respiratory particles prepared from the mosquito *Aedes aegypti* L.

2. Addition of ethylenediaminetetra-acetic acid to the isolation medium and of ethylenediamine-tetra-acetic acid and albumin either to the washing liquid or to the assay medium has been found obligatory for the exchange reaction.

3. The effect of nucleotides, respiratory inhibitors and inhibitors of oxidative phosphorylation on the exchange reaction and on the adenosine triphosphate activity has been investigated.

4. Quantitative differences exist between the response of insect and mammalian respiratory particles to 1:1:1-trichloro-2:2-di-(*p*-chlorophenyl)-ethane (DDT). 0.1 mM-DDT inhibits the exchange reaction by more than 50% in insect sarcosomes whereas the inhibition in mammalian liver mitochondria is less than 10%.

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The Biosynthesis of Porphyrins from Porphobilinogen by *Rhodopseudomonas spheroides*

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In a comprehensive review (van Niel, 1944) of the non-sulphur purple and brown bacteria or Athiorhodaceae there was described the production of a diffusible red pigment from growing cells. Some properties of this pigment were described, including some of its absorption characteristics and solubility in organic solvents. In the light of subsequent studies it seems clear that the pigment described was a porphyrin derivative. Since members of the Athiorhodaceae are photosynthetic and thus contain bacteriochlorophyll and, further, since they have been shown to be rich in haem derivatives (Elsden, 1954) they possess potent enzyme systems capable of synthesizing the porphyrin nucleus. The studies of Lascelles (1955, 1956) with whole-cell suspensions of *Rhodopseudomonas spheroides*, a typical member of the group of the Athiorhodaceae, have indeed shown that these organisms can bring about a synthesis of free porphyrins and bacteriochlorophyll from glycine together with certain intermediates of the tricarboxylic acid cycle.

Lascelles (1955, 1956) found that δ -aminolaevulinic acid was converted into porphyrins by cell suspensions of *R. spheroides* and during the initial stages of the reaction porphobilinogen diffused out of the cells. However, when porphobilinogen was added to washed cell suspensions of *R. spheroides* it was not converted into porphyrins, although it is a porphyrin precursor in other systems. (For a review of the interrelationships of δ -aminolaevulinic acid, porphobilinogen and porphyrins see Rimington, 1957.) Bogorad & Granick (1953) have shown that porphobilinogen is converted into porphyrins by suspensions of *Chlorella vulgaris* provided that they have been previously subjected to freezing and thawing. This suggests that porphobilinogen is not permeable to intact whole cells; a similar observation was made with avian erythrocytes (Dresel & Falk, 1956a).

The findings of Kikuchi, Shemin & Bachmann (1958) and of Gibson (1958), establishing the biosynthesis of δ -aminolaevulinic acid from glycine and

succinyl-coenzyme A, and the requirements for pyridoxal phosphate at this stage, in preparations of *R. spheroides*, further confirm the important role of δ -aminolaevulinic acid in porphyrin biosynthesis by this bacterium. δ -Aminolaevulinic acid dehydrase, which converts δ -aminolaevulinic acid into porphobilinogen, is widely distributed in animals, plants and micro-organisms (Gibson, Neuberger & Scott, 1955), and is presumably present in *R. spheroides*, accounting for the production of porphobilinogen by washed cell suspensions when incubated with δ -aminolaevulinic acid.

The object of this work was to study in more detail the steps involved in the biosynthesis of porphyrins from porphobilinogen. The formation of uroporphyrin from porphobilinogen has been studied with *Chlorella* (Bogorad & Granick, 1953), with spinach preparations by Bogorad (1955, 1957*a, b*) and with chicken-blood preparations (Dresel & Falk, 1956*a, b*; Lockwood & Rimington, 1957). Preliminary reports of these studies have appeared elsewhere (Heath & Hoare, 1958; Hoare & Heath, 1958*a, b*).

EXPERIMENTAL

Organism. The strain of *R. spheroides* used was obtained from Dr June Lascelles and came originally from the collection of Professor C. B. van Niel. It was maintained as described by Lascelles (1956).

Growth and harvesting of organism. The organism was grown on the 'medium S' described by Lascelles (1956) but 0.2% (w/v) of yeast extract was substituted for the pure vitamins. Cells were grown semi-anaerobically in the light in a 1 l. Roux bottle filled to the shoulder, plugged with cotton wool and illuminated by a single 40W tungsten lamp at a distance of 6–9 in.; this maintained the culture at a temperature of 30° ($\pm 2^\circ$). The cells were harvested towards the end of the logarithmic phase of growth (usually 72 hr.) by centrifuging at 12 000 g at 1° for 10 min. The packed cells were washed once with 0.9% NaCl soln. The yield was 8–9 g. wet wt. (equivalent to 1.4–1.6 g. dry wt.). The packed cells were stored at -15° unless used directly.

Growth of cells aerobically in the dark. The same medium was used as in the semi-anaerobic, light-grown cultures; 100 ml. of medium contained in 500 ml. Erlenmeyer flasks was inoculated with a cell suspension, the flasks were covered with dark paper and were clamped to a rotary shaker at room temperature (20°). The cells were harvested after reaching maximum growth in 3 or 4 days.

Buffer solutions. McIlvaine's citric acid- Na_2HPO_4 buffers, made up in de-ionized water, were used to cover the range pH 3.0–8.0 (*Documenta Geigy Scientific Tables*, 1956). Porphobilinogen (PBG), uroporphyrin I and III and coproporphyrin I and III were generously provided by Professor C. Rimington, F.R.S. Solutions were made up in a 1:10 (v/v) dilution of buffer, pH 6.5, and were stored at -15° .

Treatment of cells for enzyme experiments. (a) Frozen and thawed cell preparations. Cells were suspended in buffer

(250 mg. wet wt. of cells/ml., equivalent to 45 mg. dry wt./ml.) and were completely frozen by immersion in a glycol bath at -15° . The cell suspensions were allowed to thaw at room temperature. This procedure was repeated twice.

(b) Preparation of cell-free extracts by grinding with alumina. The method used was that described by McIlvaine (1948). Frozen packed cells (2.5 g.) were mixed with an equal weight of Microid Polishing Alumina, grade 3/50 (Griffin and George Ltd., London) in a glass mortar. All materials were previously cooled to -15° . Grinding was continued with small additions of cooled buffer at pH 6.5 until a sticky paste was obtained. A total of 10 ml. of buffer was finally added and the suspension was centrifuged at 12 000 g for 15 min. at 1°. This produced a clear dark-brown supernatant which was removed with a Pasteur pipette.

(c) Preparation of acetone-dried powders. The packed cells were suspended in water and a large excess (at least 10 vol.) of acetone, cooled to -15° , was added. After standing for 30 min. at -15° the precipitate which had formed was filtered off, washed with cold acetone and dried in a vacuum desiccator over silica gel. This produced a pale grey-green powder which was stored at -15° .

Incubation conditions. Reaction mixtures of total volume 0.5 ml., comprising 0.4 ml. of the test preparation in buffer and 0.1 ml. of PBG soln. (1 mg. of the free base monohydrate/ml.), were incubated at 37° in glass centrifuge tubes (10 cm. $\times$ 1 cm.), plugged with cotton wool.

Estimation of porphyrins. The reaction was stopped by adding 5 ml. of ethyl acetate-acetic acid mixture (3:1, v/v) and the mixture was left for 1 hr. in the dark. After centrifuging, the clear supernatant was decanted into a 60 ml. separating funnel, the residue was resuspended in a further 5 ml. of ethyl acetate-acetic acid and, after centrifuging, the two supernatants were combined. Uroporphyrin was then extracted with 3 $\times$ 5 ml. of a saturated solution of sodium acetate followed by 5 ml. of 3% (w/v) sodium acetate soln. Each extract was re-extracted in a second separating funnel with 10 ml. of ethyl acetate. When coproporphyrin was formed some was extracted into sodium acetate together with uroporphyrin, but the second ethyl acetate extraction retained the coproporphyrin. Uroporphyrin was extracted from the combined sodium acetate extracts by the cyclohexanone procedure (Dresel & Falk, 1956*a*) and was estimated in 5% (w/v) HCl. The combined ethyl acetate washings and the original ethyl acetate-acetic acid solutions were combined and extracted with 3 $\times$ 5 ml. of 15% (w/v) HCl. The combined acid extracts were adjusted to pH 4 with solid sodium acetate and the coproporphyrin was extracted into 20 ml. of ether. The aqueous phase was extracted in a second funnel with a further 20 ml. of ether. The two ether solutions were then washed in succession with saturated sodium acetate, 3% sodium acetate and water. The combined ether phases were then extracted to completion with 10% (w/v) HCl. Porphyrin concentrations in the HCl solutions were determined at the Soret maxima in a Unicam SP. 500 spectrophotometer with the correction factors of Rimington & Sveinsson (1950).

Estimation of porphobilinogen. The reaction was stopped by adding 1.5 ml. of 6.7% (w/v) trichloroacetic acid. After centrifuging, suitable portions of the clear supernatant were diluted to 2 ml. with 5% (w/v) trichloroacetic acid

and 2 ml. of a modified Ehrlich reagent (15 g. of *p*-dimethylaminobenzaldehyde dissolved in 64 ml. of 10*N*-HCl and 195 ml. of acetic acid) was added. The maximum extinction at 555 $m\mu$, usually attained in 1–5 min., was determined in a Unicam spectrophotometer. The amount of PBG present was calculated from a standard curve. ($E_{555}^{1\text{ cm.}}/0.114 = \mu\text{g.}$ of PBG monohydrate/ml. of 5% trichloroacetic acid test solution).

Chromatography of porphyrins. Identification of porphyrins and porphyrin isomers was carried out by the method of Falk & Benson (1953) and of Falk, Dresel, Benson & Knight (1956).

Separation of uroporphyrin I- and uroporphyrin III-forming enzymes. Cells grown semi-anaerobically in the light were harvested by centrifuging and washed once with 0.9% NaCl soln. The packed cells (6 g. wet wt.) were suspended in 10 ml. of water and frozen in a solid CO₂-acetone bath for 10 min. and then thawed at 15°; this procedure was twice repeated and, after centrifuging for 10 min. at 12 000 *g*, the supernatant was removed. The cell residue was resuspended in water (10 ml.) and the whole procedure was repeated a further three times. The four supernatant fractions (total volume 40 ml.) thus obtained were combined, cooled to 1° and acetone (400 ml.), previously cooled to –15°, was added. After standing at –15° for 30 min. the precipitate (110 mg.), which contained the enzyme(s) converting PBG into uroporphyrin I, was collected by centrifuging.

The final cell residue was suspended in water (10 ml.) and treated with cold acetone (200 ml.) to yield a green powder (240 mg.) containing the enzyme(s) converting PBG into uroporphyrin III.

RESULTS

Whole-cell suspensions. Cells harvested from cultures grown semi-anaerobically in the light, and washed and suspended in buffer to a concentration of 12 mg. dry wt./0.5 ml., on incubation at pH 6.2, 6.6, 6.8, 7.0 and 7.2 for 16 hr. at 37° with PBG (50 $\mu\text{g.}$) formed less than 2 $\mu\text{g.}$ of uroporphyrin and no coproporphyrin. The small amounts of uroporphyrin formed were of the same order as that formed non-enzymically.

Frozen and thawed cell suspensions. After cell suspensions in buffer had been frozen and thawed three times successively, considerable amounts of porphyrins were formed on incubation with PBG for 16 hr. at 37°. At pH 6.1–6.8 the total porphyrin formed accounted for about 50% of the PBG added. The effect of pH on porphyrin formation under these conditions is shown in Table 1. The uroporphyrin figures are not corrected for the non-enzymic conversion of PBG into uroporphyrin (Cookson & Rimington, 1954). Since maximum coproporphyrin formation occurred at pH 6.5 under the above conditions, this pH was used for the further study of porphyrin formation from PBG.

Time course of the reaction with frozen and thawed cell suspensions. Washed cells equivalent to 18 mg. dry wt. were suspended in 0.4 ml. of buffer,

pH 6.5, and the suspension was frozen and thawed three times and incubated at 37° with 0.1 ml. of PBG soln. (100 $\mu\text{g.}$). Tubes were set up in duplicate for each incubation time, one tube for the estimation of porphyrins and the other for PBG. The course of the reaction is illustrated in Fig. 1. Such preparations formed virtually only coproporphyrin; the small amounts of uroporphyrin could have been formed non-enzymically. Coproporphyrin production reached a maximum after incubation for 4 hr. Disappearance of PBG was very rapid, and was complete after 6 hr.; only half could be accounted for as porphyrin. Controls in which PBG solutions were incubated in buffer for different times without enzyme showed appreciable disappearance of Ehrlich-reacting material with only very slight formation of uroporphyrin.

Table 1. *Effect of pH on porphyrin formation by frozen and thawed suspensions of cells incubated with porphobilinogen*

Cells (25 mg. dry wt./tube) in 0.4 ml. of buffer with 0.1 ml. of PBG soln. (100 $\mu\text{g.}$). Incubation: 16 hr. at 37°.

pH	Porphyrin ($\mu\text{g.}$)		
	Uro-	Copro-	Total
4.2	1	2	3
4.6	1	7	8
5.2	3	15	18
5.6	5	22	27
6.1	4	34	38
6.3	5	34	39
6.5	2	36	38
6.8	6	33	39
7.2	3	34	37
7.5	10	31	41
8.0	11	32	43

Fig. 1. Rate of formation of uroporphyrin (O) and coproporphyrin (●) and disappearance of PBG (□). Frozen and thawed cell suspensions (18 mg. dry wt./0.5 ml.) were incubated at 37°, pH 6.5, with PBG (100 $\mu\text{g.}$).

Fig. 2. Effect of cell concentration on the formation of uroporphyrin (○) and coproporphyrin (●) and on the disappearance of PBG (□). Frozen and thawed cell suspensions were incubated for 4 hr. at 37°, pH 6.5, with PBG (100 μg.).

Table 2. Recovery of added porphyrins after incubation for 4 hr. either in buffer, pH 6.5, or in the presence of frozen and thawed cell suspensions (18 mg. dry wt./tube)

Porphyrin added	Wt. (μg.)	Porphyrin recovered (μg.)			
		Buffer		Cell suspension	
		Copro-	Uro-	Copro-	Uro-
Uro- I	44	0	36	0	30
Uro- III	46	0	39	0	33
Copro- I	45	38	0	37	0
Copro- III	44	39	0	38	0

Table 3. Separation of the frozen and thawed cell porphyrin-forming systems

Cells (45 mg. dry wt./ml.) were frozen and thawed three times and separated by centrifuging into supernatant and residual cells. Incubation: 16 hr. at 37° with 100 μg. of PBG.

	Porphyrin (μg.)		
	Copro-	Uro-	Total
Whole cells	38	7	45
Supernatant	2	29	31
Residual cells	22	13	35
Supernatant plus residual cells	41	6	47

Frozen cell suspensions in 0.9 % NaCl soln., kept at -15° for 2 weeks, showed no loss of enzyme activity when assayed for 16 hr.; 39 μg. of total porphyrin (36 μg. of coproporphyrin and 3 μg. of uroporphyrin) was formed from 100 μg. of PBG. After storage for 2 months the total porphyrin formed was 31 μg. (18 μg. of coproporphyrin and 13 μg. of uroporphyrin).

Over an incubation period of 4 hr. and a range of cell concentrations from 2 to 20 mg. dry wt./tube, the amounts of porphyrin formed and of PBG used were proportional to the concentration of cells (Fig. 2).

Coproporphyrin was not formed when uroporphyrin I or uroporphyrin III was incubated with frozen and thawed cell suspensions. Control tubes in which uroporphyrins I and III and coproporphyrins I and III were similarly incubated in buffer alone showed recoveries of 80–90 % (Table 2).

Separation of frozen and thawed cell suspensions into enzyme systems forming uroporphyrins I and III respectively. When a frozen and thawed cell suspension was centrifuged, it was found that the clear supernatant formed uroporphyrin from PBG. The residual cells, on resuspending in buffer, formed less coproporphyrin and more uroporphyrin than the original frozen and thawed preparation. However, when the residual cells were resuspended in the supernatant, coproporphyrin production was comparable with that found with the original frozen and thawed cell suspension (Table 3). If the freezing and thawing procedure and subsequent separation was repeated several times, the ability of the cell residue to synthesize coproporphyrin progressively diminished and at the same time the formation of uroporphyrin correspondingly increased (Table 4). The total amount of porphyrin formed remained fairly constant. The supernatant, however, formed very little coproporphyrin from PBG. Reconstitution of the above system by adding a boiled supernatant to the extracted cell residue gave an increase in coproporphyrin production and a decrease in uroporphyrin (Table 5). The boiled supernatant alone formed only small amounts of uroporphyrin non-enzymically. The uroporphyrins and coproporphyrins isolated from these fractions were examined chromatographically for isomer type. The frozen and thawed cells formed mainly coproporphyrin III with traces of coproporphyrin I, the uroporphyrin formed by the cell residue after removal of the supernatant was mainly type III, whereas the supernatant itself formed uroporphyrin type I.

Cell-free extracts. Cell-free extracts were prepared by grinding with alumina and extracting in buffer. The resulting clear-yellow-brown extracts converted added PBG into both uroporphyrin and coproporphyrin; the total and the relative amounts of the porphyrins depended on both the time of incubation and the enzyme concentration. The course of the reaction is shown (Fig. 3) for a concentration of extract equivalent to 18 mg. dry wt. of whole cells; the system is thus comparable with that of the frozen and thawed cells illustrated in

Table 4. *Effect of repeated extraction of cells by freezing and thawing in buffer*

A volume (30 ml.) of cell suspension (45 mg. dry wt./ml.) in buffer, pH 6.5, was frozen and thawed three times and 0.4 ml. (equivalent to 18 mg. dry wt.) was used for assay of 'original cells'; cell suspension was centrifuged and 0.4 ml. of supernatant assayed. Cell residue was resuspended in buffer to a cell concentration of 45 mg. dry wt./ml. and again frozen and thawed three times; 0.4 ml. of suspension was used for assay of 'first-treatment' cells; cell suspension was centrifuged and 0.4 ml. of the corresponding supernatant assayed. The above process was repeated on the cell residue for the second and third treatments. Incubations: 16 hr. at 37°, pH 6.5, with 100 μ g. of PBG.

	Porphyrin (μ g.)					
	Cells			Supernatant		
	Copro-	Uro-	Total	Copro-	Uro-	Total
Original cells	37	3	40	2	20	22
First treatment	27	6	33	0	15	15
Second treatment	13	25	38	1	30	31
Third treatment	6	38	44	2	21	23

Table 5. *Porphyrin formation by frozen and thawed cell suspensions partially reconstituted with boiled supernatant*

Cell suspension in water (45 mg. dry wt./ml.) was frozen and thawed three times and centrifuged; cell residue and supernatant (equivalent to 18 mg. dry wt. of original cells) were incubated for 16 hr. at 37°, pH 6.5, with 100 μ g. of PBG.

Enzyme system	Porphyrin formed (μ g.)		
	Uro-	Copro-	Total
Cell residue	11	27	38
Boiled supernatant	9	0	9
Residue + boiled supernatant	5	35	40
Residue + supernatant	5	38	43

Fig. 1. By contrast, the cell-free extracts formed more uroporphyrin initially, reaching a maximum in 2 hr. After incubation for 16 hr. at 37°, pH 6.5, formation of coproporphyrin was approximately proportional to the concentration of cell-free extract. Uroporphyrin formation, however, deviated considerably from such proportionality: at high enzyme concentrations more coproporphyrin than uroporphyrin was formed; the reverse was true at low concentrations (Table 6). Magnesium ions (mm) had no effect on the nature or quantity of porphyrins formed. Chromatographic examination of the reaction products showed the 'uroporphyrin' fraction to consist of a mixture of the I- and III-series isomers, together with small amounts of penta-, hexa- or hepta-carboxylic porphyrin; such intermediate carboxylic porphyrins were also observed in the other systems.

Compared with the frozen and thawed cell preparations, the cell-free extracts were relatively unstable. A fresh preparation formed 13 μ g. of coproporphyrin and 24 μ g. of uroporphyrin on incubation for 2 hr. with 100 μ g. of PBG, and after storage for 3 days at -15° it then formed only 5 μ g. of coproporphyrin and 11 μ g. of uroporphyrin.

Fig. 3. Rate of formation of uroporphyrin (○) and coproporphyrin (●) and disappearance of PBG (□). Cell-free extract of *R. spheroides* (0.4 ml.; 2.9 mg. dry wt., equivalent to 18 mg. dry wt. of whole cells) was incubated at 37°, pH 6.5, with 0.1 ml. (100 μ g.) of PBG soln.

Table 6. *Porphyrin formation by cell-free extracts of Rhodopseudomonas spheroides: effect of enzyme concentration*

Reaction mixture (total volume 0.5 ml.) contained cell-free extract at pH 6.5 and 100 μ g. of PBG and was incubated for 16 hr. at 37°.

Concn. of enzyme (mg. dry wt./tube)	Porphyrin formed (μ g.)		
	Uro-	Copro-	Total
2.4	11	23	34
0.6	37	6	43
0.24	27	2	29
0.06	11	0	11

Enzymic activity of acetone-dried powders. Acetone-dried powders of semi-anaerobic, light-grown cells appeared to be more active than either frozen and thawed cell preparations or cell-free extracts in

Table 7. Porphyrin formation by acetone-dried powders of (a) semi-anaerobic, light-grown and (b) aerobic, dark-grown cells of *Rhodopseudomonas spheroides*: effect of enzyme concentration

Reaction mixture (total volume 0.5 ml.) contained the acetone-dried powder in buffer, pH 6.5, and 100 µg. of PBG and was incubated for 4 hr. at 37°.

Acetone-dried powder (mg. dry wt./tube)	Porphyrins formed (µg.)		
	Uro-	Copro-	Total
	(a)		
18.0	4	30	34
4.5	33	4	37
1.8	24	1	25
	(b)		
18.0	18	18	36
9.0	29	8	37
4.5	24	2	26
1.8	7	0	7

Fig. 4. Rate of formation of uroporphyrin (O) and coproporphyrin (●) and disappearance of PBG (□). Acetone-dried powder of *R. spheroides* (18 mg.) in 0.4 ml. of buffer, pH 6.5, was incubated at 37° with 0.1 ml. (80 µg.) of PBG soln.

forming porphyrins from PBG. As with cell-free extracts, the nature and relative proportions of the porphyrins produced depended on the enzyme concentration as well as on the time of incubation. Table 7 shows that over an incubation period of 4 hr. coproporphyrin production exceeds uroporphyrin production with high concentrations of enzymes, but uroporphyrin predominates if low concentrations are employed. The time course for the reaction is illustrated in Fig. 4. The pH optimum for coproporphyrin formation over a short incubation period (75 min.) was 7.0 (Fig. 5).

Fig. 5. Effect of pH on coproporphyrin formation from PBG. Acetone-dried powder (20 mg.) in 0.4 ml. of buffer was incubated for 75 min. at 37° with 0.1 ml. (100 µg.) of PBG soln. ●, McIlvaine citric acid-Na₂HPO₄ buffer; O, borate buffer.

Fig. 6. Rate of formation of uroporphyrin (O) and coproporphyrin (●) and disappearance of PBG (□). Acetone-dried powder of *R. spheroides* grown aerobically in the dark: 18 mg. of powder in 0.4 ml. of buffer, pH 6.5, was incubated at 37° with 0.1 ml. (90 µg.) of PBG soln.

Added uroporphyrins I and III were not decarboxylated by acetone-dried powders even after incubation for 16 hr. at pH 6.5 and 37°.

Acetone-dried powders of aerobic, dark-grown cells also formed porphyrins but were less active (Table 7). A similar effect of enzyme concentration and time of incubation was observed on the relative quantities of uroporphyrin and coproporphyrin formed (Fig. 6). The uroporphyrin formed was the III-series isomer.

Preparation of enzymes converting porphobilinogen into uroporphyrin I and uroporphyrin III respectively. After repeated extraction by freezing and thawing, acetone-dried powders of the extracted cell residue and of the combined supernatants were prepared as described in the Experimental section. The cell-residue fraction converted PBG into uroporphyrin III, whereas the acetone precipitate of the combined supernatants formed uroporphyrin I. A quantity (5 mg.) of the cell-residue acetone-dried powder incubated with PBG (100 μ g.) formed 38 μ g. of uroporphyrin III in 16 hr., 5 mg. of the acetone-precipitated supernatant fraction formed 33 μ g. of uroporphyrin I in 16 hr. Acetone-dried powders of both whole cells and extracted cells after 30 min. preheating at 60–65° converted PBG into uroporphyrin I. Preheated preparation (5 mg. of acetone-dried powder of whole cells) formed 27 μ g. of uroporphyrin I after incubation with PBG for 16 hr.

DISCUSSION

The fact that PBG gives rise to porphyrins on incubation with frozen and thawed cell suspensions, but not with washed cell suspensions of *R. spheroides*, could be due to the failure of PBG to enter the intact bacterial cell. The passage of PBG through the intact cell wall appears to take place in one direction only, since PBG diffuses out of intact cells when incubated with δ -aminolaevulinic acid (Lascelles, 1956). Frozen and thawed cells differ from both cell-free extracts and acetone-dried cells in forming very little uroporphyrin at any stage. This is attributed to the fact that even in the frozen and thawed cell, the rate of penetration of PBG to the enzymes within the cell may still be the limiting step in the overall process of converting PBG into coproporphyrin. The studies on cell-free extracts and acetone-dried powders indicate that uroporphyrin formation proceeds more rapidly than coproporphyrin formation, and so some step preceding uroporphyrin formation must be rate-limiting in frozen and thawed cells, otherwise larger amounts of uroporphyrin would accumulate. The small amounts of uroporphyrin formed by frozen and thawed cells could arise non-enzymically from PBG. The fact that acetone-

dried powders are more active than frozen and thawed cells on a dry-weight basis is further evidence that the rate of access of PBG to the intracellular enzymes is limiting in the latter system.

The observation that cell-free extracts on dilution form more uroporphyrin than coproporphyrin could be due to the fact that uroporphyrin-forming activity is much greater than coproporphyrin activity in the whole micro-organism; alternatively the uroporphyrin-forming enzyme may be more readily extracted into solution after grinding with alumina. A further possibility is that coproporphyrin-forming enzyme is partially destroyed by the alumina-grinding and extraction procedure. Certainly the uroporphyrin-forming enzyme system is the more stable, since cell-free extracts on storage rapidly lost their ability to form coproporphyrin without a comparable loss of ability to form uroporphyrin.

The time course for the reaction of PBG with acetone-dried powders suggested that uroporphyrin may be a direct precursor of coproporphyrin. Since uroporphyrins I and III were not converted into coproporphyrin by such preparations, even after incubation for 16 hr., this possibility is ruled out. Some preliminary investigations have demonstrated that uroporphyrinogens I and III are converted in high yield into coproporphyrins I and III respectively by acetone-dried powders of *R. spheroides* (Hoare & Heath, 1958*a*). Bogorad (1957*b*) has demonstrated the formation of porphyrins with less than eight carboxyl groups by *Chlorella* after incubation with uroporphyrinogen III. Bashour (1956) has reported the conversion of uroporphyrinogen I into coproporphyrin and protoporphyrin by dog-liver homogenates.

The exact relationship between the uroporphyrin I- and uroporphyrin III-forming enzyme systems still remains obscure. Investigations by Bogorad & Granick (1953) on *Chlorella* and by Booiij & Rimington (1957) and Lockwood & Rimington (1957) on chick haemolysates have shown that enzyme preparations forming uroporphyrin III are heat-labile and, after heating to 50–65° for short periods of time, then form uroporphyrin I from PBG. Recently Granick & Mauzerall (1958) and Mauzerall & Granick (1958) have prepared from rabbit reticulocytes by starch electrophoresis a 'porphobilinogenase' which converted PBG into uroporphyrinogen III, but after preheating it formed uroporphyrinogen I. We have confirmed these findings with *R. spheroides*. The further observation that frozen and thawed cell suspensions of *R. spheroides* were separated by centrifuging into a supernatant fraction forming uroporphyrin I and a residue forming mainly uroporphyrin III indicates that two enzyme systems are involved. These may be analogous to the 'porphobilinogen

deaminase' and 'uroporphyrinogen isomerase' described by Bogorad (1957*a*), in which case it is assumed that 'porphobilinogen deaminase' is partially extracted into solution by freezing and thawing whereas the isomerase together with some of the deaminase remains with the cell residue. It is not considered likely that the uroporphyrin I-forming enzyme in the supernatant fraction of frozen cells is derived from a uroporphyrin III-forming enzyme by modification of the latter as a result of freezing and thawing. The residual cells retain a very active uroporphyrin III-forming enzyme after ten or more successive extractions by freezing and thawing. The uroporphyrin I-forming enzyme concentration in the supernatant is very low, since very little protein was extracted and only small amounts of uroporphyrin I were formed from PBG after short incubation periods.

Further, when repeatedly extracted cells which formed uroporphyrin from PBG were preheated at 60° for 30 min. uroporphyrin I was formed from PBG. The resolution of frozen and thawed cells into enzyme systems forming uroporphyrins I and III could be attributed to a preferential extraction of PBG deaminase forming uroporphyrin I, the residual cells still containing some PBG deaminase but with a more active 'isomerase' so that uroporphyrin III is formed from PBG. Bogorad (1957*a, b*; 1958*a, b*) has effected a partial purification of PBG deaminase and of uroporphyrinogen isomerase, and it seems clear that uroporphyrin III is formed by the combined action of the two enzymes PBG deaminase and uroporphyrinogen isomerase, the latter having no action on PBG alone. Previously Bogorad & Granick (1953) and Shemin, Russell & Abramsky (1955) postulated a reaction scheme for the formation of uroporphyrin III which involved the formation of branched tripyrromethanes. Several objections to this scheme were raised, on purely chemical grounds, by Bullock, Johnson, Markham & Shaw (1958). These authors have put forward a reaction mechanism, based on a suggestion of Robinson (1955), which involves carbonium ions in an intramolecular process. Whether such a mechanism is involved in the enzymic synthesis of uroporphyrin III is a matter to be investigated.

The separation of a frozen and thawed cell suspension into a cell residue and supernatant fraction also demonstrates that the formation of coproporphyrin from PBG, via the decarboxylation of a uroporphyrinogen, requires the presence of the supernatant solution, since the thoroughly extracted cell residue converts PBG only into uroporphyrin. The partial restoration of coproporphyrin-forming activity by the addition of a boiled supernatant to the extracted residue suggests that a heat-stable cofactor may be required for the

decarboxylation step. We have shown that both uroporphyrinogens I and III are precursors of coproporphyrins I and III in *Rhodospseudomonas spheroides*. Mauzerall & Granick (1958) have investigated the substrate specificity of a partially purified uroporphyrinogen decarboxylase from reticulocytes and have shown that all four isomers can be decarboxylated. Bogorad (1958*a-c*) has also investigated the enzymic synthesis of porphyrins from porphobilinogen and has studied coproporphyrin formation from uroporphyrinogen.

SUMMARY

1. Frozen and thawed cells of *Rhodospseudomonas spheroides*, but not whole cells, convert porphobilinogen into uroporphyrin and coproporphyrin.
2. Acetone-dried powders of whole cells are stable when stored at -15° and are the most active source of a coproporphyrin III-forming system.
3. The presence of a heat-stable cofactor necessary for coproporphyrin formation has been demonstrated.
4. A uroporphyrin I-forming system has been separated, by freezing and thawing, from a uroporphyrin III-forming system without the destruction of the latter. Stable acetone-dried powders of these two systems have been prepared.
5. The effects of change of pH, time of incubation and concentration of enzymes have been studied both with frozen and thawed cells and acetone-dried powders.
6. Neither uroporphyrin I nor uroporphyrin III is converted into coproporphyrin, either by frozen and thawed cells or by acetone-dried powders.
7. Cell-free extracts have been made by grinding with alumina. These extracts have weak and unstable coproporphyrin-forming activity.

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The Effect of Monoiodoacetate on the Aerobic Metabolism of Ox Retina *in vitro*

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Noell (1951) and Schubert & Bornschein (1951) have shown that, in a number of species, injection of sublethal doses of monoiodoacetate abolishes the retinogram (the change in potential across the retina caused by light) and destroys the visual cells. Noell found that the changes in the retinogram differed from those caused by anoxia and he suggested that the damage was due to inhibition of glycolytic reactions and that oxidative processes were unaffected. This suggestion is supported by the experiments of Graymore & Tansley (1959), in which the anaerobic glycolysis of rat retina fell to 20% of the normal value within 10 min. of injection of iodoacetate, and by Oguchi's (1938) report that respiration of rabbit and frog retina was not inhibited after injection of iodoacetate. Noell (1958) has also reported experiments which confirm Oguchi's finding, although Auricchio (1950) found that addition of iodoacetate *in vitro* did depress oxygen uptake of rabbit retina.

The lack of effect of iodoacetate on oxygen uptake of retina cannot be regarded as established, for respiration in this tissue is dependent on the composition of the incubation medium and is much faster in a bicarbonate than in a phosphate buffer (Laser, 1937; Craig & Beecher, 1943a). Krebs-Ringer phosphate solution was used for the experiments reported by Noell (1958), but Oguchi (1938)

does not state the composition of the Ringer solution which he used. Auricchio (1950) used a Ringer phosphate solution but with air as the gas phase and so introduced some carbon dioxide into his system. Reports of the effect of iodoacetate on oxygen uptake of other tissues are conflicting. Lundsgaard (1930), Stannard (1937) and Saslow (1937) found that iodoacetate had no effect on carbohydrate oxidation in frog muscle, whereas Meyerhof & Boyland (1931), who also used frog muscle, and Krebs (1931) and Quastel & Wheatley (1932), working with brain and other tissues from a variety of species, observed a decrease either in oxygen uptake or in respiratory quotient. Barker, Shorr & Malam (1939) found that the effect varied with the tissue used (cat and dog); with testis oxygen uptake was always inhibited to a greater extent than glycolysis, but with heart and brain, at concentrations of iodoacetate which inhibited anaerobic glycolysis nearly completely, the respiratory quotient was not depressed and glucose was oxidized, although in a number of experiments the amount of oxygen used was reduced.

In the experiments described below iodoacetate has been added to ox retina incubated in bicarbonate and in phosphate buffer. In bicarbonate there was a pronounced inhibitory effect but in phosphate inhibition was small and only just significant.