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A Quantitative Method for the Determination of Uroporphyrin in Pathological Urines

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The chief difficulty attending the isolation of porphyrins from urine and their separation from one another is the relatively sparing solubility of these pigments in organic solvents. Coproporphyrins I and III are extractable by the acetic acid-ether technique discovered by Saillet [1896] and adopted by Fischer [1926] and may thus be quantitatively determined. For preparative purposes, although large volumes of solvent are required, a high degree of purification may be achieved by repeated transferences between ether and dilute HCl. Uroporphyrin III is similarly extractable by ethyl acetate from solutions adjusted to a pH of 3.0-3.2 [Waldenström, Fink & Hoerbürger, 1935], but again large volumes of solvent are required for this laborious process, which is not suitable for quantitative purposes. Uroporphyrin I is not extracted by any known solvent immiscible with water.

Recourse must, therefore, be had to adsorptive procedures for the concentration and purification of the uroporphyrins prior to their spectrophotometric or fluorimetric determination. Many different materials have been proposed as suitable adsorbents for porphyrins. They include Ca and Pb phosphates, alumina, talc, BaSO₄, etc., and Waldenström [1935] has elaborated a chromatographic procedure for the adsorption of porphyrins from urine upon alumina. In this, as in all other

procedures, adsorption takes place at or near the isoelectric point of the porphyrin. At this reaction the greater part of the dark brown or red water-soluble pigment of unknown constitution, always present in porphyrinuric urines, is also adsorbed, so that from a quantitative point of view the operation is of little value, and further purification of the uroporphyrin fraction is difficult. Attempted purification of the porphyrins from urine by acidifying with acetic acid [Nebelthau, 1899] is not always successful, and in any precipitate which forms coproporphyrin is mixed with uroporphyrin.

While searching for a new adsorptive technique, it was discovered that kieselguhr has the very desirable and remarkable property of adsorbing both coproporphyrins and uroporphyrins from solution in dilute HCl whilst rejecting almost entirely the dark, brown-red, non-porphyrin pigment. Elution by suitable buffers brings about a fairly sharp separation of the coproporphyrins from the uroporphyrins, but it has not been found possible to separate by this means the I and III isomers of either pigment, no doubt owing to the fact that their isoelectric points are so similar. Based upon the kieselguhr technique, a good quantitative method has been worked out for the determination of total porphyrin, and hence of uroporphyrin, in porphyrinuric urines, thus for the first time enabling

conditions such as acute idiopathic porphyria to be subjected to accurate quantitative examination.

METHODS

Separation of pure coproporphyrin from uroporphyrin

The pigments used had been prepared from natural sources and purified by recrystallization of their methyl esters. These were saponified and the free porphyrins brought into solution in 5% HCl (w/w). Tswett columns were prepared from acid-washed kieselguhr (B.D.H.) suspended in 5% HCl (w/w) and the behaviour of the pigments studied individually on filtering their solutions through the columns, and on developing with buffer solutions. All porphyrins tested were strongly adsorbed from the acid solutions. Table 1 summarizes the results obtained with

Table 1. *Behaviour of coproporphyrins and uroporphyrins adsorbed on kieselguhr*

Developer	Effect on porphyrin	
	Uroporphyrins I or III	Coproporphyrins I or III
20% HCl	Nil	Nil
50% acetic acid	Nil	Nil
Glacial acetic acid	Slow broadening of band	Slow broadening of band
Water	Nil	Nil
M Citrate buffer pH 3.2	Nil	Nil
M Acetate buffer pH 4.0	Nil	Nil
M Acetate buffer pH 4.5	Nil	Nil
M Acetate buffer pH 4.7	Broadening of band	Nil
M Acetate buffer pH 5.0	Broad ring passing slowly downwards	Nil
M Acetate buffer pH 5.25	All porphyrin removed	Very slow broadening of band
M Acetate buffer pH 5.5	—	Rapid broadening
M Phosphate buffer pH 6.5	—	Rapid broadening
M Phosphate buffer pH 7.15	—	All porphyrin removed

pH's measured by glass electrode.

the developers. A mixture of uroporphyrin III and coproporphyrin I was adsorbed on a column from acid solution as previously described and then washed thoroughly with water. Acetate buffer of pH 5.25 was then filtered through the column, when the uroporphyrin passed readily downwards, leaving a sharply defined zone containing the coproporphyrin at the top of the column. The eluate contained no ether-soluble pigment. The coproporphyrin was finally removed from the column by a phosphate buffer of pH 7.15.

Chromatographic separation of uroporphyrin from urine of acute porphyria

The specimen of urine, which was nearly black in colour, was passed during a period of severe attack by a man suffering from acute idiopathic porphyria. HCl was added to a concentration of 1% (w/w) and the mixture filtered through a column of kieselguhr. The deeply coloured filtrate was free from porphyrin. The column was washed well with 1% HCl (w/w) and then with glacial acetic acid, which removed the remaining dark brown pigment together with a little porphyrin detectable by fluorescence in ultra-violet light. After thorough washing with water, the uroporphyrin was

then removed by acetate buffer of pH 5.25 and obtained as a deep red solution substantially free from other pigments. Upon adjustment of the pH to 3.0, the porphyrin precipitated readily, leaving a pale straw-coloured supernatant liquor. The precipitate was collected on the centrifuge, dried and esterified in the usual way. The ester crystallized from hot chloroform-methanol in fine microscopic needles, m.p. 258°.

The adsorption technique has also been used to purify the crude precipitate of mixed porphyrins obtained from a porphyritic urine by acidification with acetic acid. The mass was dissolved in dilute alkali and the pH adjusted to 5.25. Kieselguhr was then added and the mixture shaken vigorously and centrifuged. The adsorbent retained coproporphyrin, but little, if any, uroporphyrin. It is doubtful whether the method would be so effective were the proportion of coproporphyrin to uroporphyrin not so small (in the above case it was approximately 1:20), but by repeated shaking or filtration through a column it should be possible to remove substantially all coproporphyrin from uroporphyrin solutions by this simple expedient.

A quantitative method for the determination of uroporphyrin

The property possessed by kieselguhr of adsorbing uroporphyrin strongly from dilute acid solutions but of retaining very little of the accompanying urinary pigments provides the basis of a relatively simple method for the determination of uroporphyrin. The studies to be detailed below were carried out upon some specimens of urine from a case of acute idiopathic porphyria, and for his kindness in putting this material at my disposal I wish to thank Dr G. Discombe of St Bartholomew's Hospital.

Preliminary treatment of the urine. Waldenström & Vahlquist [1939] have shown that in acute porphyria a pigment precursor, porphobilinogen, is excreted, which is rapidly converted into porphyrin when the urine is acidified and warmed on the water bath [cf. Willenbücher, 1942]. During this heating, however, the urine becomes very dark owing to the formation of other, non-porphyrin, pigments and it has been found that the same full production of porphyrin is achieved without such undesirable accompaniment by rendering the specimen acid to a concentration of 1% HCl (w/w) and leaving it at room temperature overnight.

A known volume (usually 1–2 ml.) of urine containing approximately 5–50 µg. of porphyrin is thus acidified, diluted with 1% HCl (w/w) to 5 ml. and left overnight. About 0.7 g. of acid-washed kieselguhr is then added, the tube stoppered, shaken vigorously for about 1 min., and then centrifuged for 5 min. The kieselguhr packs well, allowing the brown supernatant liquid to be decanted. Control examination of this liquid by ultra-violet light should show only a greenish-blue fluorescence unmixed with red, thus indicating that the quantity of adsorbent used has been sufficient.

Elution and quantitative determination of porphyrin

The kieselguhr is now washed by stirring up and centrifuging successively twice with 1% HCl (w/w) and once with H₂O.* The porphyrin is then eluted completely by three successive washings with 0.1 N NaOH of about 7 ml. each,

* From some urines, washing with 50% acetic acid removes a further small quantity of fluorescent material without loss of porphyrin.

and centrifuging for 15–20 min. The eluates may still contain fine particles of kieselguhr in suspension, but these are disregarded since, when all the eluates are combined, made up to a volume of 25 ml. and left overnight, the turbidity subsides completely, leaving a clear solution which may be pipetted off. From this, a suitable dilution, 1/5 or 1/10, is prepared with 0.1 *N* NaOH, such that the final porphyrin concentration is near the optimal figure of 0.25 $\mu\text{g./ml.}$, and the exact concentration is determined fluorimetrically in the fluorescence comparator [Rimington, 1943].

This determination gives the total porphyrin. Coproporphyrin is conveniently determined, by the acetic acid-ether extraction method and fluorimetric analysis, in a portion of the eluate, or in the urine itself. By subtraction the quantity of uroporphyrin is calculated. It has been found more convenient to elute the total porphyrin with dilute alkali rather than to attempt a two-stage elution and separation of coproporphyrin from uroporphyrin by means of buffers. Errors due to the effect of salts upon fluorescence intensity are thereby eliminated, and, furthermore, quantitative elution by buffers was found to be rather a tedious process, requiring relatively large volumes of fluid.

Recovery of added uroporphyrin was quantitative, and the error of the method, judged by results of repeated determinations, is estimated at less than 10%. The following example may be quoted in illustration:

Urine specimen 26. (Coproporphyrin by ether extraction 147 $\mu\text{g./100 ml.}$)

		Uro- porphyrin mg./100 ml.
(1)	2 ml. urine + 2 ml. H_2O + 1 ml. 5% HCl (w/w), worked up immediately	0.96
(1a)	2 ml. urine + 2 ml. H_2O + 1 ml. 5% HCl (w/w), worked up immediately	1.08
(2)	2 ml. urine + 2 ml. H_2O + 1 ml. 5% HCl (w/w), heated on water bath for 1 hr.	1.72
(3)	2 ml. urine + 2 ml. H_2O + 1 ml. 5% HCl (w/w), left overnight at room temp.	1.67
(3a)	2 ml. urine + 2 ml. H_2O + 1 ml. 5% HCl (w/w), left overnight at room temp.	1.77
(4)	1 ml. urine + 2 ml. H_2O + 1 ml. (=20 $\mu\text{g.}$) uroporphyrin III + 1 ml. 5% HCl (w/w), left overnight at room temp. Recovery of added uroporphyrin 109%	
(5)	100 ml. pooled eluates corresponding to 8 ml. urine (=12 $\mu\text{g.}$ coproporphyrin) extracted by ether, etc. Coproporphyrin in extract 12.4 $\mu\text{g.}$ Recovery 103%	

RESULTS

Excretion of uroporphyrin and coproporphyrin in a case of acute idiopathic porphyria

The patient was a young man aged 19, under the care of Dr Geoffrey Evans of St Bartholomew's Hospital, for whose great kindness in allowing me to examine the urines and publish my biochemical findings, together with relevant data from the clinical record, I am very greatly indebted. He was admitted in April 1942, and presented the typical

clinical picture of acute idiopathic porphyria. The prominent features of his condition were malaise and marked asthenia, complicated by peripheral neuritis and muscular wasting. Relapses were characterized by a return of abdominal pain, loss of appetite and nausea. He suffered an attack of pain on 25 May, but thereafter improved until 19 August which marked the onset of another sharp attack.

Fig. 1. Daily urinary porphyrin excretion and pulse rate in case of acute idiopathic porphyria. — Consecutive daily determinations. --- Determinations at intervals of 48 hr. or more.

Urine specimens (24 hr. collections, for arranging which I am much indebted to Dr G. Discombe) were examined on the 12/13 August and 21/22 August, and thereafter daily until 5 September. This period embraced a typical attack. As the

patient's condition was improving by 5 September, analyses were performed only at 48 hr. or 72 hr. intervals until 14 September, when the investigation concluded.

Of most urine specimens, 1 or 2 ml. sufficed for the estimation of porphyrin, but of the weaker specimens 3 ml. were required, and in the case of those very rich in porphyrin 0.5 ml. was found to be ample. Table 2 presents the analytical results, together with notes upon the colour of the urines and the clinical condition of the patient. A comparison between the levels of uroporphyrin and coproporphyrin excretion from day to day and the 12-hourly records of the pulse rate (full line) and estimated 24-hourly fluctuations in pulse rate (dotted line) is set forth in Fig. 1.

of uroporphyrin follows the excursions of the pulse rate. There is no constancy in the ratio between coproporphyrin and uroporphyrin excreted or between total porphyrin and urine volume (see Table 2), although it would appear that the fluid output modifies slightly the output of pigment.

The urinary porphyrins excreted by this case were identified as uroporphyrin III, octamethyl ester M.P. 258°, and coproporphyrin III, tetramethyl ester M.P. 140°, remelt 163°, together with a small quantity of coproporphyrin I.

SUMMARY

1. Coproporphyrins and uroporphyrins of both series I and III are adsorbed by kieselguhr from

Table 2. Case: 'Peter Beresford'. Analysis of urines for porphyrin

Date	Colour of urine	Total volume ml.	Copro-porph. $\mu\text{g./100 ml.}$	Total copro-porph. mg.	Uro-porph. mg./100 ml.	Total uro-porph. mg.	Clinical condition
9/10 June	Nearly black	1420	30.6	0.435	1.9	27.0	Last attack of pain 25 May, condition improving
1/2 Aug.	Light red-brown	—	60.5	—	2.58	—	
12/13 Aug.	Dark	—	64.5	—	1.6	—	
18/19 Aug.	Light brown	1240	76.9	0.954	0.85	10.5	Pulse rate 80
21/22 Aug.	Dark	1915	108	2.068	1.87	35.7	Sudden rise of P.R. to 128 on 20th
22/23 Aug.	Deep brown	1860	100	1.860	0.76	14.14	P.R. 136
23/24 Aug.	Greenish brown	680	124	0.843	1.18	8.0	P.R. fell gradually to 85 on 23rd. Rose again to 110 on 25th; 120-130 on 26th; 140 on 27th a.m.; fell to 95 by p.m.
24/25 Aug.	Golden brown	1160	139	1.612	1.18	13.69	
25/26 Aug.	Red-brown	850	147	1.250	1.69	14.37	
26/27 Aug.	Dark purplish brown	930	68.4	0.657	0.293	2.81	P.R. 140 on 28th a.m., fluctuating, but fell to 85 by 30th. Patient lost 6½ lb. since 9 Aug.
27/28 Aug.	Dark brown	900	74.4	0.728	3.264	31.93	
28/29 Aug.	Dark brown	182	132	0.251	4.718	8.96	
29/30 Aug.	Very dark	720	130	0.969	6.745	50.25	P.R. about 100
30/31 Aug.	Dark brown	815	58	0.487	0.775	6.51	P.R. rose to 128 on 1st, falling to 80 on 2nd
31 Aug./1 Sept.	Dark brown	1420	38.7	0.568	1.128	16.58	
1/2 Sept.	Dark brown	1100	71.3	0.806	0.954	10.78	Attack subsided, according to P.R., on 16 Sept., when the rate returned to the normal range except for occasional rises to about 130. Steady slow improvement has continued since 18 Sept.
2/3 Sept.	Dark brown	1490	18	0.277	0.547	8.42	
3/4 Sept.	Light brown	1810	49.5	0.923	0.501	9.44	
4/5 Sept.	Light brown	1920	49.5	0.980	0.416	8.24	
6/7 Sept.	Light brown	2290	18	0.425	0.165	3.89	
9/10 Sept.	Light brown	1780	22.7	0.415	0.705	12.90	
11/12 Sept.	Light brown	2120	11.6	0.254	0.716	15.54	
13/14 Sept.	Light brown	2040	35.2	0.739	1.096	23.02	

No interpretation of the clinical findings or discussion of the clinical aspects of the case is intended here; the record of the pulse rate is merely given as an accompaniment to the porphyrin determinations which illustrate the potential usefulness of the kieselguhr method. With such a relatively simple and rapid technique in one's hands, it is now possible to subject the attacks and remissions of acute porphyria to a quantitative examination, and seek to gain further insight into the pathogenesis of this disease. Whilst the correspondence is not exact, it may be said, from an inspection of Fig. 1, that, in general, the quantitative excretion

acid solution (1% HCl (w/w)), and a separation of the porphyrins, but not of individual isomers, may be effected by developing the chromatogram with acetate buffer of pH 5.25. Uroporphyrins I and III are readily eluted.

2. Improvements in the preparative method of obtaining uroporphyrin from pathological urine are based upon this observation.

3. A quantitative procedure for determining the total porphyrin in urine, and hence the uroporphyrin by subtraction of the independently determined coproporphyrin, is described and its application to the analysis of consecutive 24 hr. samples

from a case of acute idiopathic porphyria, during an attack, is recorded. The provision of a quantitative technique for uroporphyrin determination

opens up possibilities for the further study of the relationship between porphyrin formation or excretion and clinical condition in this disease.

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