from uroporphyrin and coproporphyrin. Their individual separation is, however, possible and will be discussed in a separate section below.

Magnesium oxide and magnesium carbonate, grade III. Neither benzene nor chloroform possessed any appreciable eluting power with these adsorbents and no separation of esters was observed with mixtures ranging from 100% benzene to 100% chloroform. Upon the addition of methanol to the system, elution began, uroporphyrin running the most rapidly. In practice, therefore, the mixed esters may be applied in either benzene or chloroform, and development carried out progressively with mixtures of 0.5, 1 and 2% (v/v) methanol-chloroform or 4, 8 and 10% (v/v) methanol-benzene, respectively, which carry down the successive bands.

No satisfactory separation was achieved with solvent systems composed of light petroleum and chloroform, or those with methanol (or unwashed chloroform) as used by McSwiney *et al.* (1950).

Aluminium oxide, grade II. The solvent systems benzene-chloroform, benzene-methanol and chloroform-methanol produced separation of the dicarboxylic porphyrin esters as a discrete band below the uroporphyrin and coproporphyrin esters; these formed two contiguous bands which were inseparable and travelled down the column together.

Aluminium oxide, grade IV. The benzene-chloroform solvent system gave with this weaker adsorbent separation of the three groups of esters, coproporphyrin and the dicarboxylic porphyrin esters being eluted without the addition of methanol. Uroporphyrin was eluted when the latter solvent was added to the system. With chloroform-methanol and benzene-methanol, however, this adsorbent

ADSORBENTS

Solvents	MgO grade III or MgCO ₃			Al ₂ O ₃ grade IV		Al ₂ O ₃ grade II		CaCO ₃ grade V	
	No separation	No separation	No separation	No separation	No separation	No separation	No separation	No separation	No separation
Benzene									
Benzene-chloroform									
Benzene-chloroform-methanol									
Chloroform-methanol									
Benzene-methanol									
Light petroleum-chloroform									
Light petroleum-unwashed chloroform*									

* Equivalent to chloroform containing 1% (v/v) methanol.

Fig. 1. Adsorption chromatography of porphyrin methyl esters. □, uroporphyrins; ▨, coproporphyrins; ▤, dicarboxylic porphyrins; ↓ indicates movement of band on columns.

behaved in the same way as grade II aluminium oxide.

In the case of light petroleum-chloroform or the same system plus methanol, good separation of the porphyrin esters is produced with both grades of alumina (cf. McSwiney *et al.* 1950). The procedure is somewhat complicated since progressive solution of the porphyrin components takes place with increasing concentration of chloroform in the developing solvent. Fig. 2 illustrates the separation.

Fig. 2. Chromatography of esters on aluminium oxide grade II using chloroform-light petroleum mixtures. Ratio vol./vol. shown on figure. ▨, uroporphyrins; ▩, coproporphyrins; ·····, dicarboxylic porphyrins.

Calcium carbonate, grade V. Development by benzene of a chromatogram of the porphyrin esters on this adsorbent causes the dicarboxylic porphyrin esters to separate from the remainder as a mobile band. Addition of chloroform (6 vol. chloroform to 10 vol. of benzene) causes migration of the coproporphyrin band. Uroporphyrin ester is only mobilized by the addition of methanol (1 vol. methanol to 100 vol. of chloroform). If benzene-methanol or chloroform-methanol systems are used from the start, elution takes place without separation.

With a technique similar to that mentioned above for alumina, in which the porphyrins are applied to the column in light petroleum-chloroform, separation of the three classes of esters is possible.

Separation of the dicarboxylic porphyrin esters

In these experiments the esters of protoporphyrin, deuteroporphyrin and mesoporphyrin have been employed. On account of the close chemical similarity between these pigments, chromatographic separation has presented more difficulties than when dealing with porphyrins containing differing numbers of carboxyl groups. Of the three, protoporphyrin is the most firmly adsorbed, no doubt on account of its two vinyl groups. Either magnesium oxide, grade III, or calcium carbonate, grade V, may be used for separation (see Fig. 3).

Magnesium oxide, grade III. The esters are applied in chloroform solution and development performed with mixtures of 0.5:100, 2:100 and 3:100 (v/v) methanol-chloroform used successively. Mesoporphyrin, deuteroporphyrin and protoporphyrin esters are eluted in that order.

A. Magnesium oxide grade III and chloroform-methanol

Column packed in chloroform

Chloroform-methanol, 100:3

Chloroform-methanol, 100:2

Chloroform-methanol, 100:0.5

B. Calcium carbonate grade V and light petroleum-benzene-chloroform-methanol

Column packed in benzene-light petroleum, 1:4

Chloroform-methanol, 100:1

Benzene-light petroleum, 1:2

Fig. 3. Separation of dicarboxylic porphyrin esters.

Calcium carbonate, grade V. The esters are applied dissolved in benzene-light petroleum, 1:4 (v/v). For development the benzene is then increased to 2:1 (v/v), at which stage deuteroporphyrin ester passes down the column. Protoporphyrin ester is eluted with methanol-chloroform, 1:100 (v/v).

DISCUSSION

Magnesium oxide preparations constitute a valuable addition to the technical resources of porphyrin chromatography. The columns are easily packed, percolation by solvents is rapid and separations are good. In addition, the uroporphyrins are the first to be eluted from this adsorbent and its choice is therefore indicated for preparative purposes when these porphyrins are the main objective.

The separation of the naturally occurring dicarboxylic porphyrins upon magnesium oxide or calcium carbonate is a useful technical device. The combined dicarboxylic fraction may be obtained in

a primary chromatogram and then resolved by a secondary chromatogram upon one or other of these adsorbents. Filter-paper chromatography, it may be recalled, does not differentiate between the members of this group (Nicholas & Rimington, 1949).

It is unfortunate that none of the systems we have studied has been capable of separating sharply the I and III series porphyrin isomers. It may be noticed, in particular, that we failed to separate uroporphyrin esters I (from bone) and III (Waldenström ester), whereas Fischer & Hofmann (1937) claimed to have done this by chromatographing uroporphyrin ester from Petry urine upon talc. They ascribed to uroporphyrin ester I a melting point of 311° (corr.) and to the series III isomer a melting point of 261° (uncorr.), but Grinstein *et al.* (1945) have cast serious doubt upon this claim. The material with m.p. 311° is thought by them to have been a uroporphyrin metal complex (probably copper) and the existence in nature of a uroporphyrin belonging to the aetioporphyria III series is considered unproven. The Waldenström ester is thought by Grinstein *et al.* (1945) to be a mixture or molecular compound of

uroporphyrin I with a heptacarboxylic porphyrin (probably of series III).

In a later paper the application of chromatographic methods to the isolation of new porphyrins from various sources will be described.

SUMMARY

1. The chromatographic behaviour has been studied of porphyrin esters under standardized conditions.

2. Magnesium oxide and magnesium carbonate possess certain advantages for porphyrin chromatography.

3. The naturally occurring dicarboxylic porphyrins may be separated chromatographically upon calcium carbonate or on magnesium oxide columns.

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Displacement Chromatography on Synthetic Ion-exchange Resins

6. EFFECT OF TEMPERATURE ON THE ORDER OF DISPLACEMENT

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During the course of development of the work described in this series of papers it has become increasingly clear that non-ionic adsorption effects play an important part in determining the order of displacement of bases and ampholytes from columns of synthetic cation exchangers. In Part 4 of this series (Partridge, 1949b) the various factors then known to affect the order of displacement were discussed in

some detail. Previously Davies (1949) had shown, from theoretical considerations, that an ideal cation-exchange process would be expected to result in the separation of a series of monoamino-acids in an order depending upon their pK_1 values; that is to say, upon the negative logarithms of the acid dissociation constants for the reaction: $A^+ \rightleftharpoons A^\pm + H^+$. For the purposes of illustration Davies assumed a