Normal Human Urinary Porphyrins

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Chromatographic methods have recently revealed an extensive intermediary metabolism of the porphyrins, and tri-, penta-, hexa- and heptacarboxylic porphyrins have been found in pathological urines and other materials (Nicholas & Rimington, 1949, 1951). Less is known about the occurrence of these substances in normal mammalian tissues and excreta and the presence of uroporphyrin in normal material is only now becoming established. Several authors have reported the presence of an ether-insoluble porphyrin fraction in normal urine (Fischer & Zerweck, 1924; Lockwood, 1953) and shown that it probably contains uroporphyrin (Nicholas & Rimington, 1949). Schwartz (1953) has recently referred briefly to the finding of crystallizable uroporphyrin and of a number of intermediate porphyrin fractions in normal human urine. There are also negative reports (Grotepass, 1938; Brachvogel, 1950), but the methods of these authors appear to be less sensitive. In order to study the nature of the non-coproporphyrin fractions in urine, we have examined the porphyrins in the urine of healthy adult male humans and rabbits. Some of our findings have already been reported briefly (Weatherall & Comfort, 1952; Comfort & Weatherall, 1954).

METHODS

Collection of urine. Nine batches of human urine (total 118.4 l.) were obtained (Table 1). Two batches (nos. 65 and 76) were composed of the collected urine of a single individual. The urine was frozen at -20° as soon as it was passed and was stored at this temperature until it was extracted. Three batches (nos. 77, 78 and 79), collected from ten individuals, were kept at room temperature and were extracted within 48 hr. of starting the collection. All the individuals who contributed to these batches were to the best of their knowledge healthy, and (except during the collection of batch 79, where the contributors were not subject to any restriction) they consumed no alcohol and no medicines during and for 24 hr. before the period of collection. The other batches of urine (nos. 69, 74, 80 and 81) were obtained from bottles in a students' lavatory. There was no control over the contributors, and each portion of the batch was extracted within 24 hr. of beginning the collection.

Urine was collected from rabbits kept in galvanized metal metabolism cages and fed on bran, oats and cabbage. Faeces were separated by a grid of suitable mesh in the receiving area of the cages, and the urine was stored at 7° for not longer than 3 days before extraction. Dried extracts were subsequently combined to complete each batch as shown in Table 1.

Extraction of porphyrins from urine. Apart from allowing the urine to stand in daylight at room temperature during the period of collection (or the period of thawing for the

Table 1. *Sources of urine*

Species	Batch no.	Volume (litres)	No. of contributors	Duration of collection (days)	Remarks
Human	65	7.2	1	14	Frozen at -20° as soon as passed. Contributor consumed no alcohol nor medicines
	69	10.0	Unknown	2	No control over contributors
	74	19.3	Unknown	3	As batch 69
	76	14.0	1	21	As batch 65
	77	6.4	4	2	Contributors consumed no alcohol nor medicines
	78	2.5	2	1	As batch 77
	79	29.0	7*	2	Ether-extractable porphyrins of each individual's contribution $< 7.5 \mu\text{g./100 ml.}^*$ Subjects known to be healthy, but not subject to any restrictions
	80	10.6	Unknown	2	As batch 69
	81	19.4	Unknown	3	As batch 69
Rabbit	62	5.5	4	6	Total 20 rabbit days
	63	12.0	5	12	Total 54 rabbit days
	64	12.8	5	24	Total 113 rabbit days
	67	10.4	4	10	Total 40 rabbit days

* The contributors to this batch included the subject who provided batches no. 65 and 76, and three of the subjects who provided batch 77. There was no other overlap between the known contributors.

batches collected at -20°) no steps were taken to promote, or prevent, conversion of precursors into free porphyrins. The general procedure used for extracting porphyrins is shown in Fig. 1. Procedures indicated by broken lines or enclosed in brackets were used only in certain batches, and are discussed below. More detailed accounts of most of the procedures have already been given (Weatherall, 1952; Comfort & Weatherall, 1953). The quantity of porphyrin examined at one time varied from about 0.2 to 2.0 mg.

Chromatography of porphyrins and porphyrin esters. The methods of Nicholas & Rimington (1949, 1951), Nicholas (1951) and Chu, Green & Chu (1951) were used with the minor modifications already described (Comfort & Weatherall, 1953).

RESULTS

Human urine

On chromatography on aluminium oxide columns, the methyl esters from each batch of talc-extracted urine yielded two, three or occasionally four bands which fluoresced red in ultraviolet light. The separations were less consistent and the concentration of solvent required to elute each band was more variable than we have observed with porphyrins from pathological urines. This was not surprising, as the porphyrins were concentrated from larger volumes of urine and were almost certainly less pure. Similar results were obtained with grade IV Al_2O_3 prepared by us and with a specimen similar to that used by Nicholas & Rimington (1949), kindly provided for comparison by Professor C. Rimington. The first band was the largest, and it was eluted by benzene-chloroform mixtures varying in proportion

from 5:1 to 1:5 by vol. Usually it was eluted by 1:1 mixtures, and this, as well as the quantity of material, suggested that it was coproporphyrin. The later fractions were less well separated and are discussed below.

Coproporphyrin fraction. When samples of this fraction were dried, hydrolysed and chromatographed on paper with lutidine and water, they were found to behave mainly or entirely like coproporphyrin (I or III). The unhydrolysed ester on kerosene paper chromatography gave a spot like that of coproporphyrin III, and sometimes other, smaller, unidentified spots. However, no spot behaving like coproporphyrin I was found at this stage.

The coproporphyrin fractions of batches 65, 74, 76 and 77 were combined and dried. After washing with light petroleum and an unsuccessful attempt at crystallization from chloroform, the material soluble in ice-cold anhydrous ether was separated from the remainder. About half the material dissolved readily while the remainder did not go into solution; but on chromatography from kerosene mixtures both fractions gave only one spot, corresponding in position to coproporphyrin III. Chromatography of each of these fractions mixed with an approximately equal quantity of coproporphyrin I gave a good separation in the expected proportions, so that the apparent absence of coproporphyrin I in the experimental material was not simply due to the presence of interfering substances. The fractions were recombined and purified

Fig. 1. Methods of extraction and separation of porphyrins from urine. The steps indicated by broken lines, or enclosed in brackets, were performed only with certain batches, as described in the text.

by hydrolysis in 7N-HCl for 36 hr., neutralization with NaOH and sodium acetate, and repeated transfer between ether and 0.07N-HCl. The material was then washed with water, dried and re-esterified. At this stage a trace of coproporphyrin I as well as much of type III was detectable on kerosene chromatography. On crystallization from chloroform–light petroleum a few rosettes of prisms were obtained which melted at 148°, but some microscopically visible non-crystalline material was also present.

Other fractions. The fractions which were eluted from the alumina column after coproporphyrin were smaller and less well defined. With increasing proportions of chloroform in benzene, and then with methanol–chloroform (1:30, v/v), one or more fractions were obtained which yielded on hydrolysis mixtures of porphyrins all with R_F in lutidine–water in the range 0.06–0.76, i.e. in our conditions equal to or between coproporphyrin and uroporphyrin. The largest number of spots in a single sample was six. The frequency distribution of all the observed R_F values is shown in Fig. 2, and their interpretation is discussed below. Many of the fractions were too small for any further investigation. The larger ones were combined and chromatographed again on alumina. A faint trace of red fluorescent material, too small for any examination, was obtained from the column with benzene–chloroform (1:2, v/v), but otherwise no porphyrin was eluted until chloroform–methanol mixtures were used. The biggest fraction was eluted with

chloroform–methanol (200:1, v/v), and appeared to contain mainly uroporphyrin (lutidine R_F 0.09, with smaller spots at 0.18 and 0.32). Chloroform–methanol (100:1, v/v) brought down a smaller band with, surprisingly, porphyrins with a much higher R_F in lutidine–water (0.87 and 0.72 and a trace at 0.18; coproporphyrin on this paper = 0.76); and what remained on the column was eluted only with much methanol (1:1, by vol., or more in chloroform) and had a similar composition to the main band (lutidine–water R_F 0.09, 0.18, 0.31 and 0.43). These separations were not altogether satisfactory, but attempts to isolate the fractions on columns of magnesium oxide or of calcium carbonate were less successful, and the amount of material was insufficient for further analysis.

Other methods of separation. Further evidence was obtained about the minor fractions by extracting half of one 10 l. batch of urine (no. 69) with acetic acid and ether before adsorbing any remaining porphyrins on talc.

All the porphyrins were esterified and chromatographed on aluminium oxide. The ether extract yielded almost entirely coproporphyrin (R_F = 0.64) with a little porphyrin with a lower R_F (0.52) in lutidine–water. The residue after ether extraction contained a trace of coproporphyrin (R_F = 0.68) and a mixture of porphyrins with lower R_F (0.50, 0.37, 0.24 and 0.12) which were not separated on the column. The other half of the batch of urine was treated with talc without preliminary extraction, and was found to contain porphyrins corresponding to the sum of the two fractions, i.e. mainly coproporphyrin and a group of porphyrins with lower R_F values.

Towards the end of these investigations, two batches of urine were treated with talc as usual, and the talc was then washed with methanol before adding the esterifying mixture of methanol and sulphuric acid. This washing was intended to remove some of the non-porphyrin pigments and provide cleaner material for the alumina columns. However, the methanol eluted some porphyrin, which on esterification and purification on aluminium oxide columns appeared to contain a lower proportion of coproporphyrin than usual. Subsequent treatment of the talc with methanolic sulphuric acid yielded mainly coproporphyrin. This observation was not pursued further, but might offer a more satisfactory method of concentrating intermediate fractions.

Attempts to isolate the normal urinary porphyrins by adsorption on calcium phosphate precipitated from the urine by adding calcium chloride and sodium hydroxide (Dobriner, 1936; Sveinsson, Rimington & Barnes, 1949) were not successful, and in view of the good results with talc, this method was not pursued.

Fig. 2. Frequency distribution of R_F values of spots found on lutidine paper chromatography of hydrolysates of fractions obtained after elution of the main band of porphyrin esters from Al_2O_3 columns. The range of values observed with coproporphyrin fractions is shown in broken outline: the total proportion in the starting material was considerably greater than that indicated.

Other pigments. A number of other pigments appeared on the alumina column as esters. These included a bright-blue, non-fluorescent pigment eluted next after coproporphyrin ($\lambda_{\max} = 565 \text{ m}\mu$. in CHCl_3), and a substance with the colour of permanganate closely similar to the porphyrin-accompanying pigments found by one of us in marine molluscs (Comfort, 1949). A considerable amount of brownish pigments always remained at the column head, and green or yellow bands, possibly pyrrolic, came down between or with the porphyrins in several batches. None of these pigments was recovered in crystallizable amounts.

Rabbit urine

Talc extracts of rabbit urine contained a considerably larger amount of non-fluorescent pigments relative to their porphyrin content than did extracts of human urine. The most satisfactory solid chromatograms were obtained after preliminary purification of the esters in chloroform (B.P.) on a magnesium oxide column, or on an aluminium oxide column which was developed first with benzene (which removed some greenish pigments but no porphyrin) and then with chloroform (B.P.) (which removed practically all of it). Ethereal extracts of rabbit urine were cleaner, and the esterified material from these was run directly on aluminium oxide.

The fractions obtained from aluminium oxide chromatography of rabbit urine were different from those obtained from human urine. At least one porphyrin was present which could be eluted more easily than coproporphyrin: it was eluted consistently by benzene-chloroform (approx. 10:1, v/v), and on hydrolysis and lutidine paper chromatography it had an R_f suggesting a dicarboxylic porphyrin. On kerosene paper chromatography its R_f in both solvent systems was high and suggested its similarity to protoporphyrin, and also to the porphyrin A found in the urine of lead-poisoned rabbits (Comfort & Weatherall, 1953).

The main band on aluminium oxide columns behaved like coproporphyrin. On kerosene paper chromatograms it appeared to consist almost entirely of coproporphyrin III, with a minute trace of coproporphyrin I. The remainder of the material eluted from the columns contained mixtures of porphyrins and often other pigments. A zone of green pigment normally appeared in the region between porphyrin A and the coproporphyrin band, and hid any tricarboxylic porphyrin which might have been there; but traces of tricarboxylic porphyrin were sometimes detected in lutidine paper chromatograms of the eluate from this zone. Porphyrins with R_f intermediate between copro- and uro-porphyrin, but closer to that of coproporphyrin, were found by lutidine chromatography

of hydrolysates of the tail fractions from the column, and as traces in the main coproporphyrin band. Both the greenish pigment and a yellow fluorescent oil with a strong odour appeared as persistent contaminants in these fractions, even after repeated chromatography.

DISCUSSION

These results show the existence in the urine of healthy humans and rabbits of traces of several porphyrins besides coproporphyrins I and III. These porphyrins in some respects at least resemble those found in porphyric urine (Macgregor, Nicholas & Rimington, 1952; Rimington & Miles, 1951) and in the urine of lead-poisoned rabbits (Comfort & Weatherall, 1953). The quantities involved are small; at most, all the minor fractions together amount to less than 10–20 % of the total, and their combined excretion is therefore probably less than 5–10 $\mu\text{g./man/day}$. Their identification in human urine rests on their inextractibility from urine by ether (with an exception mentioned below), the behaviour of their esters on aluminium oxide columns, and particularly on their R_f value using lutidine paper chromatography. Most of them have properties intermediate between those of coproporphyrin and uroporphyrin, and it seems likely that they contain five, six or seven carboxyl groups. The simple linear relation between the number of carboxyl groups and the R_f in lutidine-water, postulated by Nicholas & Rimington (1949), cannot be applied rigidly to our material, because we have sometimes observed as many as five spots with R_f values lower than that of coproporphyrin, and because the spots we have found are not always regularly spaced. The frequency distribution of the spots shows a maximum midway between coproporphyrin and uroporphyrin; material giving a single spot in this region has been isolated and shown also to behave homogeneously on kerosene chromatography and it is provisionally assumed to be a hexacarboxylic porphyrin. There are also suggestions of smaller peaks in the frequency distribution at sites suggesting penta- and hepta-carboxylic porphyrins. As we have found a spot corresponding to a pentacarboxylic porphyrin (R_f in lutidine-water about 0.52) in ether extracts of both human and rabbit urine, without the presence of any slower running fraction, it seems probable that this also represents a separate entity. The remaining spots may include one due to a heptacarboxylic porphyrin, or be due to two isomers with the same number of carboxyl groups (cf. Falk, 1954), or be due to impurities which we have failed to eliminate from the material and which have caused splitting of homogeneous porphyrin spots. In any case, proper identification of these intermediate porphyrins is

not feasible until adequately identified standard materials are available for comparison.

The porphyrins obtained from normal rabbits appear to differ from those found in human urine. Extraction with talc has not been satisfactory for the concentration of porphyrin from large amounts of rabbit urine, owing to the heavy contamination of the product with other pigments. In view of this difficulty, the failure to find uroporphyrin may not be significant. However, the porphyrins recovered from talc appeared identical with, though less pure than, those obtained by ether extraction. They consisted chiefly of coproporphyrin with small admixture of porphyrins of lower and higher carboxyl numbers, ranging from di- and tri- to penta- and possible hexa-carboxyl porphyrins, in contrast to the human range, which appeared to extend from tetra- or possibly tri-carboxyl to octa-carboxyl porphyrins.

The isomer distribution of the human coproporphyrin was unexpected. The widespread view that coproporphyrin I regularly predominates in normal urine is not, however, very well founded in experimental evidence. Type I coproporphyrin has been identified by the melting point of its crystals by Fink & Hoerbinger (1934), Watson (1936) and Grotepass (1938). However, Watson's material melted at a temperature as low as 218–223°, and also contained some prisms which looked like the type III ester; and Grotepass crystallized almost as much type III ester as type I. The objection that Grotepass's material may have originated partly from abnormal subjects (Dobriner & Rhoads, 1940; Watson & Larson, 1947) is purely hypothetical and appears to be no more than a convenient way of dismissing an inconvenient finding. In a more recent study (Watson, Hawkinson, Schwartz & Sutherland, 1949), by the fluorescence quenching method (Schwartz, Hawkinson, Cohen & Watson, 1947), groups of normal subjects have been found in all of whom type I coproporphyrin predominated and other groups in whom type III was the main constituent. In the small groups we have examined, type III coproporphyrin always predominated, and it was only after considerable pooling of material and purification that type I could be found chromatographically at all. In one group the coproporphyrin concentration of each individual's urine was measured and found not to exceed 7.5 $\mu\text{g.}/100\text{ ml.}$; the possibility of a contributor with idiopathic type III coproporphyrinuria (Watson, Schwartz, Schulze, Jacobson & Zagaria, 1949) is therefore excluded: nor did any of the other batches of urine yield notably more porphyrin esters in relation to their size than did this one. In any case there would have to be a surprisingly large number of such 'abnormal' subjects in order to affect all the groups we examined.

It seems much more probable that the normal urinary isomer type is as variable as the evidence suggests, and that it is undesirable to postulate that either type 'normally' predominates. Since a large part or perhaps practically all of the urinary coproporphyrin is formed from a precursor or precursors after the urine has been secreted by the kidneys (Watson, de Mello, Schwartz, Hawkinson & Bossenmaier, 1951; Eriksen, 1952; Weatherall, 1952), the isomer type may depend on the kind of precursor, or it may depend on the reaction by which the precursor is converted into the free porphyrin. If the precursors are of the tetrapyrrolic porphyrinogen type, as the conversion by iodine suggests (Watson *et al.* 1951), change of isomer type is rather unlikely during the reaction. However, the precursors present in urine are not necessarily always or only of this type, and if any substantial portion of the precursor material is a monopyrrole similar to porphobilinogen (Cookson, Rimington & Kennard, 1953), the final isomer proportion may be much influenced by minor physical or chemical differences in the urinary composition or in the method of treating the urine. It has recently been found (Cookson & Rimington, 1954) that the proportions of uroporphyrin isomers formed from porphobilinogen depend on the pH of the solution, and it may therefore be of significance that Watson and his colleagues (Schwartz, Zieve & Watson, 1951; Watson *et al.* 1951) have been working with urine kept alkaline with sodium carbonate during the period of collection. It is possible that the subsidiary fractions which we and others have described are also formed to a variable extent after the urine has been secreted, and until more is known about the identity of the precursors, caution is necessary in attaching much significance to the individual porphyrins recoverable from mammalian urine.

SUMMARY

1. The porphyrins present in the urine of normal men and rabbits have been examined chromatographically.
2. Human urine contained coproporphyrin, a little uroporphyrin, and small amounts of porphyrins with, probably, five, six and seven carboxyl groups.
3. Rabbit urine contained coproporphyrin and traces of other porphyrins with, probably, between two and six carboxyl groups.
4. The coproporphyrin was in both species predominantly of type III. Statements that the normal human urinary coproporphyrin is of type I are discussed in the light of this finding.

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Studies on Sulphatases

8. THE ARYLSULPHATASE OF A STRAIN OF *ALCALIGENES METALCALIGENES* ISOLATED FROM INTER-TIDAL MUD

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Karunairatnam & Levvy (1951) showed that the β -glucuronidase activity found in sheep rumen was due to micro-organisms, and Marsh, Alexander & Levvy (1952) suggested that the high β -glucuronidase activity found in the omasum, rumen, caecum and colon of many animals might have a similar origin. It was possible that the high arylsulphatase activity of the visceral humps of marine molluscs (Dodgson, Lewis & Spencer, 1953) was due to the presence of micro-organisms, although arylsulphatase activity in bacteria is not common. Barber, Brooksbank & Kuper (1951) found activity in only two of a large number of strains of *Staphylococcus pyogenes*. Whitehead, Morrison & Young (1952) detected the enzyme in certain salmonellae and mycobacteria after studying 212 strains from a wide range of bacterial species, while Hare, Wildy, Billet & Twort (1952) found activity in only two out of ninety-nine strains of anaerobic cocci. Harada (1948) noted arylsulphatase activity in strains of *Streptococcus alcalophilus* isolated from urine and soil.

We have been unable to detect any significant arylsulphatase activity in bacteria isolated from the visceral humps of a number of different marine molluscs but have noted appreciable enzyme activity in two micro-organisms isolated from certain inter-tidal muds. The least active of these organisms, a yeast identified as *Trichosporon cutaneum*, has not been further investigated but the other, a bacterium of the genus *Alcaligenes*, is interesting, since it is an example of an arylsulphatase with an optimum pH well on the alkaline side of neutrality. A preliminary account of this work has already been given (Dodgson, Melville, Spencer & Williams, 1954).

MATERIALS AND METHODS

Arylsulphatase substrates. The following compounds were prepared: potassium *p*-acetylphenyl sulphate (Dodgson & Spencer, 1953), tripotassium phenolphthalein disulphate (Whitehead *et al.* 1952); potassium *p*-nitrophenyl sulphate (Burkhardt & Lapworth, 1926) and dipotassium 2-hydroxy-5-nitrophenyl sulphate (nitrocatechol sulphate, Roy, 1953*a*).