SUMMARY

1. The stock of rats used in the experiments upon the hexokinase reaction *in vitro* show the same behaviour regarding the absorption of sugar *in vivo* as do the rats used in experiments upon absorption described in the literature.

2. The phosphorylation-rate ratios for the sugars investigated are very similar to their absorption-rate ratios.

3. The rate of the hexokinase reaction catalysed by the intestinal mucosa is more than sufficient to account for the phosphorylation of all the sugar absorbed during the experiments *in vivo*. It is sufficient to account for the phosphorylation of at least 50% of the carbohydrate consumed by a normal 150 g. rat during 24 hr., and for the phosphorylation of 70–100% of this carbohydrate after certain legitimate corrections have been applied to the figures for the hexokinase reaction.

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A Chromatographic Study of Uroporphyrins from Cases of Cutaneous, Acute and Congenital Porphyria

By J. CANIVET* and C. RIMINGTON
Department of Chemical Pathology, University College Hospital Medical School, London, W.C. 1

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Chromatographic methods (Nicholas & Rimington, 1949, 1951; Nicholas, 1951; Chu, Green & Chu, 1951; Falk & Benson, 1953*a*, *b*) have made possible the isolation and characterization of isomeric porphyrins. A study of porphyria cases has revealed much variety in the urinary porphyrin pattern (Nicholas & Rimington, 1951; Rimington & Miles, 1951; Macgregor, Nicholas & Rimington, 1952), but final agreement has not yet been reached as to which particular isomer predominates in the different kinds of porphyria. Thus, while Waldenström (1934, 1935), Waldenström, Fink & Hoerburger (1935) and Mertens (1936, 1937) independently described the urinary porphyrin characteristic of acute porphyria as uroporphyrin III, Grinstein, Schwartz & Watson (1945) and Watson, Schwartz & Hawkinson (1945) considered that the 'Waldenström porphyrin' consisted only of uroporphyrin I together with smaller quantities of a heptacarboxylic porphyrin. Although there appears to be a good deal of clinical and chemical variability between cases of acute porphyria, nevertheless Nicholas & Rimington (1953), employing a number of available techniques (Kennard & Rimington, 1953), have confirmed Waldenström's original conclusion in several cases of undoubted acute porphyria. Porphyria cutanea tarda (Waldenström, 1937), during exacerbations, may simulate acute porphyria closely, abdominal colic with or without nervous symptoms occurring and sometimes even porphobilinogen being found. However, the history of cutaneous lesions, presence of porphyrin in the plasma during the attack and high faecal porphyrin excretion during remission serve to distinguish it from acute porphyria. Some cases of porphyria, with cutaneous symptoms appearing late in life, seem to excrete uroporphyrin continuously in the urine (although perhaps with fluctuations of

* Chargé de Recherches à l'Institute National d'Hygiène, 3 rue Leon-Bonnat, Paris, 16e.

amount) and to manifest no abdominal or nervous symptoms. In many of these some degree of hepatic damage is present, e.g. from alcoholism, and it is difficult to decide whether such cases are examples of porphyria cutanea tarda which will later progress until the symptoms become more typical, or whether they should be placed in a separate category. Several such cases were available for study and are referred to provisionally as cases of 'cutaneous porphyria'.

It was felt that a full study of their urinary uroporphyrins would be of especial interest and might, perhaps, assist in their classification. For comparison in the present work, urinary uroporphyrins from cases of acute porphyria and congenital porphyria have been examined by the same methods.

EXPERIMENTAL

Materials

Cutaneous porphyria. The five cases, Ance., Vass., Turp., Hein., Fourn. with ages from 54 to 68 have been described by Bolgert, Canivet & LeSourd (1953). They were closely similar in all respects; they had bullous skin lesions but neither colics nor nervous symptoms and excreted large quantities of urinary uroporphyrin but no porphobilinogen (repeated tests). From urines of each of these patients, total porphyrin was adsorbed on talc at pH 3·5 and esterified with methanolic HCl. The esters were chromatographed on Al_2O_3 to separate the crude uroporphyrin fractions, which were then crystallized and their m.p.'s and rates of migration on paper determined (Table 1). These fractions were then combined and this material is hereafter referred to as 'pool uroporphyrin'; it was obtained from 38 l. of urine. From two further cases, Cam. and Clem., the urinary uroporphyrin was isolated individually. A further individual isolation was made from the urine of Ance.

Acute porphyria. The uroporphyrin fraction was similarly prepared from the urines of two cases, Jo. and Hem. Before adsorption on talc, the urines were heated at pH 4·2 for 20 min. in a boiling-water bath to convert all porphobilinogen into porphyrin (Table 1).

Congenital porphyria. Two cases were available, Yve. and Jul. and their uroporphyrins were prepared in a similar manner to the pool uroporphyrin (Table 1).

Methods

Column chromatography. This was carried out according to Nicholas (1951) using MgO grade III or Al_2O_3 grade IV or V. With Al_2O_3, impurities were removed by C_6H_6:$CHCl_3$, 2:1, any metalloporphyrin present by 1:1, coproporphyrins by 1:2, pentacarboxylic porphyrin by 1:3 or 1:4 and hexa- or hepta-carboxylic porphyrin by 1:6–1:10 mixtures (all v/v); pure $CHCl_3$ eluted a porphyrin the methyl ester of which had m.p. 211–216° and which appears to be octacarboxylic, but uroporphyrin was only eluted by $CHCl_3$ containing methanol. This mixture tended to mobilize the dark layer at the top of the column, which always retained some porphyrin; therefore the uroporphyrin fraction was generally obtained by extrusion and cutting. The retained top layer usually contained portions of all the constituents of the original mixture. Difficulty of this kind was hardly ever encountered with MgO columns (from which, of course, the uroporphyrins are the first to be eluted). A re-esterification was usually advisable before proceeding with the working up of this fraction. Repeated chromatography was essential to achieve homogeneity of any fraction.

Table 1. *Origin and properties of uroporphyrins used in this investigation*

			Comparison by chromatography		
Case	No. of crystallizations ($CHCl_3$:MeOH)	M.p. of porphyrin ester (°)	No. of carboxyl groups (Nicholas & Rimington, 1949, 1951)	Types of porphyrin present (Chu *et al.* 1951)	Ratio of uroporphyrin I to uroporphyrin III (Falk & Benson, 1953*a*)*
			Cutaneous porphyria		
Ance.					
(1st batch)	1	272–5	8	Uroporphyrin only	3:1†
(2nd batch)	1	267–75	8	Uroporphyrin only	—
Vass.	2	254–7	—	Uroporphyrin only	1:3
Turp.	2	273–80	—	Uroporphyrin only	4:1†
Hein.	2	273–9	—	Uroporphyrin only	4:1
Fourn.	1	260	—	Uroporphyrin with traces of copro-porphyrins I and III	3:2†
Cam.	4	285	—	—	17:3
Clem.	4	282	8	Uroporphyrin only	17:3
Pool‡	3	271–4	—	—	—
			Acute porphyria		
Jo.	2	256–7	8	Uroporphyrin only	1:4
Hem.	1	255–7	—	Uroporphyrin only	1:3
			Congenital porphyria		
Yve.	3	281	—	—	19:1
Jul.	3	285	—	—	19:1

* Approximate proportions, estimated by comparison with chromatograms of known mixtures.
† Confirmed by X-ray diffraction pattern (Kennard & Rimington, 1953).
‡ Bulked material from first five cases (see text).

Lutidine filter-paper chromatography. This was carried out according to Nicholas & Rimington (1949, 1951) and served to control homogeneity with respect to the number of carboxyl groups and to indicate the type of porphyrin present in fractions collected from columns.

Paper-chromatographic separation of isomers. Coproporphyrins I and III were separated according to Chu *et al.* (1951) and uroporphyrins I and III according to Falk & Benson (1953*a*, *b*).

Absorption spectra. The positions of the α bands were measured in $CHCl_3$ solution by the Hartridge reversion spectroscope.

Melting points. These were observed on the electrically heated microscope stage.

Decarboxylation. This was performed by heating with 1% (w/v) HCl in sealed tubes at 180–190° for 3 hr.

RESULTS

Cutaneous porphyria

The first chromatography of the combined crude esters was made upon magnesium oxide, grade III, in chloroform. Elution with chloroform, then with a chloroform:methanol mixture (100:0·5–100:2, v/v) removed first a yellow, non-porphyrin pigment (discarded), then the porphyrin, and left a residue of brown-black material at the top of the column (discarded). The porphyrin esters, derived from porphyrins containing, 8, 6, 5 and 4 carboxyl groups, respectively, were redissolved in benzene and placed upon a column of aluminium oxide, grade V, and development was carried out with benzene: chloroform mixtures as indicated under Methods. Each eluate was evaporated to dryness, redissolved in chloroform and examined by the Hartridge reversion spectroscope and by lutidine paper chromatography. The findings are recorded in Fig. 1 which shows the progress of the fractionation. Bands from different columns, having the same characteristics, were occasionally mixed to facilitate operations.

Fig. 1 shows that the fractionation proceeded very regularly, there being always a good correlation between position on the column, α band and result of lutidine chromatography. The following materials were obtained:

Metalloporphyrin ($F_1+G_1+M_1+N_1+O_1$). Pink, spectrum in chloroform $\lambda_{max.}$ 562·6, 530·2 mμ.; not split by acids. This appears to be a complex with copper or another heavy metal and has not been further studied.

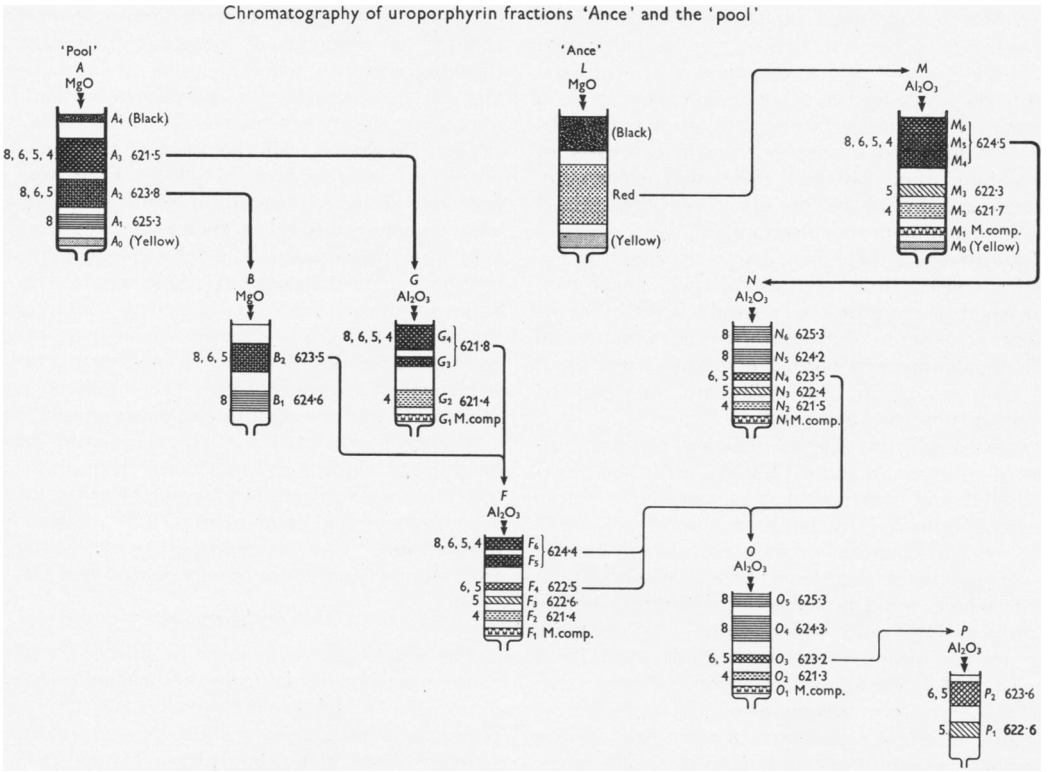

Fig. 1. Schematic representation of chromatographic separation of individual porphyrins from the crude fractions. 8, 6, 5, 4 = carboxyl numbers of lutidine chromatography; 621·5 = α band of spectrum in $CHCl_3$; M.comp. = metal complex.

Coproporphyrins (F_2, G_2, M_2, N_2, O_2). Paper chromatography (Chu *et al.* 1951) showed coproporphyrin I to predominate in F_2, G_2 and O_2 derived from the pool, whilst in M_2 and N_2 from case Ance. the series III isomer predominated. F_2, G_2 and O_2 were combined and examined; it had m.p. 243–246° and α band at 621·4 mμ. Lutidine chromatography showed that the porphyrin was tetracarboxylic, together with a trace of tricarboxylic porphyrin; isomer separation showed coproporphyrin I and a faint spot in the position of coproporphyrin III. M_2 and N_2 similarly afforded material of m.p. 136–140° and α band 621·6 mμ.; chromatography showed traces of tricarboxylic porphyrin and of series I isomer accompanying coproporphyrin III.

Pentacarboxylic porphyrin ($F_3+M_3+N_3+P_1$). In all these fractions, only pentacarboxylic porphyrin was revealed by lutidine chromatography. The material was dissolved in benzene, and the ester precipitated by addition of light petroleum; it crystallized from hot chloroform:methanol in loose aggregates of broad, curved needles, m.p. 210–214°, after recrystallization melting at 210–215°. This material, wt. 1·5 mg., had an α band at 622·5 mμ., which is typical of pentacarboxylic porphyrins. When chromatographed on paper by the dioxan method (Falk & Benson, 1953*a*), two spots formed: one, more intense, was in advance of the uroporphyrin III position, the other, less intense, was in advance of the uroporphyrin I position. Decarboxylation afforded a coproporphyrin the ester of which had m.p. 246–249° and was shown by chromatography to be mainly coproporphyrin I with a trace of coproporphyrin III.

Hexacarboxylic porphyrin. This was present in P_2, together with some pentacarboxylic porphyrin, from which it could not be entirely freed by chromatography upon aluminium oxide, magnesium oxide or calcium carbonate. The quantity was small and had an α band at 623·6 mμ. Attempted crystallization was unsuccessful.

Octacarboxylic porphyrin. During the development of columns *N* and *O* (Fig. 1), after the hexacarboxylic and pentacarboxylic porphyrin esters had been removed by benzene:chloroform (1:4–1:10, v/v), application of pure chloroform caused the separation of a distinct band (N_5 and O_4 in Fig. 1) which could be collected separately before the uroporphyrin ester, which was only mobilized by chloroform containing methanol. This material had α band 624·3–624·5 mμ. (cf. uroporphyrin ester 625·4 mμ.) and crystallized from hot chloroform:methanol in small aggregates of very fine needles somewhat resembling uroporphyrin III ester; m.p. 211–216°, unchanged by recrystallization from the same solvents, melting at 211–215° after crystallization from benzene:light petroleum and at 211–215° (remelting at the same temp.) when again recrystallized from chloroform:methanol.

Lutidine paper chromatography was carried out several times and invariably showed only one porphyrin, with R_F slightly greater than that of uroporphyrin but considerably less than would be expected for a heptacarboxylic porphyrin. That it was not uroporphyrin ester was clearly established by adding some of the material of m.p. 211–216° to uroporphyrin methyl ester and chromatographing the mixture upon aluminium oxide grade IV. Development with pure chloroform removed the ester of the new porphyrin in a compact band. It crystallized from chloroform:methanol in the usual form with m.p. 211–216° and its full visible spectrum, of aetio type, was 624·7; 571·9; 536·2; 503·9 mμ. The uroporphyrin ester which had been used in this mixture was recovered unchanged from the top of the column by elution with chloroform containing 1 % of ethanol.

Further information concerning the nature of this porphyrin ester was sought by subjecting it to a two-dimensional paper chromatography, the first solvents being those of Chu *et al.* (1951), the second the dioxan mixture of Falk & Benson (1953*a*). The porphyrin moved as a single spot, first like a uroporphyrin ester and then, with dioxan, to a position slightly in advance of uroporphyrin ester III. Chromatography together with a specimen of Watson's heptacarboxylic porphyrin methyl ester, m.p. 208°, clearly established their difference.

Decarboxylation. Of the total of 9·9 mg. of the porphyrin ester of m.p. 211–216°, 4 mg. was submitted to decarboxylation. The resultant porphyrins were transferred to ether, then to 0·1 N hydrochloric acid, precipitated, dried, esterified and chromatographed upon magnesium oxide grade III. The coproporphyrin fraction, eluted by chloroform:methanol (100:0·5, v/v) crystallized from ether in rosettes of needles, m.p. 150°, remelting at 176°; this is typical of coproporphyrin III. Only coproporphyrin III was revealed by paper chromatography.

Uroporphyrin ($A_1+N_6+O_5$). The ester crystallized from chloroform:methanol with m.p. 271–274°; this was subjected to decarboxylation, yielding a coproporphyrin ester of m.p. 220°. Paper chromatography showed coproporphyrin I together with smaller quantities of coproporphyrin III.

Acute porphyria

The uroporphyrin, isolated by similar methods to those described above from the urinary porphyrins of the case Jo., gave an ester of m.p. 256–257° and by paper chromatography (Falk & Benson, 1953*a*) consisted of about 80 % uroporphyrin III and 20 % uroporphyrin I; that from the case Hem. gave an ester of m.p. 255–257° and contained about 75 % of the series III isomer with 25 % of the series I (Table 1).

Congenital porphyria

The uroporphyrins from the two cases Yve. and Jul. yielded esters of m.p.s 281 and 285°, respectively. Assessment from the paper chromatograms indicated in each case about 95 % uroporphyrin I and about 5 % of uroporphyrin III. Decarboxylation of the Jul. specimen yielded coproporphyrin I, ester m.p. 248–251°.

DISCUSSION

The complexity of the urinary porphyrin pattern in these cases of 'cutaneous porphyria' resembles that found in acute porphyria (Nicholas & Rimington, 1951) congenital porphyria (Rimington & Miles, 1951) and some cases of porphyria cutanea tarda (Macgregor *et al.* 1952). In all the seven cases of cutaneous porphyria studied here, the uroporphyrin consisted predominantly of the I series isomer, although the proportion of I to III varied somewhat from case to case (Table 1). The melting point of the uroporphyrin ester mixture usually lay in the region of 270–280°, but in one instance (Fourn.) it was 260°. That this specimen, nevertheless, consisted mainly of uroporphyrin ester I was confirmed by its X-ray diffraction pattern (Kennard & Rimington, 1953); the low melting point is understandable by reference to the melting-point curve of mixtures of uroporphyrin methyl esters I and III established by Nicholas & Rimington (1953). In one case of the series (Vass.), a uroporphyrin was excreted of which the ester had m.p. 254–257° and which contained about 75 % of uroporphyrin III and 25 % of uroporphyrin I.

So far as the isomer type of the uroporphyrin excreted is concerned, the majority of these cutaneous porphyrias thus resemble cases of congenital porphyria more closely than they do cases of acute porphyria.

During the chromatographic operations, a fraction was obtained which was more easily eluted than uroporphyrin, could be separated from uroporphyrin in experimental mixtures, and yet which appeared to consist of octacarboxylic porphyrin only. It moved with an R_F slightly greater than that of uroporphyrin both as free porphyrin in the lutidine system and as ester in the dioxan system of Falk & Benson (1953*a*). Its ester had a constant melting point of 211–216° and decarboxylation yielded coproporphyrin III. The nature of this porphyrin remains obscure; it does not seem possible from chromatographic evidence that it can be a heptacarboxylic porphyrin and yet it is clearly different from both uroporphyrins I and III. We would emphasize that this new porphyrin has not been encountered on a single occasion only. It was isolated from different columns during the working up of the experimental material and has since been encountered in other cases of porphyria.

The pentacarboxylic porphyrin ester exhibits many points of interest. Whilst its α band at 622·5 mμ. is typical of that of other pentacarboxylic porphyrin esters which have been isolated (Grinstein *et al.* 1945; McSwiney, Nicholas & Prunty, 1950; Rimington & Miles, 1951), the melting point of this specimen is 210–215°, whereas the isomer yielding coproporphyrin I melts at 224° and that yielding coproporphyrin III melts at 180° (Watson, 1953). It would appear that in the present instance a mixture of pentacarboxylic porphyrins is present, since two spots were formed by dioxan paper chromatography and decarboxylation yielded both coproporphyrins I and III (the I series isomer predominating). It is quite clear that only by the use of these new chromatographic techniques and their further development can truly accurate knowledge be gained of the porphyrins present in porphyria urines.

SUMMARY

1. Chromatographic methods have been applied to the study of urinary uroporphyrins from seven cases of cutaneous porphyria. The patients, aged between 54 and 68, had suffered for 1–8 years from photosensitivity and bullous skin lesions. None excreted porphobilinogen or suffered from colics or nervous symptoms.

2. In six of these cases, the predominant isomer was uroporphyrin I (60–85 %) but in one there was found approximately 75 % of uroporphyrin III.

3. Urinary uroporphyrins from two cases of acute porphyria and two cases of congenital porphyria were similarly studied for comparison. The former contained 75–80 % uroporphyrin III and 20–25 % of uroporphyrin I, whilst in the congenital porphyria material about 95 % of uroporphyrin I was found.

4. A porphyrin is described, the ester of which had m.p. 211–216°, and which behaved as an octacarboxylic porphyrin on lutidine paper chromatography, but with R_F very slightly greater than that of uroporphyrin, from which it is separable by column chromatography. On decarboxylation, it yielded coproporphyrin III.

5. A hexacarboxylic porphyrin was detected but not isolated.

6. A pentacarboxylic porphyrin was isolated, with ester of m.p. 210–214°. Paper chromatography by the dioxan method afforded two spots of unequal intensity and on decarboxylation coproporphyrin I was produced together with a smaller quantity of coproporphyrin III.

7. The relation of type of porphyria to isomeric pattern of porphyrin excretion is discussed.

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The Chromatographic Separation of Free and Combined Plasma Cholesterol

By LYNDA M. H. KERR and W. S. BAULD
Department of Biochemistry, University of Edinburgh

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The standard method of estimation of plasma cholesterol, free and combined (Schoenheimer & Sperry, 1934), involves the precipitation of cholesterol as the digitonide before and after hydrolysis. Separation and purification of free and esterified cholesterol in plasma extracts and direct estimation of the two fractions would greatly simplify the procedure. Trappe (1942) and Hess (1947) have demonstrated the ease with which the separation could be achieved by chromatography on activated alumina. Both workers, however, used eluents placed rather high in the series. These might be expected to elute other components of the plasma lipid fraction, thereby diminishing the effectiveness of the purification. Contamination of the cholesterol fractions was in fact noted by Trappe (1942). Re-investigation of the method of separation was desirable.

The problem of direct colorimetric estimation of the separated fractions was solved by Trappe (1942), by using a reaction in which esterification did not alter the colour production. This condition does not hold in the more commonly used Liebermann-Burchard procedure (see Sperry & Brand, 1943). For this reason Hess (1947) hydrolysed the esters before estimation. The separated fractions could, however, be directly estimated if the effect of esterification on the colour production were independent of the fatty acid group involved. The results of Yasuda (1936) suggested that this was the case and this approach to the problem was investigated further. It is known that cholesteryl esters in plasma are mainly derived from unsaturated acids, and recently Keegan & Gould (1953) have reported the isolation of cholesteryl oleate from plasma. Accordingly, both saturated and unsaturated esters were prepared and used as models.

METHODS

Purification of reagents

Chloroform. This was washed, dried over $CaCl_2$ and used freshly distilled.

Sulphuric acid. A.R. grade (British Drug Houses Ltd.).

Acetic anhydride. The A.R. grade was distilled at constant boiling point.

Alumina (Peter Spence). This was washed with water and methanol and dried to grade II activity (Brockmann & Schodder, 1941).

Light petroleum. A.R. grade, b.p. 60–80°.

Benzene. The A.R. grade was refluxed with conc. H_2SO_4 (30 ml./l.) for 30 min., washed with NaOH and water, dried over Na and distilled.