The Synthesis of Porphyrins and Bacteriochlorophyll by Cell Suspensions of *Rhodospseudomonas spheroides*

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Free porphyrins are formed by growing cultures of some bacteria and yeasts under conditions in which formation of cytochromes is limited. With *Corynebacterium diphtheriae*, production of both porphyrin and toxin is dependent upon the concentration of iron salts in the medium; increasing this beyond a critical point decreases both porphyrin and toxin production (Pappenheimer, 1947). Cells grown with iron at concentrations sufficiently high to prevent accumulation of porphyrin contain considerably more cytochrome than those grown under conditions favouring porphyrin formation (Pappenheimer & Hendee, 1947; Rawlinson & Hale, 1949). *Bacillus cereus* excretes porphyrin (probably coproporphyrin) into the media when grown anaerobically, but only traces of porphyrin occur in aerobic cultures; the concentration of haem compounds in the cells grown anaerobically is considerably smaller than in those from aerobic cultures (Schaeffer, 1952*a, b*). There have been many observations of porphyrin formation by yeasts (see Lemberg & Legge, 1949). Stich & Eisgruber (1951) found that the amount of coproporphyrin Type I in cells of *Saccharomyces anomensis* is increased 5- to 10-fold by addition of pantothenate to the growth medium, while addition of riboflavin completely suppresses porphyrin formation. Cells grown under conditions which favour porphyrin formation contain little or no haem derivatives, whereas those grown with riboflavin and iron are rich in these substances.

Porphyrins may also be precursors of chlorophyll. Induced mutant strains of *Chlorella*, which are unable to synthesize chlorophyll, form large quantities of protoporphyrin, as well as magnesium protoporphyrin and magnesium vinyl pheophytin a_5 , when grown in the dark on a glucose-salts medium (Granick, 1948*a, b*, 1950). Another mutant produces a number of porphyrins containing up to eight carboxyl groups in each molecule, those with two such groups being predominant (Bogorad & Granick, 1953*a*; Granick, Bogorad & Jaffe, 1953).

These observations of porphyrin formation by micro-organisms have been made with growing

cultures and there is little information concerning the substrates used in the synthesis. Most of the present knowledge concerning formation of porphyrin and haem has come from experiments with red blood cells or haemolysates; isotopic techniques have shown that the initial stage of porphyrin synthesis is probably the condensation of glycine (or a derivative) with an unsymmetrical C_4 -compound derived from α -oxoglutarate resulting from utilization of acetate by the tricarboxylic acid cycle. Such work has been reviewed by Shemin (1955) and Granick (1954). Experiments with isotopically labelled δ -aminolaevulinic acid (ALA) give strong evidence that it is a product of the condensation (Shemin & Russell, 1953; Neuberger & Scott, 1953). This compound is converted into porphyrins by haemolysates and cell-free extracts of avian erythrocytes (Dresel & Falk, 1953; Shemin, Abramsky & Russell, 1954). The monopyrrole, porphobilinogen (PBG), is probably an intermediate in this reaction. Falk, Dresel & Rimington (1953) found it to be converted into porphyrin by chick haemolysates; it is also formed together with porphyrins when the haemolysates are incubated with ALA (Dresel & Falk, 1953). An enzyme, δ -aminolaevulinic acid dehydrase, which catalyses the conversion of ALA into PBG, is widely distributed in animal tissues and is present in some bacteria (Gibson, Neuberger & Scott, 1954; Gibson, 1955; Schmid & Shemin, 1955).

It seems likely that there are similar pathways in micro-organisms. [^{15}N]Glycine is incorporated specifically into the free porphyrins and intracellular haems of *C. diphtheriae* (Hale *et al.* 1950). Both glycine and acetate have been shown by isotopic techniques to be precursors of chlorophyll in the alga, *Chlorella vulgaris* (Della Rosa, Altman & Salomon, 1953). Recently Bogorad & Granick (1953*b*) have described the formation of porphyrins from PBG by cell preparations of *Chlorella*.

The aim of the present work was to study some of the factors affecting formation of free porphyrin by micro-organisms from glycine and α -oxoglutarate and from ALA. Photosynthetic bacteria were chosen since they have potent systems for forming the porphyrin nucleus; not only do they form bacteriochlorophyll but they are also rich in haem

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derivatives (Elsden, Kamen & Vernon, 1953; Vernon & Kamen, 1954; Elsdén, 1954).

The work has been carried out with *Rhodospseudomonas spheroides*, a member of the group *Athiorhodaceae*. The physiology and metabolism of these organisms has been described in detail by van Niel (1941, 1944, 1954), Gest (1951) and Elsdén (1954). Earlier experiments showed that cultures of *Rps. spheroides* contain considerable amounts of free porphyrin when grown in the light on media deficient in iron salts (Lascelles, 1955).

EXPERIMENTAL

Organism

The strain of *Rps. spheroides* used was obtained from Dr S. R. Elsdén and came originally from the collection of Professor C. B. van Niel. It was maintained in stab culture in medium *S* (see below) supplemented with Difco yeast extract (0.2%, w/v) and agar (1.5%, w/v). These were subcultured monthly and incubated for 40 hr. in the light at 32–34°; they were stored at 2°.

Medium

Cells for experiments with cell suspensions were harvested from the chemically defined medium *S*, which contained per litre final vol.: sodium L-glutamate monohydrate, 3.8 g.; DL-malic acid, 2.7 g.; KH_2PO_4 and K_2HPO_4 , 500 mg. each; $(\text{NH}_4)_2\text{HPO}_4$, 800 mg.; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 200 mg.; CaCl_2 , 40 mg.; nicotinic acid and thiamine hydrochloride, 1 mg. each; biotin, 10 μg . The pH was adjusted to 6.8 with *N*-NaOH. The medium was sterilized by autoclaving for 10 min. at 10 lb./sq.in.

Growth of organism

Light-grown cultures. Unless otherwise stated, experiments were with cells which had been grown in medium *S* in the light under semi-anaerobic conditions. The cultures were grown in 8 oz. medical flat bottles filled to the shoulder (200 ml. medium) and plugged with cotton wool. Each bottle was sown with 0.8 ml. (about 10^8 cells) of a 24 hr. culture grown in the light in medium *S* plus 0.2% (w/v) Difco yeast extract; this was derived in turn from a 40 hr. light-grown stab culture in the same medium plus 1.5% (w/v) agar. Incubation in the light was carried out in a cupboard illuminated with banks of tungsten lamps (40–60 w); the temperature was maintained between 32 and 34° by a fan and by adjustment of air vents. The intensity of light falling on the cultures was between 200 and 250 f.c.

The bottles were incubated until the end of the logarithmic phase of growth (usually 24 hr.); the yield of cells was about 1 mg. dry wt./ml. culture. Cells obtained from cultures grown anaerobically in completely filled stoppered bottles behaved similarly to those grown under the semi-anaerobic conditions normally used.

Aerobic growth in the dark. Aerobic cultures (50 ml.) were grown in tubes shaped like an inverted T, similar to those described by Monod, Cohen-Bazire & Cohn (1951), except that the horizontal limb was of 3.5 cm. external diameter and 19 cm. long. After inoculation with 0.2 ml. of a light-

grown culture developed as described above, the vessels were clamped to a rocking device (see van Heyningen & Gladstone, 1953) and shaken (36 oscillations/min.) in a water bath at 34° for 24 hr. The yield of cells was about 1 mg. dry wt./ml.

Continuous aeration by shaking was required and only very slight growth occurred in unshaken Roux bottles. In early experiments the cultures were protected from the light by covering the tubes with black cloth. This was later found to be unnecessary as the light intensity under laboratory conditions was not sufficient for photo-synthetic growth.

Procedures for the study of porphyrin synthesis

Harvesting of cultures. The cells were centrifuged, washed in half the original culture volume of 0.85% (w/v) NaCl and finally suspended in water to give 6–10 mg. dry wt./ml. The dry weight was determined by heating the samples to constant weight at 90–100°.

Synthesis from glycine. The washed cells were suspended at a final concentration of 0.6–1.2 mg. dry wt./ml. in mixture I which had the following composition: glycine, 0.01M; sodium α -oxoglutarate, 0.01M; sodium fumarate, 0.02M; $(\text{NH}_4)_2\text{HPO}_4$, 0.003M; MgSO_4 , $8 \times 10^{-4}\text{M}$; MnSO_4 , $5 \times 10^{-8}\text{M}$; CaCl_2 , $4 \times 10^{-4}\text{M}$; potassium phosphate buffer, pH 6.8, 0.006M. The pH rose from 6.8 to about 8.2 during the experimental period. Unless stated otherwise, all incubations were in the light for 20–24 hr. at 32–34° in 12×150 mm. tubes containing 10 ml. reaction mixture. Experiments carried out anaerobically in Thunberg tubes filled with H_2 or N_2 gave the same results as those under the usual semi-anaerobic conditions.

Aerobic incubation in the dark was in tubes shaped like an inverted T, the horizontal limb being 150 mm. long and 18 mm. wide (Monod *et al.* 1951); these contained 5 ml. reaction mixture. They were shaken at 34° as described above. Protection of the suspensions from the laboratory light was unnecessary.

Synthesis from δ -aminolaevulinic acid. The washed cells were suspended to a final concentration of 1–1.3 mg. dry wt./ml. in mixture II, which had the following composition: δ -aminolaevulinic acid hydrochloride, $2 \times 10^{-3}\text{M}$; MgSO_4 , $8 \times 10^{-4}\text{M}$; phosphate buffer pH 6.9, 0.1M, prepared from 0.2M- Na_2HPO_4 and KH_2PO_4 . The reaction mixtures (2.5 ml.) were incubated in the light cupboard in Thunberg tubes filled with H_2 . Aerobic incubation in the dark was under the same conditions as those described for the experiments with glycine.

Identification and estimation of porphyrins etc.

Identification of porphyrins. The porphyrins formed by the cells passed out into the suspending fluid; no free porphyrin was detected in the cells after they had been centrifuged and washed free of the supernatant fluid. After each experiment the supernatant fluids were examined with a hand spectroscope. More accurate determinations of the wavelengths of the bands in the visible spectrum were made with a Hartridge reversion spectroscope (R. and J. Beck Ltd., London, W. 1).

The component porphyrins of the mixture formed were identified by the paper-chromatographic methods of Nicholas & Rimington (1949, 1951), Eriksen (1953), Chu, Green & Chu (1951) and Falk & Benson (1953). After

adjusting the supernatant fluid to pH 1 with 2N-HCl the mixed porphyrins were adsorbed on Filter-Cel (Johns-Manville Co. Ltd., Artillery Row, London, S.W. 1). The porphyrins were eluted with 2N-NH₄OH for chromatographic separation by the methods of Nicholas & Rimington or of Eriksen. Before applying the methods of Chu *et al.* and of Falk & Benson the adsorbed porphyrins were esterified by treating the Filter-Cel overnight at 0° with methanol previously saturated with dry HCl. The esters were extracted with chloroform and the chloroform solutions were washed and applied to the chromatograms. Pure specimens of the methyl esters of coproporphyrin I and III, uroporphyrin I and III, and protoporphyrin IX were kindly provided by Professor C. Rimington. Where necessary, they were converted into the free porphyrin as described by Nicholas & Rimington (1951).

Estimation of porphyrins. The cells were removed by centrifuging and the porphyrin concentration in the supernatant was determined spectrophotometrically at 403 m μ . after dilution with 1.4N-HCl. Measurements of optical density were made with the Unicam SP. 500 or SP. 600 spectrophotometer. The values were usually expressed in terms of coproporphyrin III, since this was the major component of the porphyrin mixture formed under the conditions described. The concentrations were calculated from the extinction coefficients for this compound in 1.4N-HCl (Jope & O'Brien, 1945).

In some experiments the ratio of uro-, copro- and protoporphyrin was determined by fractionation similar to that used to separate the porphyrins formed by haemolysates (Dr J. E. Falk, personal communication; Dresel & Falk, 1956). Portions of the whole reaction mixture (cells and suspending fluid) were extracted, since protoporphyrin, when formed, tended to come out of solution and to centrifuge out with the cells. After adjusting to pH 4 with glacial acetic acid, the mixture was extracted with ethyl acetate. The uroporphyrin in the aqueous residue (together with the washings from the ethyl acetate extract) was extracted by the method of Dresel & Tooth (1954). The ethyl acetate phase was washed with 0.6M sodium acetate and water and the porphyrins were removed by shaking with 4.2N-HCl. The acid extract was adjusted to pH 4 with saturated sodium acetate and shaken with ether. After washing, the ethereal layer was extracted first with 0.3N-HCl, which removed the coproporphyrin, and then with 1.4N-HCl to obtain the protoporphyrin.

The porphyrin concentration in the HCl fractions was determined spectrophotometrically at the Soret maxima using the correction factors of Rimington & Sveinsson (1950). The results were calculated from the extinction coefficients reported by these workers for uroporphyrin, by those of Jope & O'Brien (1945) for coproporphyrin and those of Grinstein & Wintrobe (1948) for protoporphyrin IX.

Estimation of porphobilinogen. This was determined by reaction with Ehrlich's aldehyde reagent (Vahlquist, 1939; Cookson & Rimington, 1954).

Estimation of bacteriochlorophyll. The method was essentially that of van Niel & Arnold (1938) and was applied to the cells after they had been separated by centrifugation. The absorption of the bacteriochlorophyll was measured at 750 m μ . (the maximum) instead of at 667.5 m μ . The molecular extinction at 750 m μ . (1 cm. length) was calculated to be 3.8×10^4 from measurements of the absorption

at this wavelength of preparations of bacteriochlorophyll, the concentration of which had been determined by following exactly the method of van Niel & Arnold.

Estimation of intracellular haem. The bacteriochlorophyll was extracted from washed cells by stirring with methanol (30 ml./50 mg. dry wt. cells) and centrifuging; this was repeated twice. The carotenoids were then extracted in the same way with acetone. The extracted cells were suspended in water and the haem content was determined by the pyridine haemochromogen method of Elliott & Keilin (1933). These analyses were kindly carried out by Dr W. E. van Heyningen.

Manometric experiments

The conventional Warburg technique was used in manometric experiments. The manometer vessels (approx. 30 ml. volume) were incubated in a bath at 34°, illuminated from above with a bank of 100 W tungsten filament lamps.

Chemicals

δ -Aminolaevulinic acid hydrochloride and porphobilinogen, the latter isolated from human urine after ingestion of ALA (Berlin, Gray, Neuberger & Scott, 1954), were generously provided by Dr A. Neuberger and Dr J. J. Scott. Solutions of ALA hydrochloride were kept at -20° and were added to reaction mixtures without neutralization when used at concentrations of 0.002M or less; with higher concentrations equivalent amounts of NaOH were also added just before mixing. Solutions of PBG were prepared immediately before use by dissolving in a minimum volume of 0.1N acetic acid and neutralizing with 0.05N-NaOH.

Stock solutions of iron citrate (0.004M) were prepared by dissolving 157 mg. (NH₄)₂Fe(SO₄)₂·6H₂O and 236 mg. sodium citrate (dihydrate) in 100 ml. of water. Other compounds were commercial products. The sodium salts of carboxylic acids were used.

RESULTS

Porphyrin formation from glycine, α -oxoglutarate and fumarate

Course of the reaction. Cells harvested from cultures grown in the light formed considerable amounts of porphyrin when incubated in the light in mixture I (containing inorganic salts, glycine, α -oxoglutarate and fumarate). Porphyrin formation occurred after an initial lag period which varied in different experiments from 2 to 8 hr., and then continued at a linear rate for periods up to 20 hr. or more (Fig. 1a). The amount of porphyrin formed varied with different cell suspensions, but usually the concentration reached after incubation for 24 hr. was between 140 and 230 μ M-moles/ml. reaction mixture (170–290 μ M-moles/mg. dry wt. cells). Assuming that eight molecules of glycine and eight of α -oxoglutarate are utilized in the formation of one molecule of porphyrin, the yield in the standard system varied between 11 and 18% of the theoretical value.

During incubation in mixture I the bacteriochlorophyll in the cells increased 1.5- to 2-fold (Fig. 1a); the opacity of the suspensions was doubled.

Fig. 1. (a) Rate of synthesis in light, of porphyrin (full lines) and of bacteriochlorophyll (broken lines) by light-grown cells (1.1 mg. dry wt./ml.); and (b) rate of synthesis in light by aerobic, dark-grown cells (0.7 mg. dry wt./ml.). Incubation in mixture I, $\bigcirc$ - $\bigcirc$ or $\bigcirc$ - $\bigcirc$ - $\bigcirc$; incubation in mixture I with 2×10^{-3} M ALA replacing glycine and α -oxoglutarate, $\bullet$ - $\bullet$ or $\bullet$ - $\bullet$ - $\bullet$.

Fig. 2. Effect of concentration of glycine ($\bullet$), α -oxoglutarate ($\bigcirc$) and fumarate ($\times$) on porphyrin synthesis. The cells (0.7 mg. dry wt./ml.) were incubated for 24 hr. in the light in mixture I with different concentrations of substrates.

Substrates required for porphyrin synthesis. The omission of glycine, α -oxoglutarate or fumarate from mixture I reduced synthesis to 20 % or less; in the absence of both glycine and α -oxoglutarate little or no porphyrin was formed (Fig. 2; Table 1).

The amount of porphyrin formed increased as the concentration of glycine was raised from 0.0025 M to 0.01 M; at concentrations above 0.01 M it became inhibitory (Fig. 2). Similar concentrations of glycine also inhibit growth of the organism in medium S.

With the exception of threonine, no amino acid out of 15 tested replaced glycine in mixture I. Some porphyrin (about 20 % of that formed in mixture I) was synthesized when DL-threonine (0.02 M) was substituted for glycine: this suggests that threonine may be a precursor of glycine in *Rps. spheroides* as well as in animal tissues (Krasna, Peyser & Sprinson, 1952; Chao, Delwiche & Greenberg, 1953). Since serine did not replace glycine, the organism is apparently unable to convert serine into glycine; this reaction accounts for the incorporation of serine into haem by duck erythrocytes (Shemin, London & Rittenberg, 1950). Sodium glyoxylate and sodium glycollate (each tested at 0.01 and 0.001 M) were completely inactive: they are precursors of glycine in a mutant strain of *Neurospora* (Wright, 1951) and in animal tissues (Weissbach & Sprinson, 1953).

Table 1. *Requirements for porphyrin synthesis from glycine*

The complete system contained cells (0.6 mg. dry wt./ml.) suspended in 10 ml. of mixture I (see Experimental). Incubation: 24 hr., light.

Component(s) omitted	Porphyrin formed (μ m-moles/ml.)
Nil	125
Glycine	7
α -Oxoglutarate	18
Glycine and α -oxoglutarate	3
Fumarate	21
MgSO ₄	1
MnSO ₄	18
(NH ₄) ₂ HPO ₄	20
CaCl ₂	100

Both α -oxoglutarate and fumarate were needed for maximum formation of porphyrin, though considerable synthesis occurred in the presence of high concentrations (0.02 M) of α -oxoglutarate alone (Fig. 3). Little porphyrin was formed from fumarate alone, but it reduced the requirement for α -oxoglutarate.

Only succinate, of a number of carboxylic acids tested, showed activity similar to that of α -oxoglutarate; apart from L-glutamate, which had about 60 % of the activity of α -oxoglutarate, the other carboxylic acids were much less active (Table 2, Expt. a). Fumarate, however, could be replaced equally well by succinate or lactate, while malate, oxaloacetate and pyruvate were only slightly less active (Table 2, Expt. b). Acetate was

ineffective and became inhibitory at concentrations greater than 0.01M; it also inhibited synthesis from α -oxoglutarate plus fumarate. Incubation in atmospheres containing CO_2 (5% in H_2 or N_2) had no effect on synthesis with or without fumarate.

Effect of NH_4^+ , Mg^{2+} and Mn^{2+} . The optimum concentration of NH_4^+ was $4 \times 10^{-3}\text{M}$, and omission

of NH_4^+ reduced synthesis to 20% or less (Table 1); $(\text{NH}_4)_2\text{HPO}_4$ or $(\text{NH}_4)_2\text{SO}_4$ were equally effective as sources of ammonium ion.

No porphyrin formation occurred in the absence of Mg^{2+} ; omission of Mn^{2+} reduced synthesis to about 20% (Table 1). The concentration of Mg^{2+} required for maximum synthesis ($5 \times 10^{-4}\text{M}$) was 500 times greater than that for Mn^{2+} (10^{-6}M). The following ions (each tested at 10^{-5}M) neither replaced Mn^{2+} nor affected porphyrin formation in mixture I: Pb^{2+} , Co^{2+} , Cu^{2+} and Zn^{2+} . Ca^{2+} was included in mixture I, since it is needed for growth of *Rps. spheroides* as well as for other members of the Athiorhodaceae (Foster, 1944). Its omission from mixture I sometimes, but not always, reduced synthesis slightly (Table 1).

Effect of iron salts. Addition of iron citrate or ferrous ammonium sulphate to mixture I almost completely suppressed porphyrin formation (Fig. 4). There was no simple relationship between the concentration of added iron and the amount of porphyrin which failed to appear. For instance, in the experiment of Fig. 4 addition of 1, 2 and 4 μm -moles of iron citrate/ml. reduced porphyrin formation by 14, 96 and 146 μm -moles respectively. The effect of iron differs, therefore, from that found with cultures of *C. diphtheriae*, where one equivalent of porphyrin failed to appear for each equivalent of iron added (Pappenheimer, 1947).

Formation of bacteriochlorophyll was increased by the addition of iron, the concentration required to show this effect being similar to that which

Fig. 3. Effects of different concentrations of α -oxoglutarate and fumarate. The cells (0.7 mg. dry wt./ml.) were incubated for 24 hr. in the light in mixture I with various concentrations of α -oxoglutarate and without fumarate (A), or with 5, 10 and 20 μm -moles/ml. fumarate in B, C and D respectively.

Table 2. *Effect of various carboxylic acids on porphyrin synthesis from glycine*

Cells (0.7 mg. dry wt./ml.) were incubated for 24 hr. in the light in mixture I without α -oxoglutarate (system a) or without fumarate (system b) with the additions shown. The acids were added as their sodium salts.

System	Addition	Concn. (M)	Porphyrin formed (μm -moles/ml.)
(a) Fumarate (0.02M) present, but no α -oxoglutarate	Nil	—	18
	α -Oxoglutarate	0.01	141
	Succinate	0.01	120
	DL-Malate	0.01	27
	Oxaloacetate	0.01	31
	Lactate	0.01	32
	Pyruvate	0.01	46
	Acetate	0.01	37
	L-Aspartate	0.01	26
	L-Glutamate	0.01	86
	Nil	—	22
(b) α -Oxoglutarate (0.01M) present, but no fumarate	Fumarate	0.02	141
	Succinate	0.02	139
	DL-Malate	0.02	116
	DL-Malate	0.04	61
	Oxaloacetate	0.02	120
	Lactate	0.02	147
	Pyruvate	0.02	120
	Acetate	0.01	80
	Acetate	0.02	16
	L-Aspartate	0.02	40
	L-Glutamate	0.02	30

reduced porphyrin synthesis (Fig. 4). However, the increase in bacteriochlorophyll accounted for only a small proportion of the porphyrin which failed to appear; in the presence of 10^{-5} M ferric citrate porphyrin formation was reduced about 100-fold, while bacteriochlorophyll formation was increased only 8- to 10-fold (Fig. 4).

Fig. 4. Effect of iron citrate on synthesis of porphyrin (●) and bacteriochlorophyll (○). The cells (0.6 mg. dry wt./ml.) were incubated for 24 hr. in the light in mixture I with addition of iron citrate.

Light-grown cells of *Rps. spheroides* contain cytochrome pigments (Vernon & Kamen 1954), and it was possible that in the presence of iron the porphyrins were converted into cytochromes or other haem compounds. The increase in intracellular haems was therefore determined in cells incubated in mixture I with and without addition of

iron citrate. The haem concentration was increased 4-fold by incubation with iron, but again this was insufficient to account for the decrease in porphyrin formation. Some loss of haem occurred in cells incubated without iron (Table 3).

Effects of vitamins of the B-group. *Rps. spheroides* needs nicotinic acid, thiamine and biotin for growth (Hutner, 1946, 1950) and medium *S* contained each of these in excess of the requirements for maximum growth. Porphyrin formation by cells grown in this medium was not increased by the addition of these vitamins to mixture I, since they were probably rich in these compounds or in more complex related substances (Table 4). The dependence of porphyrin synthesis on each factor was shown by using cells deficient in one or other of them. Such cells were obtained by growth in medium *S* containing suboptimal concentrations of the vitamin in question; the critical concentration of each had been determined in preliminary growth experiments. The concentration of each factor used was sufficient to give about 80 % maximum growth after incubation for 40 hr.; cultures grown in media *S* with the normal concentration of each vitamin attained the same growth in 24 hr.

Porphyrin formation in mixture I by cells deficient in any one of these three vitamins was negligible; addition of the appropriate factor partially or fully restored synthetic ability (Table 4).

Addition to mixture I of the following compounds (each tested at 10^{-6} M) did not affect porphyrin formation: calcium pantothenate, riboflavin, pyridoxine, pyridoxal, *p*-aminobenzoic acid, pteroylglutamic acid, leucovorin, DL-6-thioctic acid. Vitamin B₁₂ (2 μg./ml.) was also without effect. No attempts were made to deplete the cells of these factors, all of which the organism can presumably synthesize.

Table 3. *Effect of iron citrate on the synthesis of porphyrin, bacteriochlorophyll and intracellular haems*

Cells (0.8 mg. dry wt./ml. reaction mixture) were suspended in mixture I and the suspension was divided into 3 parts (*A*, *B* and *C*; 60 ml. each). The suspensions were distributed in 10 ml. quantities in tubes (14 mm. diameter) and incubated under the conditions stated. The contents of each set of tubes were pooled and samples (10 ml.) were removed for the determination of porphyrin in the supernatant and of bacteriochlorophyll in the cells. The remainder of each suspension was centrifuged and the cells were washed once with 50 ml. 0.85 % (w/v) NaCl. After treatment of the cells to remove the photosynthetic pigments (see Experimental) the extracted cells were suspended in 5 ml. water, samples were removed for determination of dry wt. and the remainder was used for the estimation of haem.

Suspension	Incubation conditions	Porphyrin derivative (μm-moles/mg. dry wt. cells)		
		Porphyrin	Bacteriochlorophyll	Haem*
<i>A</i>	No incubation	0	10	1
<i>B</i>	Light; 24 hr. in mixture I	225	12	0.5
<i>C</i>	As <i>B</i> , with 10^{-5} M iron citrate added	3	28	4

* Concentration of haem in μm-moles/mg. dry wt. extracted cells.

Effect of light and oxygen with light-grown cells. Porphyrin formation occurred only when the suspensions were incubated in the light. Anaerobic conditions were also essential for maximum synthesis; oxygen almost completely prevented porphyrin formation in the light, while in the dark no synthesis at all occurred in atmospheres of air, N₂ or H₂ (Table 5). The light-grown cells, however, oxidized both α -oxoglutarate and fumarate, when incubated aerobically in the dark.

porphyrin and bacteriochlorophyll (Fig. 1*b* and Table 5), but only after a lag period. The length of this delay varied from 8 to 24 hr. with different batches of cells; it was always much longer than the lag period observed with the light-grown cells (see Fig. 1*a*). Formation of bacteriochlorophyll always preceded the appearance of porphyrins; these were formed only after the concentration of bacteriochlorophyll had approached its maximum value (Fig. 1*b*).

Table 4. *Effect of deficiency of B-group vitamins on porphyrin synthesis*

Normal cells were grown for 24 hr. in the light in medium *S* (see Experimental), which contained nicotinic acid, thiamine and biotin at 8×10^{-6} , 3×10^{-6} and 4×10^{-8} M respectively. Deficient cells were grown in a similar medium for 40 hr. but with 5×10^{-8} M nicotinic acid, 10^{-8} M thiamine or 10^{-8} M biotin. The cells (1 mg. dry wt./ml.) were incubated for 21 hr. in the light in mixture I (containing glycine and α -oxoglutarate) or in mixture II (containing ALA) with the supplements shown; these were added to give the same final concentration as were normally present in medium *S*.

Cells*	Supplement	Porphyrin formation (μ m-moles/ml.)			
		Mixture I		Mixture II	
		Unsupplemented	Supplemented	Unsupplemented	Supplemented
Normal	Nicotinic acid + thiamine + biotin	160	160	140	134
N-	Nicotinic acid	6	50	133	122
T-	Thiamine	2	88	130	135
B-	Biotin	3	153	140	145

* N-, T- and B- denotes cells deficient in nicotinic acid, thiamine and biotin respectively.

Table 5. *Effect of light and oxygen on synthesis from glycine and α -oxoglutarate*

The cells were incubated at 34° for 24 hr. in Warburg manometers in 2.5 ml. mixture I under the conditions shown. Dry wt. cells: Expt. 1, 0.6 mg./ml.; Expt. 2, 0.8 mg./ml.

Expt. no.	Type of cells	Incubation conditions	Porphyrin formed (μ m-moles/ml.)
1	Grown in light; semi-anaerobic (see Experimental)	Light—H ₂	159
		Light—N ₂	173
		Light—Air	3
		Dark—H ₂	0
		Dark—Air	0
2	Grown in dark; aerobic	Light—H ₂	80
		Dark—H ₂	0
		Dark—Air	0

Synthesis by cells grown aerobically in the dark. *Rps. spheroides* grows aerobically (though not anaerobically) in the dark on medium *S* almost as well as it grows anaerobically in the light, provided that the cultures are aerated. The aerobically grown cultures contain no bacteriochlorophyll and little or no carotenoid, but these pigments are rapidly developed when the cultures are transferred to the light and incubated anaerobically (van Niel, 1944).

Cells harvested from dark, aerobic cultures formed no porphyrin when incubated in mixture I in the dark, either aerobically or anaerobically (Table 5). However, when such cells were incubated anaerobically in the light they formed both

Effect of inhibitors. Cyanide, azide, 2:4-dinitrophenol, arsenite, *p*-chloromercuribenzoate and streptomycin all strongly inhibited porphyrin synthesis from glycine and α -oxoglutarate (Table 6). Streptomycin prevents chlorophyll formation in *Euglena gracilis* at concentrations which permit it to grow in the dark (Provasoli, Hutner & Schatz, 1948; Lwoff & Schaeffer, 1949). Growth of *Rps. spheroides* is, however, equally sensitive to inhibition by streptomycin under anaerobic-light and aerobic-dark conditions.

Malonate inhibited porphyrin synthesis at concentrations similar to those of the dicarboxylic acids present in mixture I (Table 6). It inhibits the incorporation of methylene-labelled succinate into

the haem of duck erythrocytes, but does not effect the incorporation of carboxy-labelled succinate (Shemin & Kumin, 1952).

Metabolism of α -oxoglutarate and fumarate by cell suspensions

Since NH_4^+ , Mg^{2+} and Mn^{2+} are all needed for porphyrin formation from glycine and α -oxoglutarate, the question arises whether they are specifically required in the reactions leading to porphyrin or whether their function is in other metabolic reactions linked to the synthetic system. Examination was made therefore of their effect on

the metabolism of α -oxoglutarate and fumarate by the cell suspensions under conditions where no porphyrin formation occurred.

The cells liberated carbon dioxide from α -oxoglutarate and fumarate when incubated in the light, but not in the dark, under H_2 or N_2 (Fig. 5*a*, *b*). The cells did not evolve H_2 from these substrates, since they had been grown on medium *S* which contained ammonium salts. *Rps. spheroides*, like other members of the Athiorhodaceae, liberates H_2 in the light from dicarboxylic acids only when grown in the absence of ammonium salts (Gest, Kamen & Bregoff, 1950; Gest, 1951).

Table 6. Effect of inhibitors on porphyrin formation

Cells (0.8 mg. dry wt./ml.) were incubated in the light for 24 hr. in mixture I (containing glycine and α -oxoglutarate) or in mixture II (containing ALA) with the inhibitors shown.

Inhibitor	Concn.	% Inhibition of porphyrin synthesis in	
		Mixture I	Mixture II
Potassium cyanide	10^{-3}M	94	55
Hydrazine	10^{-3}M	45	0
Sodium azide	10^{-3}M	98	0
2,4-Dinitrophenol	10^{-3}M	99	82
Sodium arsenite	10^{-3}M	98	0
p-Chloromercuribenzoate	$4 \times 10^{-6}\text{M}$	100	—
	10^{-4}M	100	100
Streptomycin sulphate	14 $\mu\text{g.}/\text{ml.}$	90	0
	110 $\mu\text{g.}/\text{ml.}$	100	17
	550 $\mu\text{g.}/\text{ml.}$	100	35
Sodium malonate	$2 \times 10^{-2}\text{M}$	50	—
Sodium malonate	$4 \times 10^{-2}\text{M}$	75	—

Fig. 5. (*a*) Evolution of CO_2 from α -oxoglutarate, and (*b*) from fumarate. The complete systems (2.5 ml. final vol.) contained cells (2 mg. dry wt./ml.), 0.1M-NaK phosphate buffer pH 6.9 and $8 \times 10^{-4}\text{M}$ - MgSO_4 together with: (*a*) $4 \times 10^{-3}\text{M}$ α -oxoglutarate, $5 \times 10^{-6}\text{M}$ - MnSO_4 and $3 \times 10^{-3}\text{M}$ -(NH_4) $_2\text{HPO}_4$; (*b*) $4 \times 10^{-3}\text{M}$ fumarate. Incubation was in the light in N_2 .

The maximum rate of CO_2 evolution from α -oxoglutarate occurred only in the presence of NH_4^+ , Mg^{2+} and Mn^{2+} , omission of any one of these reducing the rate by one-third or more (Fig. 5a). Neither NH_4^+ nor Mn^{2+} affected the rate of CO_2 production from fumarate; Mg^{2+} was, however, highly stimulatory (Fig. 5b).

Iron citrate (10^{-5}M) did not affect the rate of CO_2 evolution from either α -oxoglutarate or fumarate when added to the complete system containing phosphate buffer, Mg^{2+} , Mn^{2+} and NH_4^+ .

Porphyrin synthesis from δ -aminolaevulinic acid

δ -Aminolaevulinic acid (ALA) was actively converted into porphyrins when substituted for glycine and α -oxoglutarate in mixture I; it behaved as if it were an intermediate in porphyrin synthesis from these compounds since (a) it completely replaced both glycine and α -oxoglutarate (Fig. 1a), (b) the concentration needed for maximum synthesis was

Table 7. *Extent of conversion of δ -aminolaevulinic acid into porphyrin*

Cells (1.1 mg. and 0.8 mg. dry wt./ml. in Expts. 1 and 2 respectively) were suspended in mixture II with different concentrations of ALA. Incubation: 24 hr. under H_2 in the light.

ALA (μ -moles/ ml.)	Porphyrin formed (μ m-moles/ml.)		% Conversion*	
	Expt. 1	Expt. 2	Expt. 1	Expt. 2
0	0	0	—	—
0.05	4	—	64	—
0.10	8	—	64	—
0.20	14	—	56	—
0.50	34	45	54	72
1.0	75	90	60	72
2.0	150	184	60	74
4.0	188	185	38	37

* Calculated on the assumption that 8 molecules of ALA are converted into 1 of porphyrin.

less than the required concentrations of glycine and α -oxoglutarate (Fig. 2 and Table 7), and (c) it was converted into porphyrins without lag and at a rate equal to or exceeding that attained in the glycine- α -oxoglutarate system (Fig. 1a).

Requirements for synthesis. Porphyrin formation from ALA was studied in mixture II containing only phosphate buffer and Mg^{2+} . Addition of Mn^{2+} , NH_4^+ or fumarate (at the concentrations used in mixture I) did not increase synthesis, provided that incubation was in the light under anaerobic conditions. Omission of Mg^{2+} reduced synthesis by 50% (Table 8). The optimum pH was from 6.4 to 7.0 (Table 8).

Rate and extent of synthesis. Porphyrin was formed anaerobically in light at a linear rate in mixture II until the concentration reached approx. 50% of the theoretical value for complete conversion of ALA (Fig. 6). Conversion of ALA into porphyrins occurred over a wide range of substrate concentration; at lower concentrations about 60% of the added ALA was converted into porphyrin, though better yields (up to 70–80%) were obtained with some batches of cells (Table 7). It is not known whether incomplete conversion of ALA was due to its utilization in other reactions or to non-enzymic destruction. The amount of bacteriochlorophyll in the cells was not increased during incubation in mixture II; there was also no increase when ALA was substituted for glycine and α -oxoglutarate in mixture I (Fig. 1a).

Formation and utilization of porphobilinogen. The monopyrrole was formed concurrently with porphyrin during the early stages of incubation anaerobically in the light in mixture II; after reaching a peak, the concentration fell almost to zero as incubation proceeded (Fig. 6). No PBG (or porphyrin) was detected when the cells were incubated anaerobically in the dark in mixture II.

Table 8. *Requirements for porphyrin synthesis from δ -aminolaevulinic acid*

Cells (1 mg. dry wt./ml.) were suspended in 0.1M buffer with the additions shown. Incubation: 10 hr. under H_2 in the light.

Buffer		Additions		Porphyrin formed (μ m-moles/ml.)
Type	pH	MgSO_4 ($8 \times 10^{-4}\text{M}$)	ALA ($2 \times 10^{-3}\text{M}$)	
Phosphate	6.9	+	+	90
	6.9	—	+	44
	6.9	+	—	0
*Phthalate	5.0	+	+	0
*Acetate	5.5	+	+	0
Phosphate	5.8	+	+	58
	6.5	+	+	89
	6.9	+	+	90
	7.5	+	+	28
	7.8	+	+	19

* No porphyrin formed on addition of 0.04M phosphate, pH 5.8.

Although over 50% disappeared when PBG was incubated with washed cells, there was no formation of porphyrin (Table 9). Loss of PBG also occurred to almost the same extent when it was incubated with boiled cells, suggesting that PBG was being destroyed non-enzymically. The monopyrrole is unstable and decomposes on standing, even at pH 6.5. One of the products is uroporphyrin, but this accounts for only about 20% of the PBG which disappears at this pH value (Cookson & Rimington, 1954).

Since added PBG is not significantly converted into porphyrin, the disappearance of PBG formed in the early stages of incubation with ALA may have been due to non-enzymic processes rather than to its conversion into porphyrin. To test this possi-

bility, cells were first incubated in mixture II for 6 hr.; at this stage a considerable amount of PBG was present in the supernatant fluid. The cells were removed from the reaction mixture and inactivated by heating at 100°; they were then added back to the supernatant fluid and reincubated. No PBG was found at the end of the second incubation nor did the concentration of porphyrin increase. These results suggest that the PBG excreted into the medium is not utilized for porphyrin synthesis and is destroyed non-enzymically. In the parallel experiment with unheated cells, the PBG disappeared, but the porphyrin concentration increased during the second incubation, presumably due to the conversion of residual ALA.

Effect of vitamins of the B-group. Cells deficient in nicotinic acid, thiamine or biotin formed porphyrin from ALA as actively as normal cells, though they formed little or no porphyrin from glycine and α -oxoglutarate (Table 4). Addition of these vitamins did not affect synthesis in mixture II.

Effects of inhibitors. Of the compounds tested only *p*-chloromercuribenzoate, 2:4-dinitrophenol and cyanide appreciably inhibited synthesis from ALA (Table 6). Gibson *et al.* (1954) have found *p*-mercurichlorobenzoate to inhibit conversion of ALA into PBG by an enzyme preparation from liver; this reaction may also be inhibited in the bacterial system, but it is possible that the inhibitor acts at other stages in the conversion of ALA into porphyrins by *Rps. spheroides*.

Effects of light and oxygen with light-grown cells. Some porphyrin was formed from ALA when light-grown cells were incubated aerobically in the dark in mixture II. This was increased by the addition of an oxidizable substrate such as fumarate, succinate or lactate. Little or no porphyrin was formed anaerobically in the dark in the presence or absence of these substances (Table 10). Even in the presence of a carboxylic acid, the amount of porphyrin finally formed was only about 35% of

Fig. 6. Rate of formation of porphyrin (O) and porphobilinogen (●) from ALA. Cells (1.3 mg. dry wt./ml.) were incubated anaerobically in the light in mixture II.

Table 9. Utilization of porphobilinogen

Cells (1.3 mg. dry wt./ml.) were suspended in 0.1M phosphate buffer, pH 6.9 and 8×10^{-4} M-MgSO₄. The boiled cells had been heated for 10 min. at 100° before addition to the system. Incubation: H₂ in the light.

Cells	Additions (μm-moles/ml.)		Time of incubation (hr.)	Pyrroles found (μm-moles/ml.)	
	PBG	ALA		PBG	Porphyrin*
Untreated	120	0	6	102	2
	120	0	18	53	4
	0	320	6	22	18
	0	320	18	12	19
	0	0	18	0	0
Boiled	120	0	0	121	0
	120	0	6	99	0
	120	0	18	68	2

* The theoretical amount of porphyrin is 30 from 120 μm-moles PBG and 40 from 320 μm-moles ALA.

that formed in the light (Fig. 7 and Table 10). The low yield of porphyrin under aerobic conditions may have been due to inactivation of some of the enzymes involved, or ALA may have been utilized by other reactions not leading to porphyrin formation. However, cell suspensions, which had been first incubated aerobically in the dark until porphyrin formation had almost ceased, continued to form porphyrin when transferred to the light and incubated anaerobically (Fig. 7).

PBG was formed simultaneously with porphyrin aerobically in the dark; as in the anaerobic-light systems, it accumulated in the early stages of

Table 10. Effect of conditions of incubation on porphyrin formation from δ -aminolaevulinic acid

Light-grown cells (1 mg. dry wt./ml.) were incubated for 22 hr. in mixture II with the additions shown.

Incubation conditions	Additions	Porphyrin formed (μ m-moles/ml.)
H ₂ —light	None	164
H ₂ —dark	Fumarate, 10 ⁻² M	7
Air—dark	None	11
	Fumarate, 4 $\times$ 10 ⁻³ M	48
	Fumarate, 10 ⁻² M	54
	Succinate, 10 ⁻² M	52
	Lactate, 10 ⁻² M	55

Fig. 7. Rate of porphyrin synthesis from ALA anaerobically in the light and aerobically in the dark. Cells (1 mg. dry wt./ml.) were incubated in mixture II anaerobically in the light (○) or in mixture II with 0.01M fumarate aerobically in the dark (●); after incubation for 8 hr. under the latter conditions a sample (2.5 ml.) of the reaction mixture was transferred to the light and incubated anaerobically (broken line).

Table 11. Formation of coproporphyrin and protoporphyrin from δ -aminolaevulinic acid

Cells, grown either in the dark or light, were incubated under various conditions in mixture II with the additions shown.

Expt. no.	Type of cells	Incubation conditions	Additions to mixture II			Total porphyrin* formed (μ m-moles/ml.)	Copro-porphyrin (%)†	Proto-porphyrin (%)†
			Iron citrate (10 ⁻⁵ M)	Carboxylic acid				
1	Light-grown (1 mg. dry wt./ml.)	H ₂ —Light	+	—	Fumarate, 4 $\times$ 10 ⁻³ M	134	100	0
			+	—	Fumarate, 4 $\times$ 10 ⁻³ M	136	100	0
			+	—	Fumarate, 10 ⁻² M	96	97	3
			+	—	Fumarate, 10 ⁻² M	86	76	24
2	Light-grown (1 mg. dry wt./ml.)	H ₂ —Light	+	—	Fumarate, 10 ⁻² M	87	77	23
			+	—		126	100	0
			+	—		120	96	4
			+	—		93	77	23
3	Light-grown (1 mg. dry wt./ml.)	Air—Dark	+	—	Fumarate, 10 ⁻² M	107	74	26
			+	—	Succinate	93	74	26
			+	—	Lactate	90	73	27
			+	—	Butyrate	9	100	0
4	Air-grown (1.4 mg. dry wt./ml.)	Air—Dark	—	—	Fumarate, 10 ⁻² M	40	100	0
			+	—	Fumarate, 10 ⁻² M	39	100	0
			—	—	Fumarate, 10 ⁻² M	15	81	19
			—	—	Fumarate, 10 ⁻² M	44	80	20
			+	—	Fumarate, 10 ⁻² M	42	77	23

* Calculated as coproporphyrin. † Percentage of ether-soluble porphyrins.

incubation and disappeared as incubation proceeded.

Synthesis by aerobically grown cells. Cells which had been grown aerobically in the dark also formed porphyrin when incubated under the same conditions in mixture II with fumarate. The rate and extent of synthesis were similar to those found with light-grown cells incubated aerobically in the dark (see Table 11).

Effect of iron. Iron citrate or ferrous ammonium sulphate at concentrations which almost completely suppressed porphyrin formation from glycine and α -oxoglutarate did not affect synthesis from ALA in mixture II (Table 11). When, however, both iron and a carboxylic acid were present, considerable amounts of protoporphyrin were formed from ALA (see p. 89).

Types of porphyrin formed

Porphyrins formed from glycine and α -oxoglutarate. Spectroscopic examination of the supernatant fluids after incubation with suspensions of cells in mixture I showed absorption bands at alkaline or acidic pH values which corresponded closely to those of coproporphyrin. A band at 638.5 m μ . was also detected under alkaline conditions; on acidification this shifted to 621.5 m μ ., becoming weaker and more diffuse. The substance responsible for this absorption band has not been identified.

Most of the porphyrin formed in mixture I was extracted by ether or ethyl acetate at pH 4 and behaved as coproporphyrin type III on chromatographic analysis (see Experimental). The ether-insoluble porphyrin was identified as a mixture uroporphyrin of types I and III; there was no constant preponderance of either isomer. Traces of other porphyrins with probably three and six carboxyl groups were detected by chromatography of whole supernatants. Quantitative fractionation of the porphyrins showed that coproporphyrin comprised 95 % and uroporphyrin 4 % of the total. No difference was detected in the composition of the porphyrins formed after incubation for 5, 12 and 24 hr. respectively.

Porphyrins formed from δ -aminolaevulinic acid in the light. After incubation of cells anaerobically in the light in mixture II the porphyrins formed were of the same types as those formed from glycine and α -oxoglutarate. The component observed in the glycine- α -oxoglutarate systems with an absorption band at 638 m μ . (in alkali) could not be detected in the ALA systems incubated anaerobically.

When iron citrate and a carboxylic acid, such as fumarate, were added to mixture II the absorption spectrum in alkali of the porphyrins formed showed a strong band at 644.6 m μ . This was found by chromatography and by fractionation of ether

extracts with HCl to be due to protoporphyrin. This substance accounted for 20–30 % of the total porphyrins formed under these conditions, the remainder consisting mainly of coproporphyrin III, with small amounts of uroporphyrin. Both iron and a carboxylic acid were needed for maximum formation of protoporphyrin (Table 11). Total porphyrin formation under these conditions was about 70 % of that in unsupplemented mixture II; addition of carboxylic acids alone caused a similar depression in the total porphyrin formation (Table 11).

Porphyrins formed from δ -aminolaevulinic acid aerobically in the dark. The porphyrins formed by light-grown cells incubated aerobically in the dark in mixture II with fumarate consisted mainly of coproporphyrin III with small amounts of uroporphyrin. Addition of iron citrate did not cause any change (Table 11). The supernatant fluids from these aerobic systems (with or without iron) contained another component with an absorption band of 638 m μ . in alkali, which appeared to be the same as that observed in the experiments with glycine plus α -oxoglutarate.

Aerobically grown cells formed appreciable amounts of protoporphyrin when incubated aerobically in mixture II with or without fumarate; coproporphyrin III was, however, the major component of the porphyrin mixtures formed by these cells. Addition of iron citrate did not influence the amount or ratio of the porphyrins formed (Table 11).

DISCUSSION

Rps. spheroides has potent systems for forming porphyrins from glycine and derivatives of the tricarboxylic acid cycle not only in cell suspension but also during growth. The culture fluids from other photosynthetic bacteria related to *Rps. spheroides* also contain a pink pigment which may well be porphyrin (van Niel, 1944, 1954; private communication).

Accumulation of intermediates or stabilized by-products of intermediates in a biosynthetic sequence is usually associated with a biochemical lesion which may be induced by an enzymic or nutritional deficiency. Formation of porphyrins by non-photosynthetic bacteria occurs when synthesis of cytochromes is limited by iron deficiency or by anaerobic growth. *Rps. spheroides* forms both cytochromes and bacteriochlorophyll, but porphyrin excretion seems to be associated more closely with the formation of bacteriochlorophyll than with the synthesis of cytochromes. Thus, porphyrins are formed only by light-grown cultures; they appear in the later stages of growth when synthesis of bacteriochlorophyll is limited by iron deficiency (Lascelles, 1955).

Substrates. The experiments indicate that the pathway of porphyrin synthesis by *Rps. spheroides* is similar to that found in animal tissues. Both glycine and α -oxoglutarate (or succinate) are required for maximum synthesis; the situation is complicated, however, by the additional requirement for an oxidizable substrate such as fumarate.

Although fumarate alone does not replace α -oxoglutarate, the amount of the latter needed for maximum synthesis is reduced by fumarate; in the presence of low concentrations of fumarate a certain amount of α -oxoglutarate may be utilized as an energy source, thereby reducing the amount available for porphyrin formation.

The C_4 -intermediate which condenses with glycine (or a derivative) has not been identified. Succinyl coenzyme A is formed by the α -oxoglutarate oxidase of animal tissues (Sanadi & Littlefield, 1953), and is probably also a product of similar reactions in *Escherichia coli* and *Azotobacter vinelandii* (Gunsalus, 1953; Lindstrom, 1953). So far, however, there is no evidence that succinyl coenzyme A participates in porphyrin synthesis. Experiments with bacteria by cells deficient in pantothenic acid may provide information on this point.

Manganese and ammonium ions are probably required by the cell suspensions for formation of the C_4 -intermediate from α -oxoglutarate; both ions are needed for maximum porphyrin synthesis from glycine and α -oxoglutarate and both are also required for the decarboxylation of the latter compound in the light.

Between 10 and 20 % of the added glycine could be accounted for as porphyrin. It is usually not possible to make a balance sheet of the products formed from the metabolism of organic substrates by photosynthetic bacteria in the light, since they are rapidly assimilated into cell substance (Gest, 1951; Elsdon, 1954). The free porphyrins excreted by *Rps. spheroides* are not utilized further, possibly because they are unable to penetrate back into the cell.

The rapid rate of conversion of ALA into porphyrin, as well as the fact that it replaces both glycine and α -oxoglutarate, suggests that it is a product of condensation of these substances. The immediate precursor of ALA may be α -amino- β -ketoadipic acid (Shemin, 1955).

Porphobilinogen is probably an intermediate in the conversion of ALA into porphyrins, since it is found during the early stages of incubation of the cell suspensions with ALA. The PBG which can be detected in the supernatant fluid may be only a small proportion of that present inside the cells, possibly in a bound form. The monopyrrole which is excreted is not utilized for porphyrin synthesis, though it disappears as incubation proceeds. Cell

suspensions of *Rps. spheroides* did not convert added PBG into porphyrins and the compound may not pass into the cell. Whole cells of *Chlorella* are also unable to convert PBG into porphyrin, although they do so after the cell wall has been damaged by freezing and thawing (Bogorad & Granick, 1953b). Porphobilinogen was detected only under conditions which permitted porphyrin synthesis. None was found when ALA was incubated with the cells anaerobically in the dark; acetone-dried cells were similarly inactive. *Rps. spheroides* differs therefore from *C. diphtheriae* and *Bacterium cadaveris*; acetone-dried preparations of these organisms have an active ALA-dehydrase which converts ALA into PBG under anaerobic conditions (Gibson *et al.* 1954).

Effects of vitamins of the B-group. Cells deficient in nicotinic acid, thiamine or biotin formed little or no porphyrin from glycine and α -oxoglutarate, but activity was restored by the addition of the appropriate factor. Nicotinic acid and thiamine (in their coenzyme forms) are probably needed for conversion of α -oxoglutarate into the C_4 -intermediate required for condensation with glycine. Both diphosphopyridine nucleotide and thiamine pyrophosphate are required for the oxidative decarboxylation of α -oxoglutarate by cell-free preparations from *Esch. coli* and *Azotobacter vinelandii* and animal tissues (Gunsalus, 1953; Lindstrom, 1953; Sanadi, Littlefield & Bock, 1952; Sanadi & Littlefield, 1953).

Effects of light and oxygen. Significant porphyrin formation from glycine and α -oxoglutarate occurred only anaerobically in the light. The photosynthetic reaction presumably provided any energy required for synthesis. The inability of the cells to form free porphyrin aerobically in the dark cannot, however, be attributed to lack of aerobic oxidation systems since the organism can grow under these conditions and forms cytochromes. It appears, therefore, that in the light the metabolism of glycine and α -oxoglutarate is directed towards the synthesis of porphyrins. The conditions of incubation which favoured the formation of porphyrins parallel those for the formation of the photosynthetic pigments; both bacteriochlorophyll and carotenoids are formed only in the light and oxygen suppresses their synthesis (van Niel, 1944).

Although ALA is converted into porphyrins aerobically in the dark, the yield of pigment and the rate of reaction is less than is observed under anaerobic conditions in the light. Moreover, an oxidizable substrate is needed, whereas maximum synthesis is attained in the presence of phosphate and Mg^{2+} only, under anaerobic conditions in the light. Energy may be needed for the conversion of ALA into porphyrins and this could be provided by oxidation of the carboxylic acid required under

aerobic conditions; in the light sufficient energy may be generated by the photosynthetic reaction coupled to endogenous hydrogen donors in the cells. Both adenosine triphosphate and mitochondria from rat liver are needed for maximum conversion of PBG into porphyrins by *Chlorella* preparations, suggesting that energy is required in this system also (Bogorad & Granick, 1953b).

Types of porphyrin formed. Coproporphyrin III, the major component of the porphyrins formed by *Rps. spheroides*, is also formed predominantly by other bacteria which form free porphyrins. It comprises 98–99 % of the porphyrins excreted by *C. diphtheriae* (Gray & Holt, 1948), and it has been isolated from the cells of *Mycobacterium karlinskii* (Todd, 1949).

Protoporphyrin was formed from ALA either (a) anaerobically in the light, provided that iron salts and an oxidizable substrate were present, or (b) aerobically in the dark by aerobically grown cells. In the light, iron and the oxidizable substrate may be concerned at some stage in the formation of the vinyl side chains of protoporphyrin, such as, for instance, in the oxidative decarboxylation of a precursor. Since iron is not needed by aerobically grown cells, there may be a different mechanism in this case. The difference could lie in the electron transfer mechanism; oxygen is the ultimate hydrogen acceptor aerobically, whereas in the light an oxidized compound generated by the photosynthetic system may serve.

The free porphyrins are not metabolized by *Rps. spheroides*, but this may be due to lack of penetration. In preparations of *Chlorella*, uroporphyrin III is found during the early stages of incubation with PBG; it disappears as incubation proceeds and porphyrins containing fewer carboxyl groups are found. Uroporphyrin III may therefore be a precursor of the other porphyrins (Bogorad & Granick, 1953b). With *Rps. spheroides* the ratio of coproporphyrin to uroporphyrin was constant throughout the incubation period and this does not suggest that coproporphyrin is an intermediate in the conversion of uroporphyrin III into protoporphyrin IX. There is also no evidence that these porphyrins are on the direct line of synthesis of bacteriochlorophyll or haem derivatives.

It is possible that the free porphyrins are stabilized by products of actual intermediates in the biosynthesis of bacteriochlorophyll and haem derivatives. Recent work using isotopic techniques has also suggested that the free porphyrins are not intermediates in the formation of haem from glycine, ALA or PBG by haemolysates of chick erythrocytes (Falk, 1955; Dresel, 1955).

Effect of iron. Excretion of porphyrins by growing cultures of *Rps. spheroides* and related organisms occurs only in media deficient in iron

salts; under these conditions formation of bacteriochlorophyll is limited (Lascelles, 1955). Similar effects of iron were observed in cell suspensions; here iron prevented the formation of porphyrins from glycine and α -oxoglutarate, but increased the synthesis of bacteriochlorophyll. Formation of chlorophyll by other photosynthetic bacteria and green plants also depends on the concentration of iron (Larsen, 1952, 1953; Hill, 1951; Hill & Hartree, 1953).

The relation between iron and the synthesis of bacteriochlorophyll and porphyrins in the photosynthetic bacteria suggests that iron may participate in the conversion of porphyrins or derivatives into chlorophyll. The situation is similar to that with *C. diphtheriae*, where intracellular haems are increased, whilst porphyrin excretion is decreased by iron (Pappenheimer & Hendee, 1947; Rawlinson & Hale, 1949). In *C. diphtheriae* there is no evidence that the porphyrins are converted quantitatively into haems in the presence of iron, and it is possible that the metal may act indirectly, rather than as a source of the iron atom in haem. It may also act at a similar stage in the formation of bacteriochlorophyll.

The results with *Rps. spheroides* do not show whether the action of iron on the synthesis of porphyrin and bacteriochlorophyll from glycine and α -oxoglutarate is a consequence of its effect on the conversion of ALA into protoporphyrin. The dependence of bacteriochlorophyll formation upon iron may be due partly, at least, to the participation of the metal in the synthesis of protoporphyrin. This porphyrin and its magnesium derivative may be intermediates in the synthesis of chlorophyll by *Chlorella* (Granick, 1948a, b). Since increased bacteriochlorophyll does not result when the cells are incubated with ALA even in the presence of iron, it is possible that precursors of the non-pyrrole parts of the pigment (for example, the phytol residue) may be supplied by glycine and/or α -oxoglutarate but not by ALA. In the presence of iron, therefore, synthesis of bacteriochlorophyll may proceed from glycine and α -oxoglutarate via protoporphyrin (or a derivative) but may stop at this stage when ALA is the substrate. However, this possibility does not account fully for the effects of iron, since the increase in bacteriochlorophyll formed from glycine and α -oxoglutarate with iron present accounts for only about 10 % of the porphyrins which fail to appear.

Bacteriochlorophyll contains magnesium, and it might be expected that deficiency in this metal would cause excretion of porphyrins or derivatives of bacteriochlorophyll lacking magnesium. This was not observed, possibly since magnesium is needed for many cell reactions besides the formation of bacteriochlorophyll.

SUMMARY

1. Cell suspensions of *Rhodospseudomonas spheroides* convert glycine and α -oxoglutarate into porphyrins. An oxidizable substrate, magnesium, ammonium and manganese ions are also required for maximum synthesis.

2. Porphyrins are formed from glycine and α -oxoglutarate only in the light; aerobiosis suppresses the synthesis in the light, whilst no porphyrins are formed aerobically in the dark.

3. Cells deficient in nicotinic acid, thiamine or biotin form little or no porphyrin from glycine and α -oxoglutarate, but do so when these factors are added to the incubation systems.

4. δ -Aminolaevulinic acid is converted into porphyrins by the cell suspensions and behaves as if it is a product of condensation between glycine and α -oxoglutarate or derivatives.

5. Porphobilinogen accumulates during the early stages of incubation with δ -aminolaevulinic acid and disappears as incubation proceeds.

6. δ -Aminolaevulinic acid is converted into porphyrins anaerobically in light or aerobically in the dark. In the light, maximum formation of porphyrins occurs in the presence of phosphate and magnesium ions only; under aerobic conditions an oxidizable substrate is also needed.

7. Iron salts almost completely suppress porphyrin formation from glycine and α -oxoglutarate, but increase the formation of bacteriochlorophyll and haem components. The increase in these substances accounts for only 10 and 1% respectively of the porphyrins which fail to appear. Iron does not suppress porphyrin synthesis from δ -aminolaevulinic acid.

8. Coproporphyrin III is the major component of the porphyrins formed from glycine and α -oxoglutarate and from δ -aminolaevulinic acid. Uroporphyrins I and III are also formed in small quantities, and traces of other porphyrins containing three and six carboxyl groups can be detected by chromatography.

9. Protoporphyrin, comprising 20–30% of the total porphyrins, is formed from δ -aminolaevulinic acid anaerobically in the light, provided that iron and an oxidizable substrate are present. Aerobically grown cells form protoporphyrin from δ -aminolaevulinic acid under aerobic conditions in the absence of iron.

10. It is suggested that porphyrin formation by *Rps. spheroides* is associated with the synthesis of bacteriochlorophyll and that iron is concerned in the conversion of porphyrins or derivatives into bacteriochlorophyll.

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An Investigation on Rabbit Antibodies by the Use of Partition Chromatography

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In a previous paper (Porter, 1955) a method was described for fractionating rabbit γ -globulin by partition chromatography. It was found that, while no separation into distinct components could be obtained, the protein was resolved into a series of overlapping fractions. When γ -globulin from rabbits immunized against ovalbumin was examined, the antibody was confined to the more slowly moving fractions after the first course of immunization, but as the injections were con-

tinued the antibody appeared in the middle fractions as well and later these contained the greater part of the antibody.

It was of interest to find whether during immunization of the rabbit the chromatographic behaviour of the antibodies was dependent on the nature of the antigen used. In addition to the pure protein antigen used earlier, killed bacteria (pneumococcus type III) and a living virus (Influenza Melbourne strain of type A) have been