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The Biosynthesis of Porphyrins from Porphobilinogen by *Rhodospseudomonas spheroides*

2. THE PARTIAL PURIFICATION AND SOME PROPERTIES OF PORPHOBILINOGEN DEAMINASE AND UROPORPHYRINOGEN DECARBOXYLASE*

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It has been already established that preparations from the photosynthetic bacterium *Rhodospseudomonas spheroides* synthesize coproporphyrin III from porphobilinogen (Heath & Hoare, 1959*a*, *b*). It was also shown that under certain conditions porphobilinogen was converted into uroporphyrin I and uroporphyrin III and that uroporphyrinogens I and III were decarboxylated to yield, ultimately, coproporphyrins I and III respectively (Hoare & Heath, 1958).

The recent work of Bogorad (1958*a*, *b*, *c*) with enzyme preparations from spinach, wheat germ and *Chlorella*, and that of Granick & Mauzerall (1958) and Mauzerall & Granick (1958) with enzyme preparations from chick and rabbit reticulocytes, has shown that at least three enzymes are involved in the conversion of porphobilinogen into coproporphyrin III. Porphobilinogen deaminase purified from spinach extracts (Bogorad, 1958*a*) converts porphobilinogen, via unknown intermediates, into uroporphyrinogen I. In a preliminary report Lockwood & Rimington (1957) described the partial purification of an enzyme from chick erythrocytes

which converted porphobilinogen into uroporphyrin III: the enzyme was called porphobilinogenase. When porphobilinogenase was preheated to 55° for 15 min. it catalysed the formation of uroporphyrin I from porphobilinogen. Similar effects of preheating had been observed earlier with crude preparations of *Chlorella* (Bogorad & Granick, 1953) and erythrocyte systems (Booij & Rimington, 1957). Similar investigations were reported in more detail by Granick & Mauzerall (1958), who purified a porphobilinogenase from chick erythrocytes and from rabbit reticulocytes. They established that uroporphyrinogen III was the reaction product in unheated preparations and uroporphyrinogen I was formed when preheated preparations were incubated with porphobilinogen. Uroporphyrinogen isomerase isolated from extracts of wheat germ (Bogorad, 1958*b*) catalysed the formation of uroporphyrinogen III from porphobilinogen in the presence of porphobilinogen deaminase; it had no action on uroporphyrinogen I or on porphobilinogen alone. Crude preparations of *Chlorella* were found to catalyse the formation of coproporphyrin from uroporphyrinogen (Bogorad, 1958*c*). Mauzerall & Granick (1958) partially purified a uroporphyrinogen decarboxylase from chick and rabbit

* Part I: Heath & Hoare (1959*b*).

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Scheme 1

reticulocytes. The reactions involved in the biosynthesis of coproporphyrin III from porphobilinogen may be illustrated as in Scheme 1.

We have now found that the two enzymes porphobilinogen deaminase and uroporphyrinogen decarboxylase can be separated from one another in preparations derived from *R. spheroides*. Furthermore, an assay has been devised for uroporphyrinogen decarboxylase with porphobilinogen as substrate in the presence of an excess of porphobilinogen deaminase. Some properties of the two enzymes are described.

MATERIALS AND METHODS

Growth and harvest of organisms. *Rhodopseudomonas spheroides* was grown semi-anaerobically as previously described (Heath & Hoare, 1959b). The harvested cells were treated with cold acetone, as previously described, and the dried cells were stored at -15° .

Substrates. Porphobilinogen (PBG) and uroporphyrins I and III were provided by Professor C. Rimington, F.R.S. Uroporphyrins II and IV were provided by Dr S. F. MacDonald.

Preparation of uroporphyrinogens. Uroporphyrins (500–1000 μ g./ml. in 0.1N-NaOH) were reduced with a large excess (2 g.) of freshly prepared 2.5% (w/w) sodium amalgam by a similar procedure to that used by Mauzerall & Granick (1958). The porphyrins were reduced in the dark in an atmosphere of N_2 and when completely colourless the mixture was filtered through three layers of Whatman no. 42 paper into a tube shielded from the light and immersed in a freezing mixture. The tube contained solid sodium dithionite and cysteine hydrochloride to make a final concentration of 1 mM each. The filtrate was adjusted to pH 6.8 with acetic acid or with phosphoric acid and was made up to 2 ml. In some cases, as specified in the results, sodium dithionite and cysteine hydrochloride were omitted. Under ideal conditions the uroporphyrinogen preparations were colourless and non-fluorescent, although oxidation occurred to a small degree in some cases during the pH adjustment.

Preparation of stimulating factor from liver. Fresh pig liver (135 g. wet wt.) was homogenized with 500 ml. of water in a Waring Blendor. The homogenate was boiled, and cooled and strained through muslin, producing a turbid yellow extract. The extract was boiled and filtered. Barium acetate (50 g. in 100 ml. of water) was added and the mixture was adjusted to pH 8.2 with dilute aq. NH_3 soln. An equal volume of absolute ethanol was added and the

precipitate which formed was allowed to settle out overnight at -15° . The bulk of the supernatant was decanted off and the precipitate was collected by centrifuging, and was washed once with 50% (v/v) ethanol. The precipitate was suspended in water (about 10 ml.) and 3 ml. of 2N- H_2SO_4 was added dropwise with continuous stirring. The suspension was centrifuged, producing a clear yellow supernatant. The residue was re-suspended in water and was re-extracted with 2N- H_2SO_4 until the final extract (the fifth extraction) was almost colourless. The combined extracts were adjusted to pH 6.5 with 2N-NaOH and were centrifuged to produce a bright-yellow solution. The solution was made up to 130 ml. (0.1 ml. = 100 mg. wet wt. of liver) and was stored at -15° . This is referred to as 'pig-liver extract' in all the experiments described.

Preparation and purification of enzymes. Acetone-dried cells (2 g.) were suspended in 50 ml. of ice-cold McIlvaine citrate-phosphate buffer, pH 6.5, diluted 1:10 with glass-distilled water containing 0.5 mM-dimercaptopropanol (BAL). This buffer was used throughout these investigations unless stated otherwise. The suspension was stirred in the cold for 3 hr. and was then frozen overnight. The frozen suspension was thawed out and centrifuged for 15 min. at 12 000g. The slightly opalescent red-brown supernatant was decanted off and the residual cells were resuspended in 10 ml. of buffer containing 0.5 mM-BAL. The suspension was centrifuged as before and the two supernatants were combined (total volume 35–45 ml.). The cell-free extract thus obtained was fractionated with solid $(NH_4)_2SO_4$, care being taken to keep the mixture cool ($0-2^{\circ}$) by immersion in a freezing mixture. Ammonium sulphate was added in small quantities; the cell-free extract was stirred mechanically. Insoluble proteins were centrifuged for 15 min. at 12 000g and were dissolved or suspended in 10–20 ml. of buffer containing 0.5 mM-BAL. The different protein fractions were then ultrafiltered overnight, reducing the volume to less than 1 ml. The ultrafiltered fractions were made up to 2.5 ml. with buffer containing 0.5 mM-BAL and were centrifuged for 15 min. at 12 000g to remove small amounts of insoluble proteins. All enzyme fractions were stored at -15° .

Fractionation of whole acetone-dried cells for investigations on a stimulating factor for coproporphyrin formation. Acetone-dried cells were suspended in buffer, pH 6.5, containing 0.5 mM-BAL and were extracted by freezing and thawing three times successively with 20 ml. of buffer/g. dry wt. of cells. The residual cells were acetone-dried and the extract was concentrated by ultrafiltration through dialysis tubing (Grant, Rowe & Stanworth, 1958). Three fractions were obtained: (A) an extracted cell residue; (B) an ultrafiltrate; (C) an ultrafiltered residue. The fractionation procedure is outlined in the diagram (Scheme 2).

Scheme 2

Incubation conditions. The standard conditions for most experiments included a reaction mixture of 0.5 ml. total volume, which was incubated for 2 hr. in an atmosphere of N_2 . Anaerobic conditions were maintained by carrying out the reactions in small centrifuge tubes, which were inserted into Thunberg tubes containing alkaline pyrogallol. The Thunberg tubes were then alternately evacuated and flushed with O_2 -free N_2 (three times). Incubations were carried out at 37° in the dark by covering the incubation bath with a black cloth.

Estimation of porphyrins. The procedure previously used (Heath & Hoare, 1959b) was slightly modified. One drop of iodine (1%, w/v, in absolute ethanol) was added to each reaction mixture at the end of the incubation period, and this was followed within 10–15 sec. by one drop of 1% (w/v) sodium thiosulphate. Ethyl acetate–acetic acid mixture (3:1, v/v; 5 ml.) was then added and the mixture was centrifuged; the residue was re-extracted with 5 ml. of ethyl acetate–acetic acid mixture and the combined extracts were transferred to separating funnels. The uroporphyrin was extracted with saturated sodium acetate followed by 3% (w/v) sodium acetate as previously described. The combined sodium acetate extracts were acidified to pH < 1.0 with conc. HCl and were made up to a given volume (generally 75 ml.) with 5% (w/v) HCl and the extinctions at 380, 406 and 430 $m\mu$ were measured in a Unicam SP. 500 spectrophotometer. Coproporphyrin was then extracted directly from the ethyl acetate phase with 10% (w/v) HCl; the extinction of the combined acid extracts, made up to 25 ml. or 75 ml. with 10% (w/v) HCl, was read at 380, 402 and 430 $m\mu$. The correction factors of Rimington & Sveinsson (1950) were used to calculate the porphyrin concentrations.

Direct assay of porphobilinogen deaminase. Preparations of porphobilinogen deaminase which had been found to be free from uroporphyrinogen decarboxylase were assayed directly by the following procedure. Reaction mixtures of 0.5 ml. total volume contained PBG (100 $\mu g.$) in McIlvaine's citrate–phosphate buffer at pH 7.6. Incubations were carried out anaerobically for 1 hr. at 37°, after which the reaction mixture was treated with iodine and thiosulphate as before, diluted to 25 ml. (or 75 ml.) with 5% (w/v) HCl and examined directly in a spectrophotometer. The small amount of protein present did not interfere.

Enzyme activities were expressed as $\mu g.$ of porphyrin formed/mg. of protein/hr. Protein estimations were carried out by the method of Lowry, Rosebrough, Farr & Randall (1951), bovine-serum albumin being used as standard.

Paper chromatography of free porphyrins and of porphyrin esters. Porphyrin esters, prepared by esterification with 5% (v/v) H_2SO_4 in methanol, were chromatographed by the method of Falk & Benson (1953). Free porphyrins were chromatographed by the method of Falk, Dresel, Benson & Knight (1956) and by the modified procedure of Eriksen (1958).

Column chromatography of porphyrin esters. Chromatography on magnesium oxide grade III and on alumina grade IV was carried out according to the procedure of Nicholas (1951). Small columns of 1 cm. diam. and 12 cm. length were used.

Paper electrophoresis of porphyrins. Free porphyrins were applied to the cathode end of a strip of Whatman no. 3 paper (4 cm. width, 60 cm. length) equilibrated with 1% (w/v) anhydrous Na_2CO_3 and 0.1 mM-disodium ethylenediaminetetra-acetate. A current of 3 mA, 240 v was applied for 2–3 hr.; the migration of porphyrins could be observed visually. Uroporphyrin and coproporphyrin were run simultaneously as markers.

RESULTS

Ammonium sulphate fractionation of cell-free extracts

Cell-free extracts were prepared from acetone-dried cells as described in the Materials and Methods section. As has already been shown (Heath & Hoare, 1959b), the relative amounts of uroporphyrin and coproporphyrin formed by acetone-dried cells and by cell-free extracts depend upon the protein concentration and the time of incubation. It is thus difficult to assess the degree of extraction of the respective porphyrin-forming enzymes. In a typical experiment 2 g. of acetone-dried cells after extraction yielded 40 ml. of cell-free extract containing a total of 320 mg. of protein, representing 16% of the starting material. The enzyme activities are shown in Table 1.

The above-mentioned cell-free extract was fractionated with $(NH_4)_2SO_4$ and, after overnight ultrafiltration, the protein content of each fraction was determined before and after removing insoluble matter by centrifuging. Each fraction was then assayed anaerobically under the standard conditions (2 hr. at 37°). The distribution of protein

Table 1. *Extraction of protein and porphyrin-forming enzyme activity from acetone-dried cells of Rhodopseudomonas spheroides*

Acetone-dried cells, or cell-free extract, were incubated anaerobically in a total volume of 0.5 ml. with PBG (100 $\mu g.$) for 2 hr. at 37° in citrate–phosphate buffer, pH 6.5.

	Dry wt. per assay (mg.)	Porphyrin formed ($\mu g./2$ hr.)		Enzyme activity ($\mu g.$ of porphyrin/mg./hr.)		
		Uro-	Copro-	Uro-	Copro-	Total
Acetone-dried cells	2	8	2	2.0	0.5	2.5
	5	19	8	1.9	0.8	2.7
	10	28	20	1.4	1.0	2.4
Cell-free extract	1.6	40	9	12.5	2.8	15.3

Table 2. *Distribution of protein and porphyrin-forming enzyme activity in ammonium sulphate fractions from cell-free extracts of Rhodopseudomonas spheroides*

Cell-free extract (40 ml., 300 mg. of protein) was fractionated with ammonium sulphate; each fraction was ultrafiltered overnight as described in the text and assayed for porphyrin-forming enzyme activity.

(NH ₄) ₂ SO ₄ fraction (%, w/v)	Total protein (mg.)	Soluble protein (mg.)	Protein per assay (mg.)	Porphyrin formed (μg./2 hr.)		PBG deaminase activity* (μg. of uro-/mg./ hr.)
				Uro-	Copro-	
0-15	80	—	—	Discarded		—
15-30	100	85	0.5	44	2	44
30-35	40	30	0.8	27	2	17
35-40	20	17	0.4	1	2	1
40-45	17	17	0.3	0	0.5	Nil

* Assayed at pH 6.5, which is not the optimum pH.

Table 3. *Ammonium sulphate subfractionation of porphobilinogen deaminase*

Starting material (34 ml.) contained 442 mg. of protein of enzyme activity 17 μg. of uro-/mg. of protein/hr., total enzyme units 7600. Solid ammonium sulphate was added to the percentage (w/v) shown below; each precipitate was dissolved in buffer, pH 6.5, containing 0.5 mM-BAL, and was assayed for porphyrin-forming enzyme activity. Enzyme unit: mg. of protein catalysing the formation of 1 μg. of uroporphyrin/hr. under the assay conditions.

(NH ₄) ₂ SO ₄ fraction (%, w/v)	Total protein (mg.)	Protein per assay (mg.)	Enzyme activity (μg. of uro-/mg./hr.)	Enzyme units
0-15	105	0.5	2	210
15-22	119	0.6	8.5	1012
22-30	166	0.4	36.2	6009
30-36	38	0.7	17.2	654

Table 4. *Indirect assay of uroporphyrinogen decarboxylase in ammonium sulphate fractions from Rhodopseudomonas spheroides*

Test fraction was incubated anaerobically in a total volume of 0.5 ml. with an excess of PBG deaminase (0.6 mg. of protein), pig-liver extract (0.1 ml.) and PBG (100 μg.) for 2 hr. at 37° in citrate-phosphate buffer, pH 6.5.

(NH ₄) ₂ SO ₄ fraction (%, w/v)	Protein per assay (mg.)	Copro- porphyrin formed (μg./2 hr.)	Decarboxyl- ase activity (μg. of copro-/ mg./hr.)
35-40	0.4	6.4	8
40-45	0.33	5.1	7.7

and enzyme activity in the different fractions is shown in Table 2. The fraction precipitating between 0 and 15% (w/v) of (NH₄)₂SO₄ was grey-green and redissolved only incompletely in buffer solution; in every case when tested it was devoid of enzyme activity. Porphobilinogen deaminase activity was found in the protein fractions precipitating between 15 and 35% (w/v) of (NH₄)₂SO₄. Protein fractions precipitating between 45 and 55% of (NH₄)₂SO₄ were pink and a determination of the absorption spectrum at pH 6.5 showed sharp peaks at 415, 520 and 548 mμ, corresponding to a reduced cytochrome c (cf. Vernon & Kamen, 1954). Fractions containing PBG deaminase activity from several fractiona-

tion procedures applied to different batches of acetone-dried cells were combined and subfractionated with (NH₄)₂SO₄. Such a subfractionation is illustrated in Table 3. Nearly 80% of the PBG deaminase activity precipitated between 15 and 30% (w/v) of (NH₄)₂SO₄. It was completely free from decarboxylating enzymes forming coproporphyrin.

Indirect assay of uroporphyrinogen decarboxylase. Protein fractions precipitated by (NH₄)₂SO₄ concentrations greater than 35%, and which formed very little coproporphyrin from PBG, were tested for uroporphyrinogen decarboxylase by an indirect method with PBG as substrate. Use was made of the PBG deaminase preparations from earlier (NH₄)₂SO₄ fractions (15-35%), particularly after a second subfractionation, as just described, to free them from coproporphyrin-forming enzymes. On incubating test fractions with PBG, together with an excess of PBG deaminase, the formation of coproporphyrin indicated the presence of uroporphyrinogen decarboxylase. Protein fractions precipitated by (NH₄)₂SO₄ concentrations above 35% (w/v) (as shown in Table 2) were tested in this way (Table 4).

Protein fractions containing uroporphyrinogen decarboxylase free from PBG deaminase were stable on storage at -15° for 2-3 weeks in the presence of 0.5 mM-BAL (Table 5). When a number

Table 5. *Stability tests on uroporphyrinogen decarboxylase preparations*

Uroporphyrinogen decarboxylase was assayed by the indirect method, in two preparations after storage at -15° .

Enzyme preparation	Age of preparation (days)	Protein per assay (mg.)	Decarboxylase activity (μ g. of copro-/mg./hr.)
D4 [35-40% (w/v) $(\text{NH}_4)_2\text{SO}_4$]	Fresh	0.34	10
	7	—	17
	20	—	12
C5 [36-40% (w/v) $(\text{NH}_4)_2\text{SO}_4$]	Fresh	0.44	9
	13	—	11
	26	—	12

Fig. 1. Effect of time of incubation on the formation of uroporphyrin (a) and coproporphyrin (b) in the indirect assay of uroporphyrinogen decarboxylase. PBG (100 μ g.) was incubated anaerobically in citrate-phosphate buffer, pH 6.5, with an excess of PBG deaminase (1600 μ g. of protein) (●), or uroporphyrinogen decarboxylase (400 μ g. of protein) (□), or uroporphyrinogen decarboxylase together with an excess of PBG deaminase (○), in a final volume of 0.5 ml. at 37° for the times indicated.

of stored decarboxylase preparations were combined and subfractionated with $(\text{NH}_4)_2\text{SO}_4$, no purification was achieved and the treated preparations lost activity after storage for 2-3 days at -15° . Instability of the decarboxylase was also shown in the early stages of enzyme extraction. When attempts were made to improve the degree of extraction of acetone-dried cells by homogenizing in buffer in a Potter-Elvehjem glass homogenizer, the resulting extracts formed only uroporphyrin from PBG. Inactive preparations could not be reactivated by the addition of BAL.

A progress curve for coproporphyrin formation and uroporphyrin formation in the indirect assay is illustrated in Fig. 1. Control curves show that no coproporphyrin is formed from PBG by the decarboxylase or deaminase preparations alone.

Fig. 2. Effect of enzyme concentration on porphyrin formation in the indirect assay of uroporphyrinogen decarboxylase, and the effect of adding pig-liver extracts to the system. PBG (100 μ g.) was incubated for 2 hr. at 37° anaerobically at pH 6.5, with an excess of PBG deaminase (570 μ g. of protein) and different amounts of uroporphyrinogen decarboxylase (7400 μ g. of protein/ml.) in a final volume of 0.5 ml. with and without 0.1 ml. of pig-liver extract (prepared as described in the Materials and Methods section). ●, Coproporphyrin formation in the absence of pig-liver extract; ○, coproporphyrin formation in the presence of pig-liver extract; ■, uroporphyrin formation in the absence of pig-liver extract; □, uroporphyrin formation in the presence of pig-liver extract.

Fig. 2 illustrates the increase in coproporphyrin production with increasing concentration of uroporphyrinogen decarboxylase in the indirect-assay procedure; the uroporphyrin concentration correspondingly decreases.

Tests with uroporphyrinogens as substrates. Uroporphyrinogens were prepared as described in the Materials and Methods section. When tested as substrates with a partially purified decarboxylase preparation which had no effect on PBG, coproporphyrin was formed. The time course for the reaction, with uroporphyrinogen III as substrate, over a 4 hr. incubation period is shown in Fig. 3.

With a fixed concentration of uroporphyrinogen (prepared in the presence of cysteine and dithionite) and different concentrations of decarboxylase, the amount of coproporphyrin formed per hour was proportional to the enzyme concentration (Fig. 4).

Uroporphyrinogens I, II and IV were also decarboxylated to yield coproporphyrins. A direct comparison of the rates of reaction with the four uroporphyrinogens was not made. In the absence of sodium dithionite, the rate of coproporphyrin formation was independent of uroporphyrinogen III concentration over the range $8\text{ }\mu\text{M}$ – 0.16 mM .

Fig. 3. Effect of time of incubation on coproporphyrin formation from uroporphyrinogen III. Coproporphyrin formation (○) and uroporphyrin recovered (□) when uroporphyrinogen decarboxylase ($430\text{ }\mu\text{g.}$ of protein) was incubated anaerobically with uroporphyrinogen III ($23\text{ }\mu\text{g.}$) and pig-liver extract (0.1 ml.) at pH 6.5 in a final volume of 0.5 ml. at 37° for the times indicated.

Fig. 4. Effect of enzyme concentration on coproporphyrin formation from uroporphyrinogen III. Coproporphyrin formation after the incubation of uroporphyrinogen III ($40\text{ }\mu\text{g.}$) at 37° for 1 hr. anaerobically at pH 6.5, in a final volume of 0.5 ml. with different amounts of uroporphyrinogen decarboxylase ($800\text{ }\mu\text{g.}$ of protein/ml.).

Properties of partially purified porphobilinogen deaminase

pH optimum was determined in McIlvaine's citrate-phosphate buffer over the range pH 6.4–8.0; borate buffer was used up to pH 8.8. For 1 hr.

Fig. 5. pH-activity curve for PBG deaminase. PBG deaminase ($160\text{ }\mu\text{g.}$ of protein) was incubated anaerobically with PBG ($100\text{ }\mu\text{g.}$) in a final volume of 0.5 ml. for 1 hr. at 37° in different buffers (pH 6.4–9.0). ○, McIlvaine citrate-phosphate buffer; □, borate buffer.

Fig. 6. Optimum temperature for PBG deaminase. PBG ($100\text{ }\mu\text{g.}$) was incubated anaerobically for 1 hr. at pH 7.6 in a final volume of 0.5 ml. at the temperatures indicated: ○, with PBG deaminase ($190\text{ }\mu\text{g.}$ of protein); □, without PBG deaminase. Figures with PBG deaminase are corrected for uroporphyrin formed non-enzymically.

incubations anaerobically at 37° the optimum pH was 7·6 (Fig. 5).

At pH 7·6 the rate of uroporphyrin formation over a 1 hr. incubation period was measured at different temperatures. Both the enzymic and non-enzymic reactions were studied. An optimum temperature between 45 and 55° was observed under these conditions (Fig. 6). A re-determination of enzyme activity at four different pH values at 55° showed an optimum pH at 7·0. The following activities ($\mu\text{g.}$ of uroporphyrin formed/mg. of protein/hr.) were found: 248 at pH 6·5, 279 at pH 7·0, 216 at pH 7·6, 205 at pH 8·0.

In the determination of the Michaelis constant, the PBG concentration was varied over the range $5\text{ }\mu\text{M}$ – 0.1 mM . The volume of the reaction mixture was increased to 20 ml. to ensure that a measurable quantity of porphyrin was produced, leaving sufficient PBG unreacted at the end of the incubation period. K_m $40\text{ }\mu\text{M}$ was calculated from a plot of S/V against S (Fig. 7). This value was unchanged by the presence of porphobilinogen α -carboxylic acid ($50\text{ }\mu\text{M}$) in the reaction mixture.

Investigations on a stimulating factor for formation of coproporphyrin

We have previously reported evidence for the presence of a heat-stable factor which stimulates coproporphyrin formation from PBG (Hoare & Heath, 1958; Heath & Hoare, 1959*a*). When extracts of acetone-dried cells were ultrafiltered, formation of coproporphyrin from PBG was diminished but was partially restored by adding the ultrafiltrate. The ultrafiltrate was heat-stable (10 min. at 100°, pH 6·5). Similar stimulating activity was found in ultrafiltrates of chick blood and in extracts of pig liver and of baker's yeast. Since liver was found to be a convenient source of

stimulating activity, preparations were made from this according to the procedure given in the Materials and Methods section. Preparations were stored at -15° and a single preparation stored over several months was used in all experiments.

Stimulating effects with crude enzyme preparations. Acetone-dried cells were extracted and resolved into three fractions as described in the Materials and Methods section. When assayed anaerobically the extracted cell residue (*A*) had very low activity, as also had the ultrafiltrate (*B*). The ultrafiltered

Fig. 7. Lineweaver-Burk plots. Reaction mixtures, of 20 ml. final volume, containing PBG deaminase ($780\text{ }\mu\text{g.}$ of protein) were incubated at 37°, pH 7·6, for 30 min. in open tubes with the concentrations of PBG indicated. V , Uroporphyrin formed ($\mu\text{g.}$ /tube in 30 min. at 37°, pH 7·6). S , $10^5 \times$ molar concn. of PBG.

Table 6. *Stimulation of coproporphyrin formation by ultrafiltrates from cell-free extracts of Rhodopseudomonas spheroides*

Cell fractions (equivalent to 20 mg. of whole cells), prepared as described in the text, were incubated in a total volume of 0·5 ml. with PBG ($100\text{ }\mu\text{g.}$) at 37° in citrate-phosphate buffer, pH 6·5. Heated preparations were kept at 100°, pH 6·5, for 10 min. Expt. I: anaerobic incubation for 2 hr. Expt. II: aerobic incubation (in tubes open to the air, without shaking) for 4 hr. Expts. I and II were carried out with different batches of cells.

Test system	Porphyrin formed			
	Expt. I (anaerobic)		Expt. II (aerobic)	
	Uro- ($\mu\text{g.}$)	Copro- ($\mu\text{g.}$)	Uro- ($\mu\text{g.}$)	Copro- ($\mu\text{g.}$)
Extracted cell residue (<i>A</i>)	3	—	10	—
Ultrafiltrate (<i>B</i>)	—	—	—	—
Ultrafiltered residue (<i>C</i>)	28	6	36	7
(<i>C</i>) + (<i>A</i>)	15	16	24	11
(<i>C</i>) + (<i>B</i>)	17	22	20	18
(<i>A</i>) + (<i>B</i>) + (<i>C</i>)	10	18	15	24
(<i>A</i>) heated + (<i>B</i>) + (<i>C</i>)	10	17	20	16
(<i>A</i>) + (<i>B</i>) heated + (<i>C</i>)	12	18	15	24

extract (*C*) formed mainly uroporphyrin with some coproporphyrin, and on addition of the ultrafiltrate there was a stimulation of coproporphyrin formation and a decrease in uroporphyrin (Table 6). The stimulating effect of the ultrafiltrate was unchanged after heating for 10 min. at 100°, pH 6.5. The extracted cell residue (*A*), although forming very small amounts of uroporphyrin from PBG, also stimulated coproporphyrin production when added to the ultrafiltered extract (*C*). Again, the stimulating activity of fraction (*A*) was heat-stable, and since no further stimulation was obtained by the combined actions of fraction (*A*) and the ultrafiltrate (*B*), it was concluded that a single stimulating factor was present in both fractions. When uroporphyrinogen was tested as substrate in the ultrafiltered system a similar stimulation of coproporphyrin formation was observed (Table 7). Under aerobic conditions, however, all three fractions (*A*, *B* and *C*) were necessary for maximum coproporphyrin formation from PBG (Table 6). The additional stimulating effect of the cell residue (*A*) under these aerobic conditions was destroyed by

heating to 100° for 10 min. at pH 6.5. The stimulating effect of the ultrafiltrate is not due to any protecting effect on uroporphyrinogen since, after anaerobic incubation in the dark at 37° in the absence of enzymes, the same amount of uroporphyrin was recovered in the presence as in the absence of the ultrafiltrate; in both cases non-fluorescent pink pigments were formed in addition to uroporphyrin. The stimulating effect of the ultrafiltrate does not appear to be due to sulphhydryl compounds since its activity was stable towards iodine oxidation.

Stimulating activity in cell ultrafiltrates was strongly adsorbed on charcoal and on alumina. It was precipitated, or adsorbed, with insoluble barium salts at pH 8.0. Thiamine pyrophosphate (0.1 mM–10 μ M) with or without magnesium or manganese chloride (0.1 mM–10 μ M) failed to stimulate coproporphyrin formation by the ultrafiltered enzyme preparations. Ultrafiltrates were completely inactivated by ashing; they were stable to weak acid or alkali at room temperature, but were inactivated by hot concentrated HCl and aq. NH₃ soln. (sp.gr. 0.880). All attempts to adsorb and elute activity from ion-exchange resins were unsuccessful.

Stimulating effects with partially purified enzyme fractions. When PBG deaminase and uroporphyrinogen decarboxylase were separated by fractionation with (NH₄)₂SO₄, the stimulating effect of the pig-liver extract was re-examined. In the 'indirect assay', with PBG as substrate, a definite stimulation of coproporphyrin formation, with a corresponding decrease in uroporphyrin, was observed (see Fig. 2).

Attempts to demonstrate a stimulation of coproporphyrin formation by the pig-liver extract, with uroporphyrinogen as substrate, with the partially purified decarboxylase preparation have not yielded very conclusive results. A single batch of acetone-dried cells was used to provide a cell-free extract

Table 7. *Stimulation of coproporphyrin formation from uroporphyrinogen*

Ultrafiltered extract (equivalent to 20 mg. of whole cells) was incubated anaerobically in a total volume of 0.5 ml. with uroporphyrinogen for 2 hr. at 37° in citrate-phosphate buffer, pH 6.5.

Test system	Porphyrin formed (μ g.)	
	Uro-	Copro-
Uroporphyrinogen + ultrafiltered extract	42	20
Uroporphyrinogen + ultrafiltered extract + ultrafiltrate	10	52
Uroporphyrinogen control (no enzyme)	74	—

Table 8. *Stimulation of coproporphyrin production from uroporphyrinogen III or from porphobilinogen by the addition of a pig-liver extract to crude and partially purified preparations from Rhodopseudomonas spheroides*

Enzyme preparations were made as described in the text: 'crude enzyme' (540 μ g. of protein), 'purified enzyme 1' (530 μ g. of protein) or 'purified enzyme 2' (1100 μ g. of protein). These were incubated anaerobically in a total volume of 0.5 ml. with uroporphyrinogen III (100 μ g.) or with PBG (100 μ g.) in the presence of an excess of PBG deaminase (1300 μ g. of protein), with or without a pig-liver extract (0.1 ml.), for 18 hr. at 37° in citrate-phosphate buffer, pH 6.5.

Test system	Substrate	Porphyrin formed (μ g.)	
		Uro-	Copro-
Crude enzyme	Uroporphyrinogen III	77	6
Crude enzyme + pig-liver extract	Uroporphyrinogen III	69	11
Purified enzyme 1	Uroporphyrinogen III	58	24
Purified enzyme 1 + pig-liver extract	Uroporphyrinogen III	47	27
Purified enzyme 2	Uroporphyrinogen III	68	8
Purified enzyme 2 + pig-liver extract	Uroporphyrinogen III	68	11
Purified enzyme 1 + PBG deaminase	PBG	18	34
Purified enzyme 1 + PBG deaminase + pig-liver extract	PBG	3	49

('crude enzyme') part of which was retained and ultrafiltered directly; the remainder was fractionated with $(\text{NH}_4)_2\text{SO}_4$. A fraction ('purified enzyme 1') precipitating between 40 and 45% (w/v) of $(\text{NH}_4)_2\text{SO}_4$ was found to contain uroporphyrinogen decarboxylase free from PBG deaminase. A fraction ('purified enzyme 2'), precipitating between 45 and 55% (w/v) of $(\text{NH}_4)_2\text{SO}_4$, was also found to have a very weak uroporphyrinogen decarboxylase activity and was free from PBG deaminase. Both these fractions and the original cell-free extract were ultrafiltered twice, from 20 ml. of solution in buffer, pH 6.5, containing 0.5 mM-BAL, to a volume less than 1 ml. The ultrafiltered preparations were then tested for coproporphyrin formation from uroporphyrinogen both in the presence and in the absence of pig-liver extract. The results are shown in Table 8.

Examination of the porphyrin reaction products

The porphyrins formed under different incubation conditions were examined by chromatographic techniques. It has been noted before that whole fresh acetone-dried cells form largely the III series isomers of uroporphyrin and coproporphyrin from PBG. There was no evidence for the formation of porphyrins with less than four carboxyl groups. Cell-free extracts formed both the I and III series isomers, the I series isomer predominating in most cases. With both the whole acetone-dried cells and the cell-free extracts there was evidence for the formation of intermediate penta-, hexa- or heptacarboxylic porphyrins. After fractionation with $(\text{NH}_4)_2\text{SO}_4$ the fractions containing PBG deaminase formed uroporphyrin I and no trace of any other porphyrin. In the presence of the partially purified decarboxylase fraction, coproporphyrin I and intermediate porphyrins were formed in addition.

Porphyrin fractions estimated as 'coproporphyrin' according to the procedure given in the Materials and Methods section, on chromatography by the Eriksen method (1958), showed coproporphyrin I together with traces of coproporphyrin III in some cases; no other porphyrins were present. Acid extracts containing coproporphyrin, from a large number of incubations, were esterified with methanol- H_2SO_4 (95:5, v/v). Coproporphyrin ester was extracted into CHCl_3 and was washed successively with water, aq. 2N- NH_3 soln. and water. Typical crystals of coproporphyrin I tetramethyl ester were formed on concentration of the washed CHCl_3 extract. Insufficient material was available for recrystallization and determination of m.p. The combined coproporphyrin methyl esters were eluted as a single sharp band from a column of alumina grade IV by benzene- CHCl_3 (1:1, v/v).

Extracts containing uroporphyrin were concentrated to dryness, were similarly esterified and the methyl esters were chromatographed on paper (Falk & Benson, 1953). One or two spots with R_f greater than that for uroporphyrin III octamethyl ester were invariably found in addition to a strong spot corresponding to uroporphyrin I.

When the esters were hydrolysed and the free porphyrins were chromatographed by the method of Falk *et al.* (1956), in addition to uroporphyrin there were two well-defined fluorescent spots intermediate between uroporphyrin and coproporphyrin. The faster R_f component was present in much higher concentrations. In a typical chromatogram in which uroporphyrin, coproporphyrin and deuteroporphyrin were run simultaneously as markers, the following R_f values were found: deuteroporphyrin, 0.83; coproporphyrin III, 0.51; major unknown, 0.29; minor unknown, 0.10; uroporphyrin I, 0.012. The uroporphyrin always appeared as a double spot, the faster R_f component being yellow. This

Table 9. *Chromatographic separation of porphyrin reaction products*

Combined 'uroporphyrin fractions' from a number of reaction mixtures were esterified and the methyl esters were chromatographed on a column of magnesia grade III; eluates were concentrated to dryness and were hydrolysed in 6N-HCl; the free porphyrins were chromatographed by the method of Falk, Dresel, Benson & Knight (1956).

Magnesia chromatogram		Paper chromatogram
Band	Eluting solvent	R_f values
(1) Sharp and strong	CHCl_3 -0.5% methanol	0.05 (Uroporphyrin) 0.08 [Pseudo-uroporphyrin (yellow)] 0.17 (Trace of unknown intermediate)
(2) Diffuse band	CHCl_3 -0.5% methanol	0.17 (Minor intermediate) 0.30 (Major intermediate)
(3) Distinct band	CHCl_3 -1% methanol	0.33 (Major intermediate)
(4) No distinct band	CHCl_3 -5% methanol	0.33 (Traces only)
(5) No distinct band	CHCl_3 -50% methanol	0.50 (Traces of coproporphyrin) 0.033 (Uroporphyrin I marker) 0.47 (Coproporphyrin I marker)

may correspond to the 'pseudo-uroporphyrin' reported by Falk *et al.* (1956).

Similar intermediate porphyrins were observed with the partially purified decarboxylase and uroporphyrinogen as substrate. Controls with uroporphyrinogen alone showed only uroporphyrin on chromatography although other non-fluorescent pink pigments were present: these may be related to the pigments formed from PBG under certain conditions (Brockman & Gray, 1953).

Paper electrophoresis of intermediate porphyrins. 'Uroporphyrin fractions' were resolved into five components travelling as follows: uroporphyrin together with pseudo-uroporphyrin, 8–10 cm.; minor component, 5 cm.; major component, 2–3 cm.; very weak spot on origin (which may be due to traces of coproporphyrin), 0.5 cm.

Attempts to isolate intermediate porphyrins by column chromatography on magnesia. The 'uroporphyrin fractions' from a number of reaction mixtures were accumulated and esterified. The methyl esters were then chromatographed on a column of magnesium oxide grade III, mixtures of CHCl_3 -methanol being used as the eluting solvent. The eluates were concentrated to dryness and were hydrolysed with 6N-HCl for 48 hr. at room temperature. The free porphyrins were then run on paper chromatograms in lutidine-water (Falk *et al.* 1956), with uroporphyrin I and coproporphyrin I as markers. The results are summarized in Table 9. No further characterization was possible owing to the small quantities of material available.

DISCUSSION

Mauzerall & Granick (1958) and Granick & Mauzerall (1958) in their studies on porphyrin biosynthesis by chick and rabbit reticulocytes were able to separate PBG deaminase from uroporphyrinogen decarboxylase by zone electrophoresis on starch plates at pH 7.65. These two enzymes have now been separated from one another in cell-free extracts of *R. spheroides* by fractional precipitation with ammonium sulphate. We have also applied a similar fractionation procedure to the strain of *R. capsulatus* found by Cooper (1956) to produce a coproporphyrin precursor under certain cultural conditions; this also effected a separation of PBG deaminase from uroporphyrinogen decarboxylase.

The development of an indirect assay for uroporphyrinogen decarboxylase with PBG as substrate in the presence of an excess of PBG deaminase greatly facilitated the following of activity of different preparations of uroporphyrinogen decarboxylase. The indirect assay was found to be reproducible and in this respect was far more

satisfactory than the direct assay with uroporphyrinogens as substrates. Indeed assays of a single-enzyme preparation by both the direct and indirect methods did not agree (see, for example, Table 8). Activities in the direct assays with uroporphyrinogens were invariably lower. This may be attributed to the presence of inhibitors in the uroporphyrinogen preparations. It is unlikely to be due to traces of mercury from the sodium-amalgam reduction since the reduction was carried out under alkaline conditions. Lower activities were found independently of the presence or absence of cysteine or of dithionite with the uroporphyrinogen preparation. It was in many cases difficult to prevent absolutely any re-oxidation of the uroporphyrinogen preparations. It is quite likely that products of partial re-oxidation of uroporphyrinogens inhibit the decarboxylase. Non-fluorescent pink pigments invariably formed after re-oxidation of uroporphyrinogens and these unidentified products may also inhibit uroporphyrinogen decarboxylase.

Although a large number of enzyme fractionations were carried out on different batches of acetone-dried cells, the final partially purified enzyme preparations had very similar activities. Uroporphyrinogen decarboxylase activities ranged from 8 to 11 $\mu\text{g.}$ of coproporphyrin formed/mg. of protein/hr. It is difficult to compare the activity of our preparations with those of Mauzerall & Granick (1958), who used 0.5 ml. of enzyme preparation containing about 0.1 mg. of nitrogen/ml., and found 2 $\mu\text{m-moles}$ of coproporphyrin formed/hr. (These values are derived from the most active system shown in Fig. 9 of Mauzerall & Granick, 1958.) Assuming that their preparation contained 0.312 mg. of protein (equivalent to 0.05 mg. of nitrogen/0.5 ml. of enzyme) its activity would be 4.2 $\mu\text{g.}$ of coproporphyrin formed/mg./hr. Our preparations of uroporphyrinogen decarboxylase were stable to storage at -15° only in the presence of sulphydryl compounds such as BAL. This suggests that it is a sulphydryl enzyme. This is confirmed by preliminary experiments in which the enzyme was found to be inhibited by heavy metals (mercury and copper). The loss of decarboxylase activity when acetone-dried cells were homogenized in a Potter-Elvehjem glass homogenizer may be due to oxidation of essential sulphydryl groups. The few investigations on the decarboxylase from *R. spheroides* show that it is very similar in properties to the enzyme isolated from chick and rabbit reticulocytes by Mauzerall & Granick (1958). This extends to the observation that all four position-isomers of uroporphyrinogen are decarboxylated and also that the decarboxylation proceeds via the formation of intermediate hepta-, hexa- and penta-carboxylic porphyrins which have been detected chromatographically.

Table 10. Comparison of porphobilinogen deaminase activities from different sources

	Enzyme source	Activity as expressed by authors	Activity (μ g. of uro-/mg. of protein/hr.)
Lockwood & Rimington (1957)	Chick blood	μ g. of uro-/ml./hr. 133 μ g. of uro-/9 mg. of protein/hr.	14.8
Granick & Mauzerall (1958)	Chick blood	μ mole of PBG/ml. of enzyme/hr. 0.3 μ mole of PBG/2.1 mg. of protein/hr.	29.8
Granick & Mauzerall (1958)	Rabbit reticulocytes	0.3 μ mole of PBG/0.35 mg. of protein/hr.	178
Bogorad (1958a)	Spinach	1300 units/mg. of protein (1 unit: enzyme required to catalyse the consumption of 1 μ m-mole of PBG/hr.)	270
Present paper	<i>R. spheroides</i>	μ g. of uro-/mg. of protein/hr.	200*

* At pH 7.6, 45°.

The fact that intermediate porphyrins were invariably found in small amounts as products of the action of uroporphyrinogen decarboxylase and that these were extracted into the 'uroporphyrin fractions' raises the question of the validity of the figures quoted for uroporphyrin formation. It has been assumed that the contribution of intermediate porphyrins to the observed absorption could be neglected. However, it should be borne in mind that the uroporphyrin measured does include variable small quantities of other porphyrins. Cell-free extracts of *R. spheroides* are a very convenient source of PBG deaminase with a high specific activity. After only two fractionations with ammonium sulphate, preparations were obtained with an activity of the order of 200 μ g. of uroporphyrin formed/mg. of protein/hr. This compares favourably with enzyme preparations from other sources (Table 10).

PBG deaminase from *R. spheroides* is similar in properties to the enzyme isolated from blood and from spinach. It is comparatively thermostable with a temperature optimum around 45–55° under the assay conditions employed in our experiments. Rather surprisingly there was no increase in the amount of PBG deaminase extracted from acetone-dried cells when the extraction was carried out at 50°.

Although opsopyrrole carboxylic acid has been shown to be a competitive inhibitor of PBG deaminase (Bogorad, 1957; Carpenter & Scott, 1959), porphobilinogen α -carboxylic acid had no affinity for the enzyme. This suggests that the enzyme-substrate linkage involves a free (unsubstituted) α -position on the pyrrole nucleus. The Michaelis constant of 40 μ M found for the PBG deaminase from *R. spheroides* is very close to that reported for the spinach enzyme (Bogorad, 1958b).

The nature of the stimulating factor in pig-liver extracts is still obscure. Significant stimulations of

coproporphyrin formation from PBG were consistently obtained both with crude cell-free extracts after ultrafiltration and with the partially purified preparation. However, when uroporphyrinogens were used as substrates, coproporphyrin formation was stimulated by the addition of pig-liver extract to crude cell-free extracts but not with the purified decarboxylase. The failure to get a significant stimulation of coproporphyrin formation from uroporphyrinogens with the partially purified decarboxylase may be due to the presence of inhibitors in the uroporphyrinogen preparations. In this case it must be assumed that the purified enzyme is more sensitive to inhibitors than is the crude enzyme in cell-free extracts. It has already been mentioned that uroporphyrinogen decarboxylase activities determined with uroporphyrinogens as substrates are lower than those found in the indirect assay with PBG and a common factor may be involved in these two effects.

SUMMARY

1. Porphobilinogen deaminase and uroporphyrinogen decarboxylase have been separated from each other in cell-free extracts of *Rhodospseudomonas spheroides* by fractionation with ammonium sulphate.

2. A reproducible 'indirect' assay for uroporphyrinogen decarboxylase has been devised with porphobilinogen as substrate with an excess of porphobilinogen deaminase.

3. The Michaelis constant, temperature optimum and pH optimum of the partially purified porphobilinogen deaminase have been determined. The Michaelis constant with porphobilinogen as substrate is unchanged in the presence of porphobilinogen α -carboxylic acid.

4. Uroporphyrinogen decarboxylase acts on all four position-isomers of uroporphyrinogen and is unstable in the absence of sulphhydryl compounds.

5. *Rhodospseudomonas spheroides*, chick blood, yeast, and pig liver contain a heat-stable factor which stimulates coproporphyrin formation from porphobilinogen.

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The Chemistry of Xanthine Oxidase

6. VARIATIONS IN STABILITY AND THE PRESENCE OF AN INHIBITOR IN CERTAIN PREPARATIONS*

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It has been reported previously that the stability of xanthine oxidase samples varies from one preparation to another (Bergel & Bray, 1956, 1959). This paper reports results of a comparison of the properties of two preparations of the enzyme, one of which proved to be exceptionally unstable, whereas the other was rather stable. Kinetic data indicate that the stable preparation contained a competitive inhibitor of xanthine oxidase, the presence of which could account for the stability differences between the two samples.

MATERIALS AND METHODS

Xanthine oxidase solutions. These were prepared by methods described previously (cf. Bergel & Bray, 1959). Most of the experiments were carried out on two preparations, namely LXO-70 and LXO-116. The former was purified by the method of Avis, Bergel & Bray (1955) to stage M5 and had an activity/ E_{450} ratio of about 40; the latter was prepared by ammonium sulphate treatment (Haddow, de Lamirande, Bergel, Bray & Gilbert, 1958) and had an activity/ E_{450} ratio which fell from 50 to about 10

during the period over which experiments were carried out. The samples both had E_{280}/E_{450} ratios of around 10. They were stored at 0–5° as solutions containing about 1% of xanthine oxidase (calculated from E_{450}). Copper analyses were carried out by the method of Eden & Green (1940).

Chemicals and buffer solutions. These were generally the same as those used previously (Bergel & Bray, 1959). Catalase was obtained from General Biochemicals Inc., Chagrin Falls, Ohio, U.S.A., and activity measurements indicated a purity of the order of 5%; soy-bean trypsin inhibitor was obtained from Worthington Biochemical Corp., Freehold, N.J., U.S.A. Pyrophosphate buffer for the enzyme assays was prepared by mixing $\text{Na}_4\text{P}_2\text{O}_7$ and $\text{Na}_2\text{H}_2\text{P}_2\text{O}_7$; m-phosphate buffer, pH 5.8, contained 0.2M- Na_2HPO_4 and 0.8M- KH_2PO_4 ; the other phosphate buffers were diluted from this solution.

Enzyme assays and stability experiments. The methods employed were similar to those of Bergel & Bray (1959). However, the xanthine oxidase assays, apart from those in connexion with the stability tests, were carried out at 305 m μ , as solutions of xanthine have a lower absorption at this wavelength than at the one normally used, i.e. 295 m μ . At 305 m μ the value of $\Delta\epsilon$ for the conversion of xanthine into uric acid is about half of the corresponding figure at 295 m μ (cf. Avis, Bergel & Bray, 1956). In some cases it was found convenient to carry out assays in 0.5 or 4.0 cm.

* Part 5: Bray, Malmström & Vännegård (1959).