Acute Porphyrria

SOME PROPERTIES OF PORPHOBILINOGEN

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It was first noticed by Sachs (1931) that the urine of a patient suffering from acute porphyria gave an intense red colour with *p*-dimethylaminobenzaldehyde, in spite of the fact that it did not give Schlesinger's reaction, or the spectrum of urobilin. Moreover, the spectrum of the red dye showed two absorption bands in the green, whereas the product from the reaction of urobilin with *p*-dimethylaminobenzaldehyde gives only one. In addition, the dye was insoluble in chloroform. Exposed to sunlight, the urine darkened and the aldehyde reaction became negative. Waldenström (1937) studied this 'chromogen' in the urine of patients with acute porphyria and failed to isolate it, but demonstrated the darkening of an acid solution of this 'chromogen' with the formation of a red pigment (porphobilin). Waldenström & Vahlquist (1939*a*) mentioned the effect of urea in inhibiting its reaction with *p*-dimethylaminobenzaldehyde and described a method for its estimation in arbitrary units which they used in the study of the amount excreted in cases of acute porphyria (Waldenström & Vahlquist, 1944). These same authors (1939*b*) offered evidence that this colourless chromogen which they called porphobilinogen was a dipyrrolic compound and that, in accordance with the theory of Dobriner (Dobriner, Strain & Localio, 1937), two molecules of such a compound could condense to give rise to a porphyrin. Watson & Schwartz (1941) have suggested the value of the insolubility of the condensation product of porphobilinogen and *p*-dimethylaminobenzaldehyde in chloroform as a useful test for the diagnosis of acute porphyria. Porphobilinogen is not excreted in the congenital type of porphyria (Watson, Schwartz & Hawkinson, 1945).

The work here reported was undertaken first to obtain additional information on the reaction between porphobilinogen solutions and *p*-dimethylaminobenzaldehyde, secondly on the interconversion of porphobilinogen and porphobilin, and thirdly on the formation of porphyrin from porphobilinogen. Since porphobilinogen has been put forward as a precursor of the pathological porphyrin in acute porphyria and since the liver is a possible site of porphyrin formation, a search for the presence of porphobilinogen in the liver was thought to be of value.

EXPERIMENTAL AND RESULTS

Preparation of porphobilinogen solution from urine

Several preparations were made from the urine of a case studied (Prunty, 1945) by the method of Waldenström (1937).

In a typical preparation 385 ml. of fresh urine were acidified with 3.5 ml. of acetic acid and, after preliminary filtration, passed through a 10 × 3 cm. column of alumina (Savory & Moore Ltd.). 1% (v/v) acetic acid was then passed through the column until the eluate gave a positive reaction with *p*-dimethylaminobenzaldehyde, when the eluate was collected separately. 250 ml. of pale yellow solution rich in porphobilinogen were obtained. This material was rechromatographed in the same way and a clear colourless liquid obtained still rich in porphobilinogen. The acidity of this solution was brought to pH 6.6 with 10% (w/v) NaOH and the small phosphate precipitate filtered off. The urea content of this solution was only 1 mg./100 ml. The solution was kept on ice.

Preparation of porphobilinogen from liver

Other preparations of porphobilinogen were obtained from liver. 20 g. of liver obtained from the same case at autopsy were finely minced and treated with 50 ml. of 0.9% NaCl containing 1% (v/v) acetic acid and a few drops of toluene added. The suspension was shaken for 1 hr. and then refrigerated overnight. The solid residue was centrifuged down. The supernatant liquor was cleared by the addition of two-fifths of its volume of 20% (w/v) trichloroacetic acid and filtered. The filtrate was passed through an alumina column, a brown band of pigment remaining at the top of the column. The porphobilinogen was eluted by passing 10 ml. of distilled water through the column and the volume made up to 25 ml. It contained 8 units of porphobilinogen (see below), equivalent to 365 units for the whole liver.

The solution was colourless and behaved in a similar manner to that of the porphobilinogen eluates obtained from urine. The absorption bands of the product with *p*-dimethylaminobenzaldehyde were the same, namely, 575–550 and 530–515 mμ, and the solution showed no green fluorescence when tested for urobilinogen according to Schlesinger's technique.

The reaction of porphobilinogen with p-dimethylaminobenzaldehyde

The *p*-dimethylaminobenzaldehyde reagent used was that employed by Waldenström & Vahlquist (1939*a*). A solution of 20 g. *p*-dimethylaminobenz-

aldehyde in 500 ml. of 36 % (w/w) HCl was diluted with 500 ml. of distilled water. Equal volumes of porphobilinogen solution and the reagent were mixed and allowed to stand at room temperature for 80 sec. The intensity of the colour was measured with a Hilger absorptiometer using Ilford filter 605. 1 unit/ml. was taken as $E_{1\text{ cm}}^{550^\circ} = 0.545$ (Waldenström & Vahlquist, 1939a).

Fig. 1 shows that the light absorption of the product of the reaction of porphobilinogen with *p*-dimethylaminobenzaldehyde slightly deviates from Beer's law.

Fig. 1. Calibration curve for the reaction of porphobilinogen with *p*-dimethylaminobenzaldehyde.

Fig. 2a. Effect of inhibiting substances on the reaction of porphobilinogen with *p*-dimethylaminobenzaldehyde. 1. Porphobilinogen from urine partially purified by chromatography. 2. Urine from acute porphyria diluted to 1/17 (urea concentration 0.02 M). 3. Porphobilinogen solution (1) plus two-fifths of the volume of normal urine (urea concentration 0.07 M). 4. The same porphobilinogen solution containing 0.33 M-urea. 5. Porphobilinogen solution prepared by chromatography from liver of acute porphyria.

Influence of interfering substances on the course of the reaction

The rate of reaction of solutions of porphobilinogen with *p*-dimethylaminobenzaldehyde at room temperature is shown in Fig. 2a. It will be seen that urea both depresses the rate of reaction and

Fig. 2b. 1. Porphobilinogen solution after chromatography. 2. The same solution containing 0.01 M-ascorbic acid. 3. The same solution containing 0.01 M-oxidized ascorbic acid.

Fig. 3. Ascorbic acid concentration and the inhibition of the reaction of porphobilinogen with *p*-dimethylaminobenzaldehyde. 1. 50 sec. after the addition of *p*-dimethylaminobenzaldehyde. 2. 90 sec. after the addition of the reagent.

inhibits the complete formation of the colour. This is also demonstrated after the addition of urine. Creatine and creatinine were found to have no such effect.

The solution of porphobilinogen obtained from the liver clearly contains some substance accelerating the rate of fading, but this was not identified.

Ascorbic acid (Fig. 2*b*) also has a marked effect on inhibition of the reaction and acceleration of fading. An equivalent amount of ascorbic acid which had been quantitatively oxidized by iodine is without effect (Fig. 2*b*, curve 3). The relation of inhibition to concentration of ascorbic acid is given in Fig. 3.

Hydrogen peroxide causes almost complete inhibition of the reaction. The addition of hydrogen peroxide to a solution of *p*-dimethylaminobenzaldehyde gives an unstable light blue product. 10 ml. of H_2O_2 (20 vol.) added to 5 ml. of porphobilinogen solution, and the whole diluted to 25 ml., gave a very transitory red coloration, rapidly turning to green.

The effect of reduction with Terwen's solution on the reaction

The reduction of urobilin to urobilinogen can be effected by means of a suspension of ferrous hydroxide in alkaline solution (Terwen, 1925). The effect of this method of reduction on the urinary pigments of acute porphyria was therefore investigated. Fresh specimens of urine so treated gave a more intense reaction with *p*-dimethylaminobenzaldehyde, but the effect was only small, and no appreciable reduction of the amount of pigment in the urine was apparent. On the other hand, if the urine had been allowed to stand for 24 hr. before treatment with the reducing agent, there was actually a decrease in the intensity of the reaction with *p*-dimethylaminobenzaldehyde (Table 1).

Table 1. *The effect of Terwen's reduction on porphobilinogen reaction of urine*

		$E_{1\text{ cm.}}^{550}$	
		Before reduction	After reduction
Fresh urine diluted to 1/50	(a)	0.385	0.401
	(b)	0.167	0.180
Urine after 24 hr. standing diluted to 1/50	(c)	0.261	0.200
	(d)	0.340	0.305

The effect of heating with alkali on the course of the reaction of porphobilinogen with p-dimethylaminobenzaldehyde

Fig. 4 shows the results obtained on: (i) a specimen of urine diluted to 1/25; (ii) the same urine reduced with Terwen's reagent and diluted to 1/25; (iii) the filtrate after the reduction, containing 0.06M-NaOH, heated 15 min. in a boiling water-bath and diluted to a final dilution of 1/25. Heating with alkali causes a slight reduction in the colour intensity after reaction with *p*-dimethylaminobenzaldehyde, but a marked change in the absorption spectrum of the product. Solution (ii) showed the typical spectrum produced with porphobilinogen (575–550 and

530–515 $m\mu$), but (iii) showed the band at 575–550 $m\mu$ persisting with the disappearance of the band at 530–515 $m\mu$.

Fig. 4. Effect of treatment of porphobilinogen with alkali on its reaction with *p*-dimethylaminobenzaldehyde. 1. Urine of acute porphyria diluted to 1/25. 2. The same urine after treatment with Terwen's reagent. 3. The same material as (2) after heating 15 min. in the boiling water-bath with 0.06M-NaOH.

Conversion of porphobilinogen to porphobilin

Relation to pH of solution. This experiment was carried out in a 0.5M-phosphate, 0.25M-citric acid buffer. To 100 ml. of a porphobilinogen solution were added 43.25 ml. of N-NaOH to neutralize the solution, giving 0.302M-acetate. The small phosphate precipitate which carried down a trace of porphobilin was filtered off.

To 5 ml. of this filtrate were added 2.5 ml. of 36% (w/w) HCl and the mixture diluted to 25 ml. For the other pH values 5 ml. amounts were added to 10 ml. buffer solution and diluted to 25 ml. 3 ml. quantities of these solutions were (a) treated with 3 ml. *p*-dimethylaminobenzaldehyde reagent and (b) heated 10 min. in a boiling water-bath and, after cooling, treated with the reagent. At the same time the absorption density of the solution was determined before and after heating, using Ilford filter 603 (maximum absorption at 490–500 $m\mu$). In this way comparative data for the porphobilin content were obtained. Almost complete separation of the porphobilinogen-*p*-dimethylaminobenzaldehyde compound and porphobilin was possible with the two Ilford filters employed (605 and 603 respectively). Fig. 5 shows the relation of porphobilinogen loss to pigment production, 100% pigment formation being taken arbitrarily as the amount of pigment found in the most acid solution. The control curve 3 shows that a small part of this is due to humin. The

solutions after heating showed the typical band of porphobilin at 500m μ .

Fig. 6 indicates the rate of formation of porphobilin at the optimum pH of 5.2.

Fig. 5. The relation of pH of a porphobilinogen solution to the formation of porphobilin. Heated 10 min. in a boiling water-bath. 1. Porphobilin produced. 2. Porphobilinogen loss. 3. Pigment formation in a specimen of normal urine (control).

Fig. 6. The rate of formation of porphobilin at the optimum pH (5.2). Porphobilinogen solution heated in a boiling water-bath. 1. Porphobilin produced. 2. Porphobilinogen loss.

Effect of ascorbic acid and hydrogen peroxide. 5 ml. amounts of porphobilinogen solution were diluted with 10 ml. buffer (pH 5.2) and volumes made up to 25 ml. The solutions were heated 5 min. in a

boiling water-bath, except where otherwise stated. The data in Table 2 show the effect of ascorbic acid and hydrogen peroxide on the pigment production. Owing to the interfering action of both ascorbic acid and hydrogen peroxide in the *p*-dimethylaminobenzaldehyde reaction with porphobilinogen, no quantitative assessment of the porphobilinogen loss under these conditions was made.

Table 2. Conversion of porphobilinogen to porphobilin

(Solutions were heated for 5 min. at 100°.)

	Increase in density with Ilford filter 603 (% of density after 30 min. heating)
Control solution (pH 5.2)	73
Control solution with 5 ml. normal urine	78
Control solution with 50 mg. ascorbic acid at pH 5.2	60
Control solution with 10 ml. H ₂ O ₂ (20 vol.)	Nil
Do., stood 30 min. at room temperature in daylight	Nil
Do., 60 min.	Nil

Porphyrin formation from porphobilinogen

In vitro experiment. In one experiment two quantities of 5 ml. of a porphobilinogen solution containing 1.5 units/ml. were buffered to pH 5. In one, the control, 1 ml. conc. HCl was added to adjust acidity to pH 1 and the solution immediately treated with 1 g. Keiselguhr and allowed to stand for 2 min. The suspension was then dealt with as described by Rimington (1943*b*). The fluorescence intensity of the final product in 0.1N-NaOH was determined by means of a Hilger fluorimeter with a Chance no. 3 filter in the secondary beam. The fluorescence was not the red of a porphyria but largely greenish in colour, the intensity of which gave a control reading equivalent to 21 μ g. of uroporphyrin in the solution.

The second quantity was heated in a boiling water-bath under an air condenser for 30 min., by which time much porphobilin had been formed. After cooling it was treated in the same manner as the control. The fluorescence now obtained was equivalent to 71 μ g. of uroporphyrin, but it was not quite a pure red, being contaminated with the greenish fluorescence of the control. The difference observed between the heated solution and the control indicated therefore a formation of 50 μ g. of porphyrin, and the solution showed very weak absorption bands at approximately 570 and 535 m μ . Acidification with acetic acid and ether extraction gave no detectable coproporphyrin in the ether extract.

In vivo experiment. A healthy adult male rabbit was maintained on the laboratory stock diet in a metabolism cage for a 5-day control period. Urine and faeces were collected. The rabbit was then given 20 ml. of a porphobilinogen solution intraperitoneally for a 3-day period. This solution was prepared as outlined above and after neutralization to pH 6.6 with 10% NaOH, it was Seitz-filtered and kept in the refrigerator. The porphobilinogen content was 2 units/ml. On account of the observation that treatment of a patient suffering from acute porphyria with methionine suggested that this amino-acid might possibly enhance porphyrin production (Prunty, 1945), the rabbit was given, for a final 3-day period, 20 ml. of this solution and in addition 0.5 g. of methionine intraperitoneally.

The porphyrin excretion in urine was determined by the method of Rimington (1943 *a, b*) and in the faeces by that previously described (Prunty, 1945).

ascorbic acid studied in some detail. Preliminary dilution of the urine, as advocated by these authors, reduces the effect of such interfering substances to a minimum.

Waldenström & Vahlquist (1944) have shown that the colour of the urine of patients with acute porphyria depends upon its pH, and Waldenström (1937) found that porphobilinogen was transformed to porphobilin in acid solution, or in the presence of hydrogen peroxide in daylight. Fig. 5 shows the effect of acidity on the amount of porphobilin produced in partially purified solution of porphobilinogen, and indicates that there is an optimum at pH 5.2. There is a parallel between the amount of pigment formed and the loss of porphobilinogen from the solution in the acidic medium. In the alkaline medium, the porphobilinogen breakdown continues in the absence of pigment formation. Heating a solution of porphobilinogen with alkali

Table 3. *Coproporphyrin excretion in male rabbit receiving porphobilinogen*

Period	Urine (ml./3 days)	Faeces (g./3 days)	Coproporphyrin (μ g./3 days)		Total copro- porphyrin (μ g./3 days)
			Urine	Faeces	
I. Control	116	69	8.8	33	41.8
II. Porphobilinogen injected	145	51.5	9.8	21	30.8
III. Porphobilinogen and methionine injected	159	27	7.5	18	25.5

Table 3 records the results obtained. During the experimental period the appetite of the rabbit diminished, but there were no other untoward effects. This probably explains the drop in the faecal output, and in turn the diminishing coproporphyrin excretion. The urine analysis and faecal chromatograms afforded no evidence of the presence of uroporphyrin, in spite of the fact that observation of such chromatograms in ultra-violet light is a very sensitive test for porphyrin. The urine during the periods of porphobilinogen injection contained small amounts of porphobilinogen identified spectroscopically after reaction with *p*-dimethylaminobenzaldehyde, but the amounts were too small to make estimation practicable in the concentrated urine. At the conclusion of the experiment the animal was sacrificed, but showed no changes on post-mortem examination.

DISCUSSION

Isolation of porphobilinogen has not, so far, been possible, owing largely to its complete insolubility in organic solvents (Waldenström, 1937). The results show that the present method of determining relative amounts of porphobilinogen in urine is only approximate on account of the interfering activity of substances present in the urine. The results of Waldenström & Vahlquist (1939*a*) with urea have been confirmed and extended and the effect of

reduced the intensity of the colour produced with *p*-dimethylaminobenzaldehyde, and was accompanied by the disappearance of the weaker of the two spectral bands shown by the porphobilinogen-*p*-dimethylaminobenzaldehyde compound, thus indicating the formation of some new product. This fact has also been observed by Waldenström & Vahlquist (1939*b*). The results indicate that the reaction porphobilinogen-porphobilin is not reversible under the conditions investigated. In addition it has been found that simple oxidation of porphobilinogen with hydrogen peroxide, at the pH which is optimal for the formation of porphobilin in the presence of air, does not lead to pigment production, although ascorbic acid seems to be slightly effective in inhibiting pigment formation. Terwen's solution did not lead to significant reduction of porphobilin.

Attempts to convert porphobilinogen to porphyrin have only partially confirmed Waldenström's findings. There was evidence of production of uroporphyrin on heating at pH 5, but no evidence of porphyrin formation when porphobilinogen was injected into the rabbit. Waldenström & Wendt (1939) were successful in obtaining uroporphyrin by similar means. In a case of acute porphyria studied by the author (Prunty, 1945), some evidence was obtained which suggested the possibility that methionine administration favoured the production of uroporphyrin, but the experimental evidence in

the rabbit, to investigate this point, was negative. That porphobilinogen has here been demonstrated to be present in the liver in acute porphyria and that uroporphyrin has been obtained from liver and from faeces (Grinstein, Schwartz & Watson, 1945; Prunty, 1945), is of considerable significance. These facts do not accord with the view put forward by Waldenström & Vahlquist (1944) that the porphyrin formation in this condition is purely a secondary event, taking place after the formation of the urine.

SUMMARY

1. The preparation of purified solutions of porphobilinogen from the urine and from the liver

of a patient suffering from acute porphyria is described.

2. The reaction of these solutions with *p*-dimethylaminobenzaldehyde is studied. Urea and ascorbic acid inhibit the reaction. Creatine and creatinine are without effect.

3. The formation of porphyrin from porphobilinogen heated in acid solution is confirmed. Quantitative data afford no evidence for formation of porphyrin from porphobilinogen injected into the rabbit.

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Studies on the Metabolism of Semen

1. GENERAL ASPECTS. OCCURRENCE AND DISTRIBUTION OF CYTOCHROME, CERTAIN ENZYMES AND COENZYMES

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Semen as ejaculated is composed of two parts, the spermatozoa or 'sperm' and the seminal fluid or 'plasma'. There are numerous analytical data on the chemical composition and the metabolic activities of semen, but for the most part they refer to whole semen without drawing a clear distinction between the sperm and the plasma (for references see Anderson, 1945). Yet, from many points of view, the two components of the semen must be considered separately, in the same sense as, for instance, the blood cells and blood plasma.

The spermatozoa exhibit two outstanding biological properties, fertility and motility, and they possess a characteristic metabolism of their own, maintained by a number of intracellular catalysts. In the presence of oxygen they show a considerable respiratory activity which is correlated significantly

with both the number of cells and the intensity of movement; at the same time, the rate of respiration gives a quantitative measure of the viability of spermatozoa and is therefore used by some investigators as an indicator in assessing the potential fertilizing capacity of the semen (Walton, 1939). In the absence of oxygen, the activity of spermatozoa is maintained by glycolytic processes. Recent research indicates that the glycolysis, like the respiration, is correlated with the motility and fertility of spermatozoa. There seems to be little doubt that in whole semen the spermatozoa utilize glucose as the substrate for glycolysis (cf. MacLeod, 1943 *a*). This is supplied by the plasma which in itself is devoid of a glycolyzing system. It is frequently said that the seminal plasma acts towards the spermatozoa both as a fluid vehicle for their transport and as a