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Adaptation to Form Bacteriochlorophyll in *Rhodopseudomonas spheroides*: Changes in Activity of Enzymes concerned in Pyrrole Synthesis

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(*Received* 12 *January* 1959)

Rhodopseudomonas spheroides, like other members of the Athiorhodaceae, is able to grow either anaerobically in the light or aerobically in the dark (van Niel, 1944). Organisms cultured under the former conditions are heavily pigmented and are rich in bacteriochlorophyll and carotenoids, while aerobically grown organisms contain only traces of these pigments (van Niel, 1944; Cohen-Bazire, Sistrom & Stanier, 1957).

Growth under anaerobic conditions occurs only in the light and is therefore dependent upon the intracellular pigments concerned in photosynthesis (photopigments), in particular bacteriochlorophyll. Kinetic studies with exponentially growing cultures of *R. spheroides* have shown that transfer of aerobically growing organisms to anaerobic conditions in the light results in the adaptation of all these organisms in the inoculum to form the photopigments, and synthesis of these substances precedes cell division (Cohen-Bazire *et al.* 1957).

Cultures of *R. spheroides* grown anaerobically in the light contain 40–100 times as much bacteriochlorophyll as those grown aerobically. The concentration of haematin compounds is similar in both types of organism, but is only a small fraction (1–5 %) of the amount of bacteriochlorophyll

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(Kamen & Vernon, 1955; Lascelles, 1956*a*). Adaptation to form the photopigment must therefore involve considerable diversion of intermediates derived from metabolism of the carbon and nitrogen sources of the medium towards synthesis of bacteriochlorophyll.

There is good evidence that the early stages in the formation of bacteriochlorophyll by *R. spheroides* are identical with those leading to porphyrins and that protoporphyrin, or a derivative, is an intermediate common to the synthesis of both bacteriochlorophyll and intracellular haematin compounds (Lascelles, 1956*a*, *b*). The conditions which permit synthesis of free porphyrins by suspensions of *R. spheroides* from glycine and α-oxoglutarate paralleled those which promote formation of bacteriochlorophyll, both pigments being formed from these substrates only anaerobically in the light. This could suggest that the change found in the amount of bacteriochlorophyll in cultures grown under the different conditions might be reflected in the activity of enzymes concerned in that part of the biosynthetic pathway which is common to the synthesis of porphyrins and bacteriochlorophyll. Changes in the levels of activity of these enzymes may be connected with the adaptation to form bacteriochlorophyll, and the present work was undertaken with this possibility in mind; particular attention was given to the enzymes involved in the synthesis of δ-aminolaevulic acid and its conversion into porphobilinogen.

Recently, synthesis of δ-aminolaevulic acid, the first demonstrable intermediate in porphyrin formation, has been demonstrated in cell-free extracts of *R. spheroides* (Kikuchi, Shemin & Bachmann, 1958*b*; Gibson, 1958; Kikuchi, Kumar, Talmage & Shemin, 1958*a*; Sawyer & Smith, 1958). The substrates are glycine and succinyl-coenzyme A, added as such or generated by succinate thiokinase from succinate, adenosine triphosphate and coenzyme A or by the action of the α-oxoglutarate dehydrogenase system. Pyridoxal phosphate is essential for the reaction. The enzyme, δ-aminolaevulic acid dehydrase, which converts δ-aminolaevulic acid into porphobilinogen, is also present in extracts of *R. spheroides* (Neuberger, Scott & Shuster, 1956).

In the present work, conditions have been established under which suspensions of *R. spheroides* harvested after aerobic growth in the dark become adapted to form bacteriochlorophyll within a short period of time. Extracts prepared from organisms at various stages during adaptation have been examined for their ability to form δ-aminolaevulic acid and porphobilinogen. Other enzymic activities have also been followed, to see whether the observed increases in the enzyme systems associated with pyrrole formation reflect a general increase in enzymic activity during adaptation. Comparison has also been made between enzymic activities in extracts of organisms freshly harvested after aerobic growth in the dark and anaerobic growth in the light.

EXPERIMENTAL

Chemicals

Coenzyme A (CoA, 70–75% pure), adenosine triphosphate (ATP) and 8-azaguanine were obtained from the Sigma Chemical Co., St Louis, Mo., U.S.A.; pyridoxal 5-phosphate from Roche Products Ltd., Welwyn Garden City, Herts; glutathione from British Drug Houses Ltd., Poole, Dorset; methylphenazonium methosulphate and DL-*p*-fluorophenylalanine from L. Light and Co., Colnbrook, Bucks; chloramphenicol from Parke, Davis and Co., Hounslow, Middlesex.

Succinyl-coenzyme A (succinyl-CoA) was prepared by the method of Simon & Shemin (1953) and was used immediately. The values quoted for succinyl-CoA are based on the amount of CoA used, assuming it to be 70% pure.

Phosphate buffers were made from KH_2PO_4 and K_2HPO_4. Tris buffers were prepared from 2-amino-2-hydroxymethylpropane-1:3-diol and HCl according to Gomori (1955). Other chemicals were commercial samples of the purest grade available or were prepared as described previously (Lascelles, 1956*a*).

Organisms

Unless stated otherwise the strain of *Rhodopseudomonas spheroides* used [National Collection of Industrial Bacteria (N.C.I.B.), no. 8253] was that described previously (Lascelles, 1956*a*). Another strain, no. 2.4.1. from the collection of Professor C. B. van Niel, was used in some experiments. Stock cultures of these organisms were maintained as previously described (Lascelles, 1956*a*).

Preparation of suspensions and extracts of organisms

Growth and harvesting. Unless stated otherwise, the medium (medium MS) was similar to that described previously (Lascelles, 1956*a*) with the following modifications: $MnSO_4$ (5 μM) was added and the concentration of L-glutamate was 0·01 M instead of 0·02 M. Ferric citrate (10 μM) was added where stated in the text. Details of the inoculum and of the methods used to grow organisms anaerobically in the light have been described previously (Lascelles, 1956*a*). Most experiments were with organisms which had been grown aerobically in the dark. These cultures were grown in 2 l. Erlenmeyer flasks containing 500 ml. of medium; they were incubated at 30° in a reciprocating, gyrorotary shaker (New Brunswick Scientific Co., New Brunswick, N.J., U.S.A.) operating at 100 rev./min. The organisms were harvested by centrifuging at the end of the logarithmic phase of growth (14–20 hr.) and were washed in one half the original culture volume of 0·02 M-phosphate buffer, pH 7·0. They were finally suspended to the required concentration in 0·04 M-phosphate buffer (pH 7·0) and 0·001 M-$MgSO_4$.

Experiments with suspensions of organisms. The harvested organisms were incubated in a mixture (standard mixture) containing the following (in μmoles/ml.): potassium phosphate buffer (pH 7·0) 6; $(NH_4)_2HPO_4$, 3; $MgSO_4$, 1; $CaCl_2$, 0·4; iron citrate, 0·01; $MnSO_4$, 0·005; sodium fumarate, 20; glycine and sodium α-oxoglutarate, 10 each. In studying the synthesis of bacteriochlorophyll in the dark, three methods of incubation were used according to the aims of the particular experiment: (1) To determine the effect of oxygen partial pressure, organisms (3–5 mg. dry wt.) were incubated in 5 ml. of the standard mixture in tubes shaped like an inverted T ('small T-tubes') similar to those described by Monod, Cohen-Bazire & Cohn (1951); the horizontal limb containing the suspension was of 1·5 cm. internal diameter and 15 cm. long. The vertical limb was closed with a bored rubber stopper with connexions of glass and rubber tubing, so that the vessels could be evacuated and filled with gas mixtures. The T-tubes were clamped to a rocking device (see van Heyningen & Gladstone, 1953) and shaken in a water bath at 36 oscillations/min. (2) The effect of components of the reaction mixture and of inhibitors was determined with organisms (3–5 mg. dry wt.) suspended in 5 ml. of reaction mixture in 25 ml. Erlenmeyer flasks; these were shaken in a Dubnoff metabolic incubator shaker (Precision Scientific Co., Chicago, Ill., U.S.A.) at 65 oscillations/min. under an atmosphere of $O_2 + N_2$ (6:94). (3) When the enzymic activity at various stages of adaptation was to be determined suspensions were set up on a larger scale. Organisms (150–200 mg. dry wt.) were suspended in 50 ml. of the standard mixture and were shaken as described above in big T-tubes of 3·3 cm. internal diameter and 19 cm. long.

Incubation in the light was in 1·5 cm. × 15 cm. tubes containing 5 ml. of suspension, or in 2 oz. medical flat bottles containing 50 ml. of suspension; anaerobic conditions were obtained by forcing the cotton plug into the tube or the neck of the bottle, placing on it a few crystals of pyrogallol and 2 drops of saturated K_2CO_3 solution and sealing with a rubber stopper. The vessels were incubated in front of a battery of 40–60 w tungsten lamps (Lascelles, 1956*a*). All incubations were at 34°.

Preparation of cell-free extracts. Extracts were prepared either from organisms freshly harvested and resuspended as described above, or from suspensions of organisms which had been first incubated in big T-tubes (see above). In this case the suspensions were centrifuged, the organisms washed in an equal volume of 0·02 M-potassium phosphate buffer (pH 7·0) and finally suspended in 10–20 ml. of 0·04 M-potassium phosphate buffer (pH 7·0) and 0·001 M-$MgSO_4$. These suspensions, containing 10–20 mg. dry wt. of organisms/ml., were disrupted by ultrasonic vibration for 4 min. at 25 kc./sec. on a Mullard ultrasonic generator type E 7590B (Mullard Ltd., London, W.C. 1); to avoid overheating, the transducer assembly was immersed in a rapidly flowing stream of tap water. Whole cells and debris were removed by centrifuging at 25 000 *g* for 10 min. at 0°. The supernatant was spun for a further 90 min. at 105 000 *g* in a Spinco ultracentrifuge, model L. This brought down a particulate fraction containing the bulk of the pigment; after removal of the clear supernatant the particles were suspended in 5–10 ml. of a mixture of 0·04 M-potassium phosphate buffer (pH 7·0) and 0·001 M-$MgSO_4$, and both fractions were kept at 0° until required for assays of enzymic activity.

Estimations

Dry weight of organisms. The extinction of suspensions was measured at 680 mμ and the dry wt. calculated from a calibration curve; absorption due to the photopigments is minimal at this wavelength (Cohen-Bazire *et al.* 1957).

Protein. The concentration of protein in extracts was determined colorimetrically by a method based on the biuret reaction (Layne, 1957) after removal of photopigments by treatment with hot acid–ethanol (Vernon & Kamen, 1953). Crystalline bovine plasma albumin (Armour Laboratories, London) was the standard.

Porphyrins and bacteriochlorophyll. Porphyrins were estimated spectrophotometrically in the supernatant fluid after removal of organisms by centrifuging (Lascelles, 1956*a*). The bacteriochlorophyll in the organisms was extracted with methanol and measured spectrophotometrically by the method of Cohen-Bazire *et al.* (1957).

Assay of enzymic activities

Synthesis of δ-aminolaevulic acid (ALA-synthesis system). The method used to determine the ability to form ALA depended on the presence in the extracts of succinic thiokinase, which formed succinyl-CoA (required for condensation with glycine) from succinate, ATP and CoA. This activity was constant in all extracts tested (see below). The reaction mixture for assay of the ALA-synthesis system contained: extract, 2–5 mg. of protein; tris buffer (pH 7·5), 75 μmoles; $MgSO_4$, 10 μmoles; $MnSO_4$, 0·025 μmole; ATP, 7·5 μmoles; pyridoxal phosphate, 0·1 μmole; glutathionine, 1·5 μmoles; CoA, 0·05 μmole; sodium succinate and glycine, each 150 μmoles; H_2O to 1·5 ml. Incubation was at 34° for 1–2 hr. in 1·5 cm. tubes. The reaction was stopped by addition of 0·5 ml. of 20% (w/v) trichloroacetic acid, and the precipitated protein removed by centrifuging. The ALA in 0·05–0·4 ml. samples of the supernatants was estimated colorimetrically after condensation with acetylacetone by the method of Mauzerall & Granick (1956).

Formation of porphyrins, porphyrinogens or porphobilinogen was not detectable under the conditions used and synthesis was not increased by anaerobic incubation in Thunberg tubes. The addition of glutathione improved synthesis slightly, provided that the final concentration did not exceed 1 mM. The amount of ALA formed was proportional to the amount of extract added over a range from 1 to at least 5 mg. of protein and the rate of synthesis by all types of extracts tested was linear for at least 3 hr.

The material estimated as ALA was confirmed to be this compound by paper chromatography in butanol–NH_3 and butanol–acetic acid by the methods of Mauzerall & Granick (1956). The pyrrole formed on condensation with acetylacetone had the same R_F values as those obtained with an authentic specimen of ALA.

δ-Aminolaevulic acid dehydrase. This enzyme was assayed by a slightly modified version of the method of Gibson, Neuberger & Scott (1955). The system contained: extract, 0·5–1·5 mg. of protein; potassium phosphate buffer, pH 7·0, 100 μmoles; $MgSO_4$, 10 μmoles; L-cysteine, 25 μmoles; ALA hydrochloride, 5 μmoles; H_2O to 2·5 ml. Incubation was at 34° in Thunberg tubes filled with H_2. The ALA was added from the side arm after 30 min. preincubation, and the incubation continued for a further 60 min. After deproteinization and destruction of SH

groups by the method of Gibson *et al.* (1955) the porphobilinogen in the supernatant was determined by the method of Mauzerall & Granick (1956).

The rate of reaction was dependent on the amount of protein only up to a concentration of about 0·6 mg./ml., and was linear with time for about 1 hr. On more prolonged incubation, porphyrinogens were also detectable.

Succinic thiokinase. This was assayed by a method similar to that of Kaufman (1955). The system contained: extract, 1–3 mg. of protein; tris buffer (pH 7·5), 100 μmoles; $MgSO_4$, 10 μmoles; sodium succinate, 200 μmoles; ATP, 5 μmoles; CoA, 0·05 μmole; L-cysteine, 20 μmoles; hydroxylamine (added as a hydroxylamine hydrochloride–NaOH mixture, pH 7·0), 800 μmoles; H_2O to 2·0 ml. Incubation was in 1·5 cm. tubes at 34° for 1 hr. The succinohydroxyamate formed was estimated by the method of Lipmann & Tuttle (1945).

Succinyl-CoA deacylase. This was assayed by a method similar to that described by Rendina & Coon (1957) for assay of deacylases of other esters of CoA. The system contained: extract, 0·2–0·4 mg. of protein; tris buffer (pH 7·5), 100 μmoles; $MgSO_4$, 10 μmoles; succinyl-CoA, 1 μmole; H_2O to 1·0 ml. Incubation was at 34° for 10 min. in Thunberg tubes filled with H_2, the extract being added from the side arm. The reaction was stopped by addition of 0·2 ml. of 9% (w/v) metaphosphoric acid. After centrifuging the precipitated protein, free SH groups were estimated by the method of Grunert & Phillips (1951). The amount of succinyl-CoA decomposed by the extracts was calculated after subtraction of the values obtained in controls without substrate and without extract. The rate of the reaction was linear for periods up to 20 min.

Succinic dehydrogenase and succinoxidase. Manometric techniques were used to assay these activities. They were assayed in the particulate fraction of extracts. The dehydrogenase was determined by following O_2 uptake at 34° in the presence of methylphenazonium methosulphate by the method of Singer & Kearney (1957). Succinoxidase was assayed by measurement of O_2 uptake in manometer vessels containing: particles, 3–6 mg. of protein; potassium phosphate buffer (pH 7·5), 100 μmoles; $MgSO_4$, 10 μmoles; sodium succinate, 100 μmoles; H_2O to 2·5 ml. Incubation was at 34° in air.

RESULTS

Adaptation to form bacteriochlorophyll by suspensions of aerobically grown organisms

Organisms harvested after growth under strongly aerobic conditions in medium MS with or without added Fe contained 0·2–0·5 μm-mole of bacteriochlorophyll/mg. dry wt. These low values are to be compared with those found in photosynthetically grown organisms which contain from 12–15 to 25–30 μm-moles/mg. when grown respectively without and with addition of Fe.

The first object was to establish conditions for synthesis of bacteriochlorophyll by suspensions of aerobically grown organisms.

Effect of atmosphere. Adaptation to form bacteriochlorophyll occurred only after a lag of 16 hr. or more when the harvested organisms were incubated anaerobically in the light in the standard mixture of salts, fumarate, α-oxoglutarate and glycine (Table 1). The long delay is to be expected since under anaerobic conditions these organisms depend entirely upon the photolysis of water to obtain energy, and the rate of this reaction depends on the concentration of intracellular bacteriochlorophyll.

Cohen-Bazire *et al.* (1957) observed that O_2 suppresses synthesis of photopigments by *R. spheroides*; pigment synthesis by cultures growing under continuous illumination ceased abruptly when the gas phase of N_2 was replaced by air, though the rate of growth was unchanged. On the basis of this and other observations they proposed that synthesis of photopigments can occur in the dark provided that the partial pressure of O_2 is reduced to sufficiently low levels. The effect of incubation of the suspensions under a reduced partial pressure of O_2 was therefore examined in the present work.

Rapid synthesis of bacteriochlorophyll occurred in the dark provided that incubation was under a low partial pressure of O_2 (Table 1). Under the experimental conditions described in Tables 1–4, where the concentration of organisms was 0·6–1 mg. dry wt./ml., maximum synthesis occurred in atmospheres containing 3–6% of O_2; little or no pigment was formed in atmospheres containing 10% or more of O_2. The optimum amount of O_2 had to be determined for each set of conditions since, as might be expected, it varied with the concentration of organisms, the surface area/volume ratio of the fluid and the rate of shaking. Suspensions containing 3–4 mg. dry wt. of organisms/ml. formed bacteriochlorophyll rapidly when shaken in air in large T-tubes (see Fig. 2).

Suspensions which had been first incubated in

Table 1. *Effect of atmosphere on adaptation to form bacteriochlorophyll*

Aerobically grown organisms (0·9 mg. dry wt./ml.) were suspended in 5 ml. of the standard mixture (see Experimental). They were incubated either in the dark in small T-tubes under mixtures of N_2 and O_2 or anaerobically in the light in 1·5 cm. tubes. Initial bacteriochlorophyll, 0·3 μm-mole/ml.

Incubation conditions	O_2 in gas phase (%)	Bacteriochlorophyll formed (μm-moles/ml.)
Dark, 6 hr.	0	0
	1	10
	3	18
	6	22
	10	0·8
	20	0
Anaerobic–light, 16 hr.	0	0
Anaerobic–light, 24 hr.	0	24

the dark in an atmosphere of 6% of O_2 until the level of bacteriochlorophyll had increased by a factor of 10 or more continued to synthesize the pigment and at a faster rate on transfer to anaerobic conditions in the light (Fig. 1). The total amount of pigment formed by suspensions treated in this way was 2–4 times that formed by suspensions incubated only in the dark under a low partial pressure of O_2.

Some growth, as judged by increase in extinction at 680 mμ, occurred during incubation, but whereas the extinction increased by less than a factor of 2, there was over a 100-fold increase in bacteriochlorophyll.

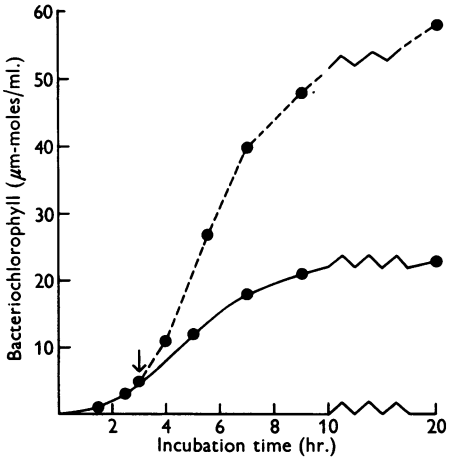

Fig. 1. Rate of synthesis of bacteriochlorophyll. Aerobically grown organisms (0·9 mg. dry wt./ml.) were incubated in 5 ml. of standard mixture in 25 ml. flasks in the Dubnoff shaker under an atmosphere of O_2+N_2 (6:94) (—). Flasks were removed at intervals and the bacteriochlorophyll was estimated as described in the text. At the time indicated by the arrow, suspensions were transferred to 1·5 cm. tubes and incubation continued anaerobically in the light (– – –). Initial bacteriochlorophyll, 0·2 μm-mole/ml. of suspension.

Requirements for synthesis of bacteriochlorophyll. The standard mixture (see Experimental) was based upon that found previously to promote maximum synthesis of porphyrins by light-grown cells under other experimental conditions (Lascelles, 1956*a*). Glycine, fumarate, α-oxoglutarate, Mg^{2+}, NH_4^+ and iron were needed for maximum synthesis of bacteriochlorophyll by suspensions of aerobically grown cells incubated under a decreased partial pressure of O_2 (Table 2). With the exception of iron, these requirements are similar to those previously found necessary for the formation of porphyrins.

The effect of iron was examined in more detail since previous work had shown that iron increased synthesis of bacteriochlorophyll but prevented formation of porphyrins by light-grown cells. The initial rate of synthesis by the aerobically grown cells incubated under a low partial pressure of O_2 did not differ greatly with or without added iron, but in its absence synthesis soon ceased. If, after preliminary incubation under a low partial pressure of O_2, the suspensions without iron were transferred to the light and incubated anaerobically,

Table 2. *Requirements for synthesis of bacteriochlorophyll*

Aerobically grown organisms (0·7 mg. dry wt./ml.) were suspended in the standard mixture with omission of the components as shown. Incubation was in 25 ml. flasks containing 5 ml. of suspension in the Dubnoff shaker under an atmosphere of O_2+N_2 (6:94). Initial bacteriochlorophyll, 0·5 μm-mole/ml.

Component omitted	Bacteriochlorophyll formed (μm-moles/ml.)
Nil	20
Glycine	6
α-Oxoglutarate	13
Fumarate	5
$MgSO_4$	2
$(NH_4)_2HPO_4$	10
Ferric citrate	5

Table 3. *Effect of iron on synthesis of bacteriochlorophyll and porphyrin*

Aerobically grown organisms (0·9 mg. dry wt./ml.) were suspended in the standard mixture with addition of ferric citrate (10 μM) as indicated. The suspensions (5 ml.) were incubated in the dark in 25 ml. flasks in the Dubnoff shaker under an atmosphere of O_2+N_2 (6:94). After preliminary incubation under these conditions, they were transferred to 1·5 cm. tubes and incubated anaerobically in the light for the periods indicated. Initial bacteriochlorophyll, 0·3 μm-mole/ml.

First incubation dark O_2+N_2 (6:94) (hr.)	Second incubation Anaerobic–light (hr.)	Bacteriochlorophyll (μm-moles/ml.)		Porphyrin* (μm-moles/ml.)	
		−Fe	+Fe	−Fe	+Fe
2	—	4·5	5	0	0
20	—	7	22	0	0
2	4	10	43	10	1
2	20	11	53	150	3

* Coproporphyrin III.

Table 4. *Effect of inhibitors on synthesis of bacteriochlorophyll*

Aerobically grown organisms (0·7 mg. dry wt./ml.) were suspended in the standard mixture with the additions shown. In Expt. 1 the suspensions (5 ml.) were incubated for 8 hr. in 25 ml. flasks in the Dubnoff shaker under an atmosphere of $O_2 + N_2$ (6:94). In Expt. 2 suspensions were first incubated without inhibitor for 4 hr. as in Expt. 1. The contents of the flasks were pooled, the organisms centrifuged and resuspended in fresh standard mixture. Samples (5 ml.) were incubated in 1·5 cm. tubes anaerobically in the light for 6 hr. with the additions shown.

Initial bacteriochlorophyll: Expt. 1, 0·3 μm-mole/ml.; Expt. 2, 6 μm-moles/ml.

Expt.	Inhibitor	Concn. (mM)	Bacteriochlorophyll formed (μm-moles/ml.)	Inhibition (%)
1	Nil	—	20	—
	Chloramphenicol	0·01	0	100
	DL-*p*-Fluorophenylalanine	2	7	65
	8-Azaguanine	2	6	70
2	Nil	—	57	—
	Chloramphenicol	0·01	0	100
	DL-*p*-Fluorophenylalanine	2	9	84
	8-Azaguanine	2	17	70

there was a small increase in bacteriochlorophyll and considerable quantities of coproporphyrin III appeared in the external fluids (Table 3). Suspensions with added iron when transferred to the light continued to form bacteriochlorophyll and only very small amounts of porphyrin were detected (Table 3). Porphyrin formation in the absence of iron was not observed until the suspensions had been transferred to anaerobic conditions in the light; these were the conditions which promoted maximum synthesis of bacteriochlorophyll with added iron.

Effect of inhibitors of protein and nucleic acid synthesis. Synthesis of bacteriochlorophyll was inhibited by low concentrations of chloramphenicol, which specifically inhibits synthesis of protein (Gale, 1953). Synthesis was also inhibited by the amino acid analogue, DL-*p*-fluorophenylalanine, and the purine analogue, 8-azaguanine (Table 4). These compounds prevented formation of bacteriochlorophyll when added to suspensions of organisms incubated under a low tension of O_2. The increased synthesis of pigment which occurred on subsequent transfer to anaerobic conditions in the light was also sensitive to these compounds, added after preliminary incubation under a low tension of O_2 (Table 4). These results suggest that formation of bacteriochlorophyll is associated with synthesis of both protein and nucleic acid.

Enzyme activity in cell-free extracts of harvested organisms

Before the activity of enzymes in organisms as they were becoming adapted to form bacteriochlorophyll were studied the enzyme systems were examined in extracts prepared from organisms freshly harvested after aerobic growth in the dark or anaerobic growth in the light. In these experiments the organisms were grown on medium MS with the addition of 10 μM-ferric citrate, and were harvested at the end of the logarithmic phase of growth (16–20 hr.). The photosynthetically grown organisms contained 40–100 times as much photopigment as those grown aerobically.

Distribution of enzymes in extracts. Extracts prepared by ultrasonic oscillation were separated by centrifuging into particulate and supernatant fractions (see Experimental) and the enzymic activity of each fraction was examined. The ALA-synthesis system, ALA dehydrase, succinic thiokinase and succinyl-CoA deacylase were found in the supernatant fraction only, while the succinic dehydrogenase and succinoxidase systems were confined to the particulate fraction. No differences in the distribution of any of these systems were found in extracts prepared from aerobically or photosynthetically grown organisms. The findings with respect to the ALA-synthesis system, ALA dehydrase and succinic thiokinase in *R. spheroides* are in agreement with those of Gibson (1958) and of Kikuchi *et al.* (1958*a*, *b*). The distribution of the other enzyme systems is in accord with observations made with a number of different organisms (Alexander, 1956).

Comparative activity of extracts. With the exception of succinyl-CoA deacylase (assayed in one extract of each type only) each enzyme system was assayed in extracts prepared from at least three different cultures of organisms grown under each condition, i.e. aerobically in the dark or anaerobically in the light. There was little variation in activity between different extracts prepared from organisms grown under the same conditions; the results shown in Tables 5 and 7 are from single typical experiments with extracts of organisms grown under the different conditions.

The activities of the ALA-synthesis system and of the ALA dehydrase were 4–5 times higher in

Table 5. *Enzyme activities in extracts of aerobically and photosynthetically grown organisms*

Organisms were grown on medium MS with addition of 10 μM-ferric citrate either aerobically in the dark or anaerobically in the light. The supernatant fractions obtained by centrifuging the cell-free extracts (see Experimental) were assayed for enzyme activity as described. The results are expressed as μmoles of product formed/mg. of protein/hr.

Enzyme activity assayed	Product determined	Activity in extracts from organisms grown: Aerobic–dark	Anaerobic–light
ALA synthesis	ALA	0·034	0·143
ALA dehydrase	Porphobilinogen	0·048	0·212
Succinic thiokinase	Succinohydroxamate	3·1	3·0
Succinyl-CoA deacylase	Free SH	1·35	1·4

Table 6. *Ability of mixtures of extracts to form δ-aminolaevulic acid*

R. spheroides N.C.I.B. no. 8253 and *R. spheroides* no. 2.4.1. were grown as in Table 5. The supernatant fractions from extracts were tested for ability to form ALA in the assay system (final vol. 1·5 ml.) described in the Experimental section. The ALA formed was estimated after incubation for 1 hr. at 34°. In col. 2 'aerobic' refers to extracts from organisms grown aerobically in the dark; 'light' refers to extracts from organisms grown anaerobically in the light.

Strain of *R. spheroides*	Extract (mg. of protein/tube)		ALA formed (μmole/tube)
N.C.I.B. no. 8253	Aerobic	2·3	0·076
	Light	1·1	0·143
	Aerobic + light	2·3 + 1·1	0·240
No. 2.4.1.	Aerobic	2·4	0·155
	Light	3·0	0·530
	Aerobic + light	2·4 + 3·0	0·691

Table 7. *Succinic dehydrogenase and succinoxidase in extracts of aerobically and photosynthetically grown organisms*

Organisms were grown as in Table 5. Succinic dehydrogenase and oxidase activities were determined in the particulate fractions of the extracts. Details of the assay methods are given in the Experimental section.

Growth of organisms	Activity of extracts (μl. of O_2/mg. of protein/hr.): Succinic dehydrogenase	Succinoxidase
Aerobic–dark	487	107
Anaerobic–light	373	20

extracts of the photosynthetically grown organisms (Table 5). Since the assay of the ALA-synthesis system depended upon the ability of the extracts to form succinyl-CoA from succinate, ATP and CoA, differences in the level of succinic thiokinase could lead to apparent differences in the activity of the ALA-synthesis system. The succinic thiokinase activity was, however, constant in extracts from aerobic and photosynthetic organisms (Table 5). Also, the differences in activity between the two types of extract were reflected in one experiment in which succinyl-CoA (2 μmoles in 1·5 ml.) was substituted for succinate, ATP and CoA in the standard assay. The ALA formed (μmole/mg. of protein/hr.) by extracts of aerobic and photosynthetic organisms was 0·018 and 0·075 respectively. The succinyl-CoA deacylase activity was high in both types of extract (Table 5).

Experiments in which known amounts of ALA (0·1–0·5 μmole) were added to extracts from aerobically and photosynthetically grown organisms showed little disappearance of this substance. Incubation for 2 hr. in the mixture used to assay the ALA-synthesis system (but without succinate and glycine) resulted in a loss of 20 % of the added ALA, and there was no difference between the two types of extract.

Kikuchi *et al.* (1958*a*) have reported, without experimental detail, that extracts from *R. spheroides* grown anaerobically in the light formed ALA more actively than those prepared from aerobically grown organisms. They also found that synthesis of ALA by extracts of organisms grown anaerobically in the light was inhibited by addition of extracts from organisms grown aerobically in the dark. Extracts from the strain of *R. spheroides* used in the present work (N.C.I.B. 8253) were cross-tested to see whether evidence for inhibition could be obtained. However, the amount of ALA formed by mixtures of each type of extract was additive (Table 6). Similar results were obtained with extracts of the strain of *R. spheroides* (no. 2.4.1., van Niel Collection) used by Kikuchi *et al.* (1958*a*) (Table 6). The difference between the present results and those of the American workers may be due to different methods of growing the organisms and of preparing the extracts.

Extracts of aerobically grown organisms had slightly greater succinic dehydrogenase activity, while the succinoxidase system was about five times as active (Table 7). Increased oxidase activity in extracts from aerobically grown *Rhodospirillum rubrum* has been observed by Crook & Lindstrom (1956).

Table 8. *Enzyme activities in suspensions during adaptation to form bacteriochlorophyll*

Organisms were grown aerobically on medium MS. Aerobic–dark incubation was with 50 ml. of suspension in the standard mixture shaken in air in large T-tubes. Where indicated these were transferred to medical flat bottles and incubated anaerobically in the light. Incubation with O_2 was effected by blowing O_2 through the suspension (50 ml. in a 150 ml. flask) with a sparger. Incubation under H_2 was in a stoppered flask filled with this gas. After incubation samples were taken for determination of dry wt. and bacteriochlorophyll; extracts were prepared from the washed organisms as described in the Experimental section. Enzyme activities were assayed in the supernatant fractions of the extracts as described.

Expt.	Incubation of suspensions	Dry wt. of organisms (mg./ml.)	Bacteriochlorophyll in suspensions (μm-moles/ml.)	Enzyme activity (μmoles of product/mg. of protein/hr.) ALA synthesis	ALA dehydrase	Succinic thiokinase
1	Nil	3	0·8	Not tested	0·034	2·75
	Aerobic–dark for 2 hr.	2·8	10	Not tested	0·049	2·6
	Aerobic–dark for 2 hr.; then anaerobic–light for 5 hr.	4·4	56	Not tested	0·094	2·7
2	Nil	3·3	0·3	0·033	0·049	2·7
	Aerobic–dark for 2 hr.	3·4	11	0·118	0·062	2·5
	Aerobic–dark for 2 hr.; then anaerobic–light for 3·5 hr.	4·3	35	0·109	0·077	2·5
	O_2–dark for 2 hr.	3·8	0·3	0·021	0·046	2·6
3	Nil	3·7	0·7	0·029	0·038	3·0
	Aerobic–dark for 2 hr.	3·8	13	0·118	0·051	3·0
	H_2–dark for 2 hr.	3·7	0·7	0·024	0·039	3·1

Enzymic activities of extracts from organisms becoming adapted to form bacteriochlorophyll

Enzymic activities were determined in extracts of organisms at various stages during the adaptation to form bacteriochlorophyll.

In these experiments relatively heavy suspensions of aerobically grown organisms (3–4 mg. dry wt./ml.) were shaken in air in the standard mixture in large T-tubes (see Experimental for full details); rapid synthesis of bacteriochlorophyll occurred under these conditions (Fig. 2). There was only a slight increase in cell mass as judged by determination of extinction at 680 mμ (see Tables 8–10).

Enzymes concerned in pyrrole synthesis. An increase in the ability to form ALA was found in extracts of organisms after 20 min. incubation under the above-mentioned conditions; this increase occurred before there was detectable synthesis of bacteriochlorophyll. The activity increased steadily and after 90 min. reached a maximum level, which was 4–5 times that in extracts from the unadapted organisms (Fig. 2). The ALA dehydrase activity increased only slightly (1·2–1·6-fold in different experiments) during the aerobic incubation.

The enzyme activities were also examined in extracts prepared from organisms which had received a first incubation aerobically in the dark followed by a second one anaerobically in the light. No further increase in the activity of the ALA-synthesis system occurred on transfer to anaerobic conditions in the light (Table 8, Expt. 2). The ALA dehydrase did show a further increase which varied in different experiments, sometimes reaching a level three times that originally present (Table 8).

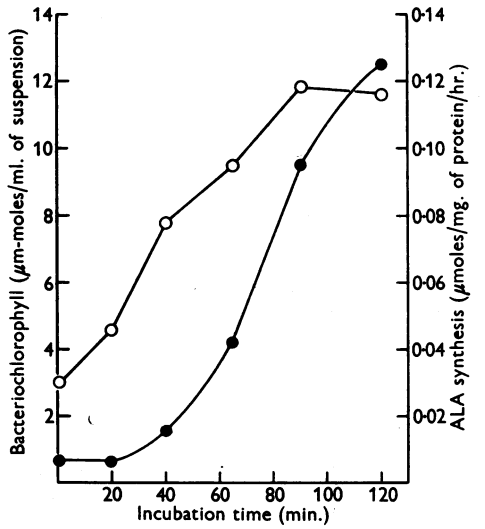

Fig. 2. Increase in ALA-synthesis activity during adaptation to form bacteriochlorophyll. Aerobically grown organisms (3·3 mg. dry wt./ml.) were incubated in 50 ml. of the standard mixture in large T-tubes aerobically in the dark. Tubes were removed at intervals, samples taken for determination of bacteriochlorophyll (●) and the organisms centrifuged and washed. Extracts were prepared and the ALA-synthesis system (○) was assayed in the supernatant fractions as described in the text.

The increase in enzyme activity occurred only under conditions which permitted formation of bacteriochlorophyll. There was no increase in either the ALA-synthesis system or in ALA dehydrase when the suspensions were subjected to

a high partial pressure of O_2, nor when they were incubated anaerobically in the dark; under neither condition was there synthesis of bacteriochlorophyll (Table 8, Expts. 2 and 3).

The increase in the activity of both enzyme systems was completely prevented by 0·1 mM-chloramphenicol, which also prevented synthesis of bacteriochlorophyll (Table 9).

Activity of other enzyme systems. No change was observed in either the succinic thiokinase (Table 8) or succinyl-CoA deacylase (Table 9) activity during adaptation to form bacteriochlorophyll. The succinoxidase activity decreased during adaptation, particularly on transfer of the suspensions to anaerobic conditions in the light (Table 10). There was a less marked decrease in the succinic dehydrogenase activity.

DISCUSSION

The adaptation of *R. spheroides* to form photopigments is an interesting example of metabolic regulation and has been discussed in great detail by Cohen-Bazire *et al.* (1957) and by Stanier & Cohen-Bazire (1957). The observation that suspensions of *R. spheroides* form bacteriochlorophyll in the dark under a low partial pressure of O_2 supports their hypothesis that synthesis of the photopigments is controlled by the state of oxidation of a carrier in the electron transport system; this, in turn, is thought to be influenced by the partial pressure of O_2 or, under anaerobic conditions, by the light intensity. The mechanism by which this hypothetical factor exerts its affect on metabolism is quite unknown.

Knowledge of the general metabolism of the Athiorhodaceae is still rather fragmentary. There is good evidence for the existence of the tricarboxylic acid cycle in *Rhodospirillum rubrum* (Eisenberg, 1953; Elsden, 1954; Elsden & Ormerod, 1956; Ormerod, 1956) and there is no reason to assume that the metabolism of *R. spheroides* differs from this organism to any major extent. The intracellular levels of succinyl-CoA or succinate may play an important role in the adaptation to form bacteriochlorophyll. Under highly aerobic conditions oxidation or assimilation of succinate via the tricarboxylic acid cycle may be enhanced, whereas under low partial pressures of O_2 diversion of these intermediates towards synthesis of porphyrins and bacteriochlorophyll may be favoured.

An increase in the activity of the enzyme system responsible for the formation of ALA from succinyl-CoA and glycine might also contribute to the diversion of succinyl-CoA towards synthesis of pyrrole derivatives. The activity of this system was observed to increase before there was detectable synthesis of bacteriochlorophyll (Fig. 2), and this is consistent with the possibility that adaptation to form this pigment is dependent upon the ability to

Table 9. *Enzyme activities in suspensions incubated with chloramphenicol*

Organisms were grown as in Table 8. Suspensions (50 ml.) were incubated aerobically in the dark in large T-tubes in the standard mixture with addition of chloramphenicol as shown. After removal of samples for determination of dry wt. and bacteriochlorophyll, extracts were prepared and the enzyme activities assayed in the supernatant fractions as described.

Incubation of suspensions	Chloramphenicol (mM)	Dry wt. of organisms (mg./ml.)	Bacteriochlorophyll in suspensions (μm-moles/ml.)	Enzyme activity (μmoles of product/mg. of protein/hr.) ALA synthesis	ALA dehydrase	Succinyl-CoA deacylase
Nil	—	3·4	0·7	0·037	0·039	1·6
Aerobic–dark for 2 hr.	0	3·8	13	0·153	0·064	1·7
	0·1	3·4	0·6	0·034	0·035	1·6

Table 10. *Succinic dehydrogenase and succinoxidase activities in suspensions during adaptation*

Organisms were grown as in Table 8. Suspensions were incubated in 50 ml. of the standard mixture aerobically in the dark in large T-tubes; where indicated they were transferred to medical flat bottles and incubated anaerobically in the light. After removal of samples for determination of dry wt. and bacteriochlorophyll, extracts were prepared and the enzyme activities assayed in the particulate fraction as described in the Experimental section.

Incubation of suspensions	Dry wt. of organisms (mg./ml.)	Bacteriochlorophyll in suspensions μm-moles/ml.)	Enzyme activity (μl. of O_2/mg. of protein/hr.) Succinic dehydrogenase	Succinoxidase
Nil	3·4	0·3	133	44
Aerobic–dark for 2·5 hr.	3·6	16	144	30
Aerobic–dark for 2·5 hr.; then anaerobic–light for 4 hr.	4·1	53	99	24

form ALA. Also, the activity of this enzyme system was enhanced only under conditions where formation of bacteriochlorophyll occurred; there was neither an increase in enzyme activity nor synthesis of pigment under a high partial pressure of O_2 or under anaerobic conditions in the dark. The increase in ALA dehydrase was less marked but it may also be concerned with the adaptation process since it, too, occurred only under conditions which permitted synthesis of bacteriochlorophyll.

There are at least three possibilities which could account for the increased ability to form ALA, observed both in suspensions during adaptation and also in organisms harvested after anaerobic growth in the light: (*a*) An inhibitor of the enzyme system may be inactivated by incubation under a reduced partial pressure of O_2. Such an inhibitor may be formed by organisms under highly aerobic conditions but not by those growing anaerobically in the light at the relatively low intensities used in the present work. Evidence for such an inhibitor in extracts of aerobically grown organisms was not found. This is in disagreement with the observations of Kikuchi *et al.* (1958*a*), but the differences may reside in the physiological state of the organisms and in the methods used to prepare extracts. (*b*) Enzymes concerned in the metabolism of ALA by pathways that do not lead to porphyrins and bacteriochlorophyll may be increased under highly aerobic conditions. Little evidence for this was found; there was only a slight disappearance of ALA added to extracts incubated under the conditions used to assay the ALA-synthesis system. Gibson (1958) found ALA to be rapidly metabolized to unknown products by fresh extracts of *R. spheroides*. Again, these discrepancies may be due to differences in the methods of preparation of the extracts. (*c*) Synthesis of the enzyme(s) concerned in the condensation of succinyl-CoA and glycine may increase under a low partial pressure of O_2 but may be suppressed by high partial pressures. This possibility is favoured by the observation that chloramphenicol completely prevented the increase in activity of the ALA-synthesis system when added to suspensions incubated under a low partial pressure of O_2. Also, there was no increase in activity when organisms were incubated under completely anaerobic conditions in the dark; under these conditions the organisms would have no means of obtaining energy for synthesis of protein.

Definite conclusions about the nature of the observed increases in activity can be reached only when there is more information about the mechanism of synthesis of ALA.

Free porphyrins accumulate in cultures of *R. spheroides* only when grown anaerobically in the light, and are not detectable during aerobic growth in the dark (Lascelles, 1955). This is consistent with the observation that both the ALA-synthesis system and the ALA dehydrase are 4–5 times as active in organisms which have been grown anaerobically in the light as in those grown aerobically in the dark.

The experiments do not give any idea of the mechanisms by which the levels of these enzymes are controlled. It is possible that the regulating factor in the electron-transport chain postulated by Cohen-Bazire *et al.* (1957) may act either indirectly or directly by some type of 'repressor' or 'feedback' mechanism, such as those found to operate in other biosyntheses (Vogel, 1957; Yates & Pardee, 1957; Umbarger & Brown, 1958).

The increased enzyme activity observed in these experiments can account only partially for the ability of *R. spheroides* to form photopigments under the appropriate conditions. The synthesis of carotenoids as well as the formation of the phytol moiety of bacteriochlorophyll must be considered. It seems likely that these substances have a common origin, but as yet there is no information on the early stages of their synthesis by photosynthetic bacteria. Also, the formation of chromatophores, the submicroscopic units to which the photopigments are bound in these organisms (Schachman, Pardee & Stanier, 1952), may play an important part in the adaptation process. The fact that synthesis of bacteriochlorophyll by suspensions of *R. spheroides* requires the presence of a nitrogen source (in addition to glycine) and is inhibited by compounds which interfere with protein formation suggests that synthesis of both pigment and protein are closely interwoven.

Oxygen influences the formation of pyrrole pigments in many organisms. The level of cytochromes increases with diminishing partial pressures of O_2 in cultures of pseudomonads (Lenhoff, Nicholas & Kaplan, 1956; Rosenberger & Kogut, 1958) and in *Aerobacter aerogenes* (Moss, 1956). Similar effects have been found in animals. For instance, some invertebrates synthesize more haemoglobin under conditions of low aeration than they do in a highly aerobic environment (Fox, 1955). The regulatory mechanisms operating in photosynthetic bacteria may be similar to those concerned in the formation of pyrroles by other organisms.

SUMMARY

1. *Rhodopseudomonas spheroides* when grown aerobically in the dark forms only traces of bacteriochlorophyll. Suspensions of such organisms synthesize bacteriochlorophyll when incubated in the dark under a low partial pressure of oxygen in a mixture containing salts, glycine, α-oxoglutarate and fumarate. The rate of synthesis and the total

amount of bacteriochlorophyll formed are increased by subsequent incubation of the suspensions under anaerobic conditions in the light.

2. Iron is necessary for maximum synthesis of bacteriochlorophyll; in its absence coproporphyrin III accumulates.

3. Synthesis of bacteriochlorophyll by the suspensions in the dark under a low partial pressure of oxygen or on subsequent transfer to anaerobic conditions in the light is inhibited by chloramphenicol, *p*-fluorophenylalanine and 8-azaguanine.

4. Enzyme activities were assayed in cell-free extracts prepared at various stages during adaptation of the suspensions to form bacteriochlorophyll. There is a four- to five-fold increase in the activity of the system responsible for synthesis of δ-aminolaevulic acid from glycine and succinyl-coenzyme A during adaptation, and the δ-aminolaevulic acid dehydrase activity also increases. The succinic thiokinase and succinyl-coenzyme A deacylase activities remain unchanged and succinic dehydrogenase and succinoxidase decrease slightly.

5. The δ-aminolaevulic acid synthesis system and the δ-aminolaevulic acid dehydrase in extracts prepared from organisms harvested after anaerobic growth in the light are 4–5 times as active as in extracts from organisms grown aerobically in the dark.

6. The level of activity of enzymes concerned in the initial stages of pyrrole synthesis may influence the ability of *Rhodopseudomonas spheroides* to become adapted to form bacteriochlorophyll.

It is a pleasure to thank Professor D. D. Woods, F.R.S., for his interest and encouragement in this work.

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