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Separation of Uroporphyrin Esters I and III by Paper Chromatography

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The separation of free porphyrins according to the number of carboxyl groups on their substituent side chains has been achieved by paper chromatography (Nicholas & Rimington, 1949, 1951*a*). This method is very sensitive, and has already revealed the presence of hitherto unknown porphyrins in pathological urines (McSwiney, Nicholas & Prunty, 1950; Nicholas & Rimington, 1951*a*; Rimington & Miles, 1951); it does not, however, separate the position isomers coproporphyrins I and III or uroporphyrins I and III. The method of Chu, Green & Chu (1951), in which the porphyrin esters are used, allows the identification of protoporphyrin, coproporphyrin I, coproporphyrin III and uroporphyrin I in mixtures. But these workers did not study uroporphyrin III and we have found that the uroporphyrin isomers do not separate under their conditions.

The identification of a uroporphyrin isolated from natural sources as the series I or series III isomer has relied in the past mainly on comparison of the melting point of the esterified specimen with the melting points of the supposedly pure isomers. Only since the isolation by Nicholas & Rimington (1951*b*) of unequivocal uroporphyrin III from turacin, has the melting point (264°) of pure uroporphyrin III ester been established beyond doubt. Even so, reliable estimates of the composition of a specimen from natural sources cannot be made simply from melting-point comparisons, because of the unexpected form of the mixed melting-point curve recently established in this laboratory for uroporphyrin esters I and III (Nicholas & Rimington, 1953). A powerful tool has been provided, however, by the use of this curve coupled with the specific decarboxylation of uroporphyrin mixtures to the corresponding coproporphyrins, with subsequent determination of the proportions of the latter (cf. Rimington & Miles, 1951).

In this paper we describe a paper-chromatographic method which gives good separation of the uroporphyrin esters I and III. By a combination of this method with that of Chu *et al.* (1951) all five main natural porphyrins (uroporphyrins I and III,

coproporphyrins I and III and protoporphyrin) can be separated on a single paper square (see preliminary note, Falk & Benson, 1953).

Some results of the application of the method to the analysis of natural uroporphyrin mixtures are described, the significance of the results being discussed more fully by Nicholas & Rimington (1953).

EXPERIMENTAL

Materials

Solvents. The kerosene was commercial heating paraffin, distilled over $CaCl_2$; the fraction boiling at 190–250° was used. Chloroform, B.P., was washed three times with water and dried over $CaCl_2$. Ethanolic chloroform: to chloroform treated as above, ethanol was added to a concentration of 1% (v/v). Dioxan was purified by the method of Eigenberger (1931). *n*-Propanol was redistilled and had b.p. 95–97°. Melting points were corrected.

Specimens from pathological sources. Sample 4 (Table 1) was collected from band *Ci* of Fig. 1 of Rimington & Miles (1951); sample 2 was material *B* of Table 1 of the paper of Rimington & Sveinsson (1950). Samples 5–9 were all 'Waldenström' esters prepared from the urine of cases of acute porphyria in relapse, as described by Nicholas & Rimington (1953). The isolation from a case of porphyria cutanea tarda of sample 10 was described by Macgregor, Nicholas & Rimington (1952). Sample 11 was isolated from the urine of the same patient during a different attack.

Methods

Chromatography was by the ascending method in closed glass jars, at 22–23°. The solvent to saturate the atmosphere was put in the bottom of the jar and the developing-solvent mixtures in a Petri dish in which the paper square, rolled into a cylinder, was stood. 20 cm. squares of Whatman no. 1 paper were used, the porphyrin esters being applied from freshly prepared solutions in chloroform as spots along a base-line 2 cm. from one edge. The positions of spots after development were determined by their fluorescence in ultraviolet light.

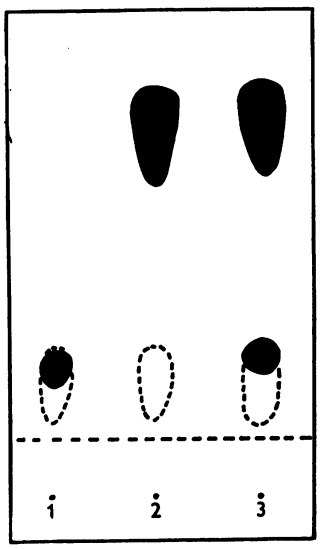

Fig. 1.

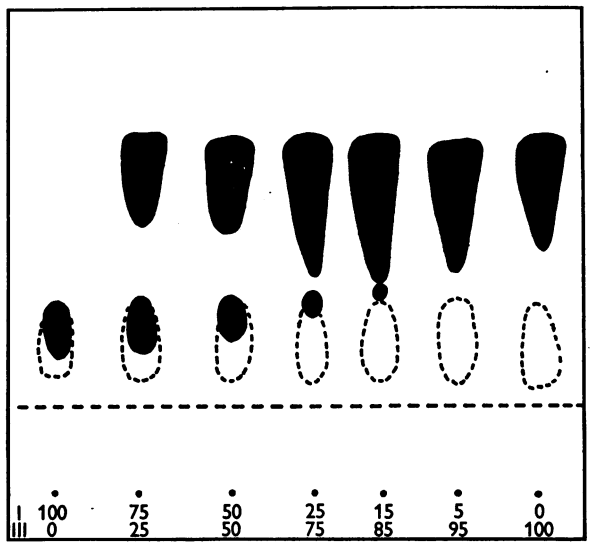

Fig. 2.

Fig. 1. Paper chromatogram obtained using method A. (1) Uroporphyrin I, (2) uroporphyrin III, (3) equal quantities of uroporphyrins I and III. Broken lines show positions of spots before final development. (For details of method, see text.)

Fig. 2. Paper chromatogram obtained by application of method A to a series of mixtures of pure uroporphyrin esters I and III. (For details of method, see text.)

Standard reference substances. Uroporphyrin I ester, m.p. 293° (Table 1, sample 3) was fraction *Ai* shown in Fig. 1 of the paper by Rimington & Miles (1951); further data concerning it are given by Rimington & Sveinsson (1950). Uroporphyrin III ester, m.p. 264° (sample 1), was prepared from turacin by Nicholas & Rimington (1951*b*). Coproporphyrin I ester, m.p. 250–251°, was isolated from calf meconium and coproporphyrin III ester, m.p. 160/178° from *Corynebacterium diphtheriae*. Protoporphyrin IX ester, m.p. 230°, was prepared from haemin by the method of Grinstein (1947).

A. *Chromatography of methyl esters of uroporphyrins* (Figs. 1 and 2)

(1) Kerosene-ethanolic chloroform (see Materials section) development (atmosphere, chloroform). The paper was developed by a mixture of kerosene (4 ml.)

and ethanolic chloroform (6 ml.), until the porphyrin spots had moved about a third of the way up the paper. This was then removed and dried. Uroporphyrin esters I and III and mixtures of them move at the same rate, and no separation is achieved in this process, which merely serves to move the porphyrin esters away from the base-line and from traces of free porphyrins or other non-mobile impurities. The base of the paper was now cut off 0·5 in. below the spots to eliminate non-mobile material and to save time in the next development.

(2) Kerosene-dioxan development (atmosphere, dioxan). The cut-down square was developed in the same direction as before, with a mixture of kerosene 4 ml. and dioxan 1·5 ml., the solvent front being allowed to reach the top of the paper.

With different batches of dioxan, in spite of the most careful purification and fractionation, it was necessary to determine experimentally the proportion of dioxan to kerosene for optimal separation of the isomers.

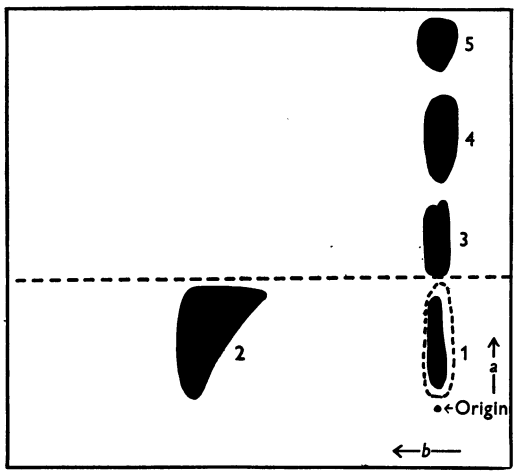

Fig. 3. Paper chromatogram obtained using method B. (1) Uroporphyrin I, (2) uroporphyrin III, (3) coproporphyrin I, (4) coproporphyrin III, (5) protoporphyrin. Developed in direction (*a*) after Chu *et al.* (1951) and the lower portion then developed in direction (*b*) with kerosene-dioxan. (For details of method, see text.)

B. *Chromatography of mixtures of uroporphyrins, coproporphyrins and protoporphyrin* (Fig. 3)

The above method was combined with the method of Chu *et al.* (1951) to analyse mixtures of the esters of the uroporphyrin isomers, the coproporphyrin isomers and protoporphyrin on a single paper square. After development by the method of Chu, the uroporphyrins remained together near the base-line. The paper was cut off parallel with the base-line, just above the uroporphyrins, leaving the separated coproporphyrins I and III and protoporphyrin on the top portion of the paper. The lower portion was then developed with kerosene-dioxan as above, but in a direction at right angles to the direction of the previous developments.

The method of Chu *et al.* (1951) involves development with kerosene-chloroform (without addition of ethanol) followed by a kerosene-propanol development. Since the uroporphyrins are moved just clear of the base-line in this process, it was not necessary to include the kerosene-ethanolic chloroform development, though this can be done if desired.

RESULTS

Uroporphyrin esters I and III move in the above processes with R_F values of about 0·02 and 0·5 respectively, the ratios being calculated from the positions of the spots before and after the last (kerosene-dioxan) development. Uroporphyrin I forms a relatively small, round, spot, very strongly fluorescent, while the uroporphyrin III spot is elongated and rather fan-shaped (Fig. 1); since the material occupies more space on the paper the fluorescence is less intense. In mixtures of the pure isomers, a 15% admixture of either was easily distinguished (Fig. 2).

Table 1 compares the chromatographic assessment of the proportions of isomers in uroporphyrin mixtures from pathological sources with that deduced from the melting point of the mixture.

DISCUSSION

The provision of a paper-chromatographic method for the separation of the isomeric uroporphyrins I and III is a considerable help in the study of the porphyrins excreted by patients with porphyria.

Uroporphyrins I and III have not yet been synthesized, and thus for the standard markers it was necessary to use carefully purified materials from natural sources. The constitution of these pigments had been established by part-decarboxylation and characterization of the resulting coproporphyrins.

It was found better to apply the materials to the paper in solution in newly washed and dried chloroform than in acetone solution. If acetone is used (cf. Chu *et al.* 1951) it is important that the solutions be freshly prepared. Decomposition sufficient to show on the chromatograms may occur within 24 hr. and becomes spectroscopically obvious after a few days.

From the paper chromatograms it is possible to make a rough estimate of the proportions of the isomers present in a natural mixture. The limit of sensitivity is about 10%; a 15% admixture of either isomer is clearly distinguishable.

Application of the method to a number of 'Waldenström' esters, the esterified uroporphyrin

Table 1. *Composition of naturally occurring uroporphyrins*

Sample	Source of material*	M.p. (corr.) (°)	Interpretation of paper chromatograms	Composition deduced from position of melting point on melting-point curves: Uroporphyrin curve†	Coproporphyrin curve‡
1	Turacin	264	III only	III only	—
			Congenital porphyria		
2	Bone	293	I only	I only	—
3	Lev.	293	I only	I only	—
4	Lev.	244	I ≧ III	—	—
			Acute porphyria		
5	Beres.	253–4	25% I	15–20% I	20–25% I
6	Salisb.	255–7	20% I	15–20% I	20% I
7	Glasg.	260	Trace I	15% I	15% I
8	Evan.	245–58	Little I	18–36% I	—
9	McLaugh.	257–8	10% I	15–20% I	20–23% I
			Porphyria cutanea tarda		
10	L. Walk.	259–61	III only	5% I	—
11	L. Walk.	256–60	25% I	14–24% I	20–25% I

* Abbreviations apply to patients' names; these are included for ease of cross-reference to the accompanying papers (Nicholas & Rimington, 1953; Kennard & Rimington, 1953).

† Cf. Nicholas & Rimington (1953).

‡ Coproporphyrin from decarboxylation of corresponding uroporphyrin sample; composition by comparison with the melting-point curve of Jope & O'Brien (1945).

fraction from acute porphyria urines, showed the presence of both isomers, with uroporphyrin III always predominating in this type of porphyria (samples 5–9). In the case of porphyria cutanea tarda studied, the III isomer was again present in the higher proportion (samples 10 and 11), while in the congenital porphyrias (samples 2–4) mainly uroporphyrin I was excreted.

By reference to Fig. 1 of the paper of Rimington & Miles (1951) it will be seen that fraction *A* i (sample 3 of the present paper) was the main uroporphyrin fraction, and that fraction *C* i (sample 4 here), a relatively very much smaller fraction, separated from the main band. The isomer analysis of these two fractions shows not only the predominance of uroporphyrin I in congenital porphyria but provides an interesting example of the behaviour of certain mixtures of the isomers as an entity, perhaps due to 'molecular compound' formation (cf. Nicholas & Rimington, 1953).

It is evident that the proportion of the two isomers varies somewhat between different cases of acute porphyria. The samples from a case of porphyria cutanea tarda show clearly that the proportions of the uroporphyrins excreted may vary, in this disease, from one attack to another.

SUMMARY

1. A paper-chromatographic method is described which separates the isomeric uroporphyrin esters I and III.

2. In conjunction with the method described by Chu, Green & Chu (1951), this new method allows the identification, on a single paper square, of the main natural porphyrins: uroporphyrins I and III, coproporphyrins I and III and protoporphyrin.

It is a pleasure to acknowledge the encouragement we have received from Prof. Rimington and his kindness in providing the pure samples and the natural mixtures.

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