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Studies on the Biosynthesis of Porphyrin and Bacteriochlorophyll by *Rhodopseudomonas spheroides*

1. THE EFFECT OF GROWTH CONDITIONS

BY K. D. GIBSON, A. NEUBERGER AND G. H. TAIT*
Department of Chemical Pathology and Medical Research Council Research Group in Enzymology, St Mary's Hospital Medical School, London. W. 2

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The various enzymes concerned with the synthesis of porphyrins from glycine and succinyl-coenzyme A (succinyl-CoA) have been found in plants and micro-organisms as well as in animal tissues. Thus the enzyme (aminolaevulate synthetase) responsible for the formation of δ-aminolaevulate has been shown to occur in the photosynthetic micro-organism *Rhodopseudomonas spheroides* (Gibson, 1958; Kikuchi, Kumar, Talmage & Shemin, 1958) and the enzyme aminolaevulate dehydratase catalysing the conversion of δ-aminolaevulate into porphobilinogen has been detected in extracts of *Rps. spheroides* (Neuberger, Scott & Shuster, 1956), of *Chlorella* (Bogorad & Granick, 1953) and of spinach (Bogorad, 1955). The enzymic formation of uroporphyrinogen has been shown in extracts of wheat germ (Bogorad, 1958*a*) and its decarboxylation to coproporphyrinogen in extracts of *Chlorella* (Bogorad, 1958*b*). The conversion of coproporphyrinogen III into protoporphyrin IX has been studied in detail only in liver mitochondria (Sano & Granick, 1961), but it seems reasonable to assume that a similar enzyme is present in all cells containing haem proteins or chlorophyll.

Granick (1948*a*, *b*; 1950) found that protoporphyrin, magnesium protoporphyrin and magnesium vinyl phaeoporphyrin a_5 accumulated in different mutants of *Chlorella* which were unable to form chlorophyll. He proposed that these substances were intermediates in the conversion of protoporphyrin into chlorophyll and that their accumulation was caused by the absence of the enzymes required for their further metabolism. Granick (1960, 1961) has identified magnesium protoporphyrin monomethyl ester and protoporphyrin monomethyl ester in another mutant of *Chlorella* and has also detected the former substance in barley seedlings incubated with aminolaevulate. Although the enzymes involved were not known, Granick (1960) suggested a biosynthetic pathway from protoporphyrin to chlorophyll which fits the evidence at present available. Tait & Gibson (1961) reported briefly on the conversion of magnesium protoporphyrin into magnesium protoporphyrin monomethyl ester by chromatophore preparations of *Rps. spheroides*.

Factors controlling porphyrin and bacteriochlorophyll biosynthesis in *Rps. spheroides* have been studied by Lascelles and by Stanier and his co-workers. Lascelles (1956) found that, on illumination of organisms in a medium containing α-oxoglutarate and glycine, porphyrins (mainly coproporphyrin) were excreted into the medium. Organisms previously grown in a medium deficient in biotin, nicotinic acid or thiamine did not form

* Some of this work forms part of a Ph.D. Thesis submitted to the University of London in July 1961 by G. H. Tait.

porphyrins under these conditions unless the missing factor were added. However, the ability of organisms to form porphyrins from added aminolaevulate was not impaired by vitamin deficiency.

Cohen-Bazire, Sistrom & Stanier (1957) showed that organisms grown aerobically in the dark in a medium containing malate as carbon source contained little bacteriochlorophyll or carotenoid, but that those grown anaerobically in the light in the same medium were highly pigmented. When the organisms were transferred from the dark to the light, pigment synthesis occurred after a lag phase. Addition of oxygen to organisms growing and forming pigment anaerobically in the light inhibited pigment synthesis immediately, but did not inhibit growth; removal of oxygen then resulted in an immediate synthesis of pigment. Increasing the light intensity had the same effect as addition of oxygen, and lowering the light intensity had the same effect as removal of oxygen. On the basis of these results Cohen-Bazire *et al.* (1957) suggested that pigment synthesis is controlled by the state of oxidation or reduction of some components of the electron-transport chain. Lascelles (1959, 1960*a*) analysed these conditions more closely and found that organisms grown aerobically in the dark at a high oxygen pressure not only contained much less pigment than those grown photosynthetically, but also showed only a quarter of the specific activity of aminolaevulate synthetase and aminolaevulate dehydratase. On transference of these organisms to anaerobic conditions in the light or to low oxygen pressure in the dark, pigment was formed and the specific activities of these enzymes increased. This adaptation could be blocked by various inhibitors of protein synthesis.

Lascelles (1956) reported that addition of iron to suspensions of *Rps. spheroides* illuminated in a medium containing α-oxoglutarate and glycine inhibited porphyrin excretion, and also (Lascelles, 1960*a*) that haemin or aminolaevulate added to cultures growing anaerobically in the light repressed the synthesis of aminolaevulate synthetase and aminolaevulate dehydratase.

These investigations have implicated several factors which may control the biosynthesis of porphyrins and bacteriochlorophyll in *Rps. spheroides*, and further results on this subject are presented in this paper. The work of Lascelles (1956) indicated that biotin might be involved in the production of aminolaevulate but not in its further conversion into porphyrins. In this paper it is shown that the activity of aminolaevulate synthetase is reduced in biotin-deficient organisms, whereas a number of other enzymes are not affected. This is not a specific effect of biotin, since other growth conditions which are unfavourable to bacteriochlorophyll synthesis also cause a fall in the activity of aminolaevulate synthetase. Some of this work has been reported earlier (Neuberger, 1961; Gibson, Matthew, Neuberger & Tait, 1961).

MATERIALS AND METHODS

Chemicals

NAD^+, α-oxoglutaric acid and ATP were obtained from Boehringer und Soehne, Mannheim, Germany; CoA (70% pure) and avidin [2·5 units/mg. of protein: 1 unit of avidin combines with 1 μg. of biotin (Eakin, Snell & Williams, 1941)] from Nutritional Biochemicals Corp., Cleveland, Ohio; preparations of avidin (4·9 and 9·6 units/mg. of protein) were kindly supplied by Dr N. M. Green; pyridoxal 5-phosphate and D-biotin were obtained from Roche Products Ltd., Welwyn Garden City, Herts. δ-Aminolaevulic acid was prepared by the method of Shemin (1957). Aminoacetone hydrochloride was prepared by catalytic hydrogenation (Pd–charcoal at atmospheric pressure) of oximinoacetone (A. Neuberger & J. J. Scott, unpublished work); it was recrystallized from dry ethanol by adding ether. Ovalbumin and ovomucoid were kindly supplied by Dr R. D. Marshall. γδ-Dioxovaleric acid was prepared by Dr A. T. Carpenter by a modification of the method of Nemeth, Russell & Shemin (1957). Succinyl-CoA was prepared by the method of Simon & Shemin (1953), acidified to pH 3 with HCl, and kept frozen until required. 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 and tris–HCl buffers according to Gomori (1955).

Growth and harvesting of organisms

The strain of *Rps. spheroides* was obtained from Dr J. Lascelles (National Collection of Industrial Bacteria, no. 8253) and was maintained in stab culture as described by Lascelles (1956).

In all experiments unless otherwise stated, organisms were grown semi-anaerobically in the light at 32–34° for 44 hr. in 'medium S' of Lascelles (1956) in 500 ml. or 1000 ml. conical flasks filled almost to the top. No special precautions were taken to make the conditions strictly anaerobic. The illumination was supplied and the temperature maintained by 60w bulbs at an appropriate distance from the flasks. The vitamin concentrations in medium S are: biotin, 40 μmM; nicotinic acid, 8 μM; thiamine, 3 μM. Vitamin-deficient organisms were grown in medium S containing concentrations of the appropriate vitamin low enough (in general, concentrations of biotin lower than 5 μmM, of thiamine lower than 10 μmM and of nicotinic acid lower than 0·5 μM) to give suboptimum growth. For growth in acetate the medium was as described above except that sodium acetate (0·04M) plus $NaHCO_3$ (0·005M) replaced the sodium malate (0·02M). Aerobic growth in the dark was carried out in medium S as described by Lascelles (1956).

After growth the organisms were harvested by centrifuging at 0°, washed with half the original culture volume of cold 0·05M-phosphate buffer, pH 7·4, and suspended at a concentration of 80 mg. dry wt./ml. This suspension was used immediately or kept frozen at −20° until required.

Procedure for the study of porphyrin and bacteriochlorophyll synthesis with whole cells

Synthesis from α-oxoglutarate and glycine. The washed cells were suspended at a final concentration of 1·0 mg. dry wt./ml. in mixture I of Lascelles (1956) and incubated semi-anaerobically in the light for 18–24 hr. at 32–34° in 10 mm. × 100 mm. tubes containing 5 ml. of reaction mixture. This medium is referred to below as 'mixture I', and the above conditions as 'standard illumination conditions'.

Synthesis from succinate and glycine. Incubations were carried out as described above except that succinic acid (0·01 M) replaced α-oxoglutaric acid (0·01 M) ('mixture IS').

Synthesis from aminolaevulic acid. The illuminations were carried out in the same way in 'mixture II' of Lascelles (1956).

Preparation of cell-free extracts

Suspensions of organisms (80 mg. dry wt./ml.) were disrupted in the Hughes (1951) press and diluted with 2 vol. of 0·05 M-phosphate buffer, pH 7·4. The suspensions were centrifuged for 90 min. at 105 000*g* in the Spinco Model L ultracentrifuge. The clear supernatants were kept at −20° until required and used without further purification.

Estimations

Dry weight of organisms. The extinction of suspensions was measured at 680 mμ and the dry weight calculated from a calibration curve. Absorption due to pigments is minimal at this wavelength (Cohen-Bazire *et al.* 1957). The term 'dry weight' used follows general practice, although the turbidity, which was measured, is not necessarily proportional to either cell number or cell mass.

Bacteriochlorophyll and carotenoids. These pigments were extracted from the organisms and measured spectrophotometrically by the methods of Cohen-Bazire *et al.* (1957).

Porphyrins. Porphyrins excreted into the medium by illuminated suspensions of organisms were estimated after removal of the organisms by centrifuging, as described by Lascelles (1956).

Protein. The concentration of protein in extracts of organisms was measured by the biuret reaction (Layne, 1957), with crystalline bovine plasma albumin (Armour and Co. Ltd.) as standard.

Aminoketones. The amount of aminolaevulate and aminoacetone was estimated colorimetrically after condensation with acetylacetone by the method of Mauzerall & Granick (1956) as modified by Gibson, Laver & Neuberger (1958).

Assay of enzymic activities

δ-Aminolaevulate synthetase. The reaction mixture contained enzyme extract (0·05–0·3 mg. of protein), phosphate buffer (pH 7·4, 37·5 μmoles), glycine (12·5 μmoles), succinyl-CoA (0·2 μmole), pyridoxal phosphate (0·05 μmole) and water to 0·375 ml. Incubation was at 37° for 30 min. The reaction was stopped by addition of 0·175 ml. of 20% (w/v) trichloroacetic acid. The precipitated protein was removed by centrifuging and washed with 0·45 ml. of 5% (w/v) trichloroacetic acid. The aminolaevulate was estimated in the combined supernatants. The amount formed was proportional to the amount of extract added over the range stated. Under the conditions of this assay no porphobilinogen or porphyrins were detected. Activity is expressed as μmoles of aminolaevulic acid/mg. of protein/hr.

δ-Aminolaevulate dehydratase. This enzyme was assayed by the method of Gibson, Neuberger & Scott (1955). The production of porphobilinogen was proportional to the amount of extract added over the range 1–5 mg. of protein. Activity is expressed as μmoles of porphobilinogen/mg. of protein/hr.

L-*Threonine dehydrogenase.* The properties of this enzyme have been described briefly by Neuberger & Tait (1960). The assay mixture contained enzyme extract (0·1–0·6 mg. of protein), DL-threonine (50 μmoles), NAD^+ (0·3 μmole) and tris buffer (pH 8·8, 75 μmoles), in a volume of 0·75 ml. Incubation was at 37° for 45 or 60 min. The reaction was stopped by addition of 0·25 ml. of 20% trichloroacetic acid and aminoacetone was estimated in the supernatant after centrifuging. The aminoacetone produced was proportional to the amount of extract added. Activity is expressed as μmoles of aminoacetone/mg. of protein/hr.

D-*β-Hydroxybutyrate dehydrogenase.* This enzyme was detected in extracts of *Rps. spheroides* prepared as described above (A. Neuberger & G. H. Tait, unpublished results). Its presence has been reported in *Rps. spheroides* by Carr & Lascelles (1961) and in *Rhodospirillum rubrum* by Doudoroff, Merrick & Contopoulou (1961). The assay mixture contained DL-β-hydroxybutyric acid (10 μmoles), NAD^+ (1 μmole), tris buffer (pH 8·8, 300 μmoles), enzyme extract (45–200 μg. of protein) and water to 3·0 ml. The reduction of NAD^+ was followed spectrophotometrically at 340 mμ in cells with 1 cm. light path held at 37° in a thermostatically-controlled cell carriage. The production of NADH was linear for 3 min. and the quantity formed was proportional to the amount of extract added over the range stated. Activity is expressed as μmoles of NADH/mg. of protein/hr.

L-*Alanine–γδ-dioxovalerate aminotransferase* (*transaminase*). This enzyme was detected in extracts of *Rps. spheroides* (A. Neuberger & G. H. Tait, unpublished results). The assay mixture contained enzyme extract (0·1–0·4 mg. of protein), L-alanine (10 μmoles), γδ-dioxovaleric acid (1 μmole) and phosphate buffer (pH 7·4, 75 μmoles), in a total volume of 0·75 ml. After incubation at 37° for 15 min. the reaction was stopped with 0·25 ml. of 20% trichloroacetic acid and the aminolaevulate was estimated. Activity is expressed as μmoles of aminolaevulic acid/mg. of protein/hr. Although this assay is valid for comparative purposes it is doubtful whether the results obtained are quantitatively reliable since chromatographic analysis showed that preparations of radioactive γδ-dioxovaleric acid, stored for prolonged periods, contain other compounds. γδ-Dioxovaleric acid also combines readily with bovine plasma albumin and presumably with other proteins (F. F. Richards, personal communication).

RESULTS

Growth and formation of pigments

Lascelles's (1956) work suggested that formation of aminolaevulate from α-oxoglutarate and glycine is blocked in organisms deficient in nicotinic acid, thiamine or biotin, but that its conversion into porphyrins is not affected. She suggested that deficiency of nicotinic acid or thiamine might prevent the conversion of α-oxoglutarate into

succinyl-CoA, but offered no explanation for the role of biotin. This work has been confirmed and extended (Table 1). The formation of porphyrins and bacteriochlorophyll by aerobically-grown biotin-deficient organisms illuminated in the presence of succinate and glycine (mixture IS) requires the addition of biotin in the same way as formation from α-oxoglutarate and glycine (mixture I). Lascelles (1959) stated that adaptation occurs before aerobically grown organisms can form pigment on illumination. To test the possibility that the effect of biotin might be exerted on this process, the experiments in Table 1 were repeated with organisms grown anaerobically in the light. The results were identical and in subsequent experiments the organisms were grown in this way.

The formation of porphyrins and bacteriochlorophyll by normal organisms (i.e. organisms grown in standard medium S) illuminated in mixture I or IS with no further addition of vitamins is accompanied by growth to the extent that the dry weight is approximately doubled in 24 hr. Biotin-deficient organisms, which form pigments on illumination in these mixtures only when biotin is added, also grow in the presence of biotin (Table 1). Qualitatively the association between growth and formation of bacteriochlorophyll has been observed consistently in a large number of experiments, although the quantitative increases vary from one batch of organisms to the other. The production of porphyrins in general follows the same pattern, although with larger fluctuations; however, there are some notable exceptions which are discussed by Gibson, Neuberger & Tait (1962). Thus organisms deficient in nicotinic acid or thiamine, which do not form porphyrins in mixture I unless the appropriate vitamin is added (Lascelles, 1956), also grow and form bacteriochlorophyll but only in the presence of the vitamin (Table 2). Further, growth inhibitors such as *p*-fluorophenylalanine and

Table 1. *Growth and synthesis of porphyrin and bacteriochlorophyll by biotin-deficient organisms*

Organisms grown aerobically in the dark in medium S containing 0·8 μmM-biotin were harvested, and then illuminated under standard conditions for 24 hr. Biotin (40 μmM) was added where stated. For estimations of dry weight, porphyrin and bacteriochlorophyll see the Methods section. In this and subsequent Tables the concentrations of bacteriochlorophyll and porphyrin are expressed as μm-moles/ml. of illumination mixture. Non-illuminated controls were kept at 0° in the dark; under these conditions the concentration of bacteriochlorophyll did not alter.

Medium	Biotin	Dry wt. of organisms (mg./ml.)	Porphyrin (μm-moles/ml.)	Bacteriochlorophyll (μm-moles/ml.)
Mixture I	−	1·00	1·3	4·1
Mixture I	+	2·73	33·8	12·9
Mixture IS	−	0·87	3·6	3·7
Mixture IS	+	1·86	28·2	8·1
Mixture II	−	0·87	11·8	2·3
Mixture II	+	0·85	10·7	1·2
Non-illuminated control	−	0·93	0·0	1·8

Table 2. *Growth and synthesis of porphyrin and bacteriochlorophyll by organisms deficient in nicotinic acid or thiamine*

Organisms were grown anaerobically in the light: normal organisms in standard medium S, nicotinic acid-deficient organisms in the presence of 0·45 μM-nicotinic acid and thiamine-deficient organisms in the presence of 5 μmM-thiamine. They were then illuminated in mixture I under standard conditions for 24 hr. with the additions stated.

Expt.	Organisms	Addition	Dry wt. (mg./ml.)	Porphyrin (μm-moles/ml.)	Bacteriochlorophyll (μm-moles/ml.)
1	Normal	—	2·04	19·2	35·7
	Normal (not illuminated)	—	1·14	0·0	24·9
	Nicotinic acid-deficient	—	1·44	5·1	24·5
	Nicotinic acid-deficient	Nicotinic acid (8 μM)	2·44	6·0	33·4
	Nicotinic acid-deficient (not illuminated)	—	1·14	0·0	19·5
2	Normal	—	2·50	14·0	37·5
	Normal (not illuminated)	—	1·18	0·0	26·0
	Thiamine-deficient	—	1·58	2·3	21·3
	Thiamine-deficient	Thiamine (3 μM)	2·50	7·5	37·6
	Thiamine-deficient (not illuminated)	—	1·00	0·0	17·0

8-azaguanine reduce the synthesis of porphyrins and bacteriochlorophyll (Lascelles, 1959; Gibson *et al.* 1962). Other conditions which reduce growth, such as omission of ammonia from the illumination mixture, also inhibit the formation of porphyrins; substitution by another source of nitrogen, such as certain amino acids, allows both processes to occur (Table 3). Similarly, when organisms are grown with just enough biotin to permit maximum growth, they can neither grow nor form pigment on subsequent illumination in mixture I unless biotin is added (Table 4). An increase in the concentration of biotin in the growth medium to 40 μmM allows both growth and pigment synthesis to occur on subsequent illumination in mixture I, without further addition of biotin, suggesting that under these conditions the organisms can retain enough of the excess of biotin in the growth medium to allow a further increase in dry weight. Similar observations were made with nicotinic acid and thiamine.

The experiments presented above show a correlation between the formation of porphyrins and bacteriochlorophyll and the ability to grow. However, when organisms are grown in media deficient in biotin the decrease in dry weight is less than the decrease in bacteriochlorophyll and carotenoids, giving a lower amount of these pigments/unit dry wt. of cells (Table 5). This might suggest that there is some specific relation between biotin and the synthesis of these pigments. However, this effect is not confined to biotin, since deficiency of nicotinic acid or thiamine reduces the content of bacteriochlorophyll/unit dry wt. It appears that substitution of malate in medium S by acetate also leads to a reduction in the concentration of bacteriochlorophyll and carotenoids. Organisms grown in acetate are pale pink in colour, but those grown in malate are deep red.

The concentrations of the two major pigments of *Rps. spheroides*, bacteriochlorophyll and carotenoids, appear to change in parallel fashion under various conditions of growth [Table 5 and Cohen-Bazire *et al.* (1957)]. Since these pigments are contained in the chromatophores it was thought that some of the unfavourable growth conditions, including biotin deficiency, might reduce pigment synthesis by inhibiting formation of chromatophores. Chromatophores, both light and heavy particles, were therefore isolated from normal and biotin-deficient organisms and their gross composition was determined (Table 6). The heavy-particle fraction may in fact consist of chromatophores attached to cell-wall material (Newton & Newton, 1957); however, this has no bearing on the points under discussion. The only components markedly reduced in biotin deficiency are bacteriochlorophyll and the yellow carotenoid.

Table 3. *Effect of substitution of ammonia by amino acids in mixture I*

Organisms previously grown anaerobically in the light were illuminated in mixture I without $(NH_4)_2HPO_4$, under standard conditions for 20 hr. The concentration of added $(NH_4)_2HPO_4$ or amino acids was 12 mM.

Nitrogen source	Dry wt. (mg./ml.)	Porphyrin (μm-moles/ml.)
None	1·75	4·1
$(NH_4)_2HPO_4$	2·21	42·0
Asparagine	1·94	30·5
Glutamine	2·90	65·0
Alanine	2·10	56·0
Arginine	1·78	32·5
Aspartic acid	2·07	8·3
Glutamic acid	2·12	11·5
None (not illuminated)	1·00	0·0

Enzyme activities in cell-free extracts

Extracts of organisms grown under different conditions were assayed for δ-aminolaevulate synthetase, δ-aminolaevulate dehydratase, L-alanine–γδ-dioxovalerate aminotransferase, L-threonine

Table 4. *Requirement for biotin by organisms grown in the presence of various concentrations of biotin*

Organisms were grown anaerobically in the light in medium S containing the concentration of biotin stated. Growth was optimum in all three cultures. Organisms were then illuminated in mixture I for 23 hr. and biotin (0·8 μmM) was added where stated.

Concn. of biotin in growth medium (μmM)	Biotin added to mixture I	Dry wt. (mg./ml.)	Porphyrin (μm-moles/ml.)	Bacteriochlorophyll (μm-moles/ml.)
40	–	2·21	13·1	41·2
40	+	2·21	9·3	42·6
40*	–	0·99	0·0	25·9
2	–	1·59	3·8	25·4
2	+	2·25	9·6	42·0
2*	–	0·99	0·0	25·9
0·8	–	1·10	0·9	17·2
0·8	+	2·26	7·1	35·3
0·8*	–	0·99	0·0	19·2

* Non-illuminated control.

Table 5. *Growth and pigment content of organisms grown in standard medium S and in vitamin-deficient medium S*

Organisms were grown in medium S anaerobically in the light. The concentration of each vitamin is the same as in standard medium S except where stated otherwise.

Vitamin (concn.)	Dry wt. of organisms (mg./ml. of culture)	Bacterio-chlorophyll (μm-moles/mg. dry wt.)	Carotenoids (μg./mg. dry wt.)	
			Yellow	Red
Biotin (40 μmM)	1·50	23·9	3·09	1·99
Biotin (0·8 μmM)	1·04	16·7	1·63	1·28
Biotin (0·4 μmM)	0·80	13·7	1·14	1·16
Nicotinic acid (8 μM)	1·31	21·8	—*	—
Nicotinic acid (0·45 μM)	0·89	18·4	—	—
Nicotinic acid (0·15 μM)	0·39	13·5	—	—
Thiamine (3 μM)	1·39	19·8	—	—
Thiamine (10 μmM)	0·85	17·3	—	—
Thiamine (5 μmM)	0·76	13·4	—	—

* Not determined.

Table 6. *Composition of chromatophores from normal and biotin-deficient cells*

Chromatophores, both light and heavy particles, were isolated by the method of Newton & Newton (1957) from normal and biotin-deficient (0·8 μmM-biotin in growth medium) organisms grown anaerobically in the light. Estimations were carried out as follows: protein, by the method of Lowry, Rosebrough, Farr & Randall (1951); total carbohydrate, by the anthrone method of Ashwell (1957); total haemin, after extraction with acetone–methanol according to Keilin & Hartree (1951); total P, by the method of Bartlett (1959); lipid phosphorus, by extracting lipids according to Bligh & Dyer (1959) and determining phosphate by the method of Bartlett (1959); bacteriochlorophyll and carotenoids, by the method of Cohen-Bazire *et al.* (1957). Contents of particles are expressed/mg. of protein.

Component	Units	Expt.	Heavy particles		Light particles	
			Normal	Biotin-deficient	Normal	Biotin-deficient
Bacteriochlorophyll	μm-moles	1	119	34	117	23
		2	79	18	112	11
Yellow carotenoid	μg.	1	13·6	4·9	6·1	2·1
		2	13·1	2·3	16·5	2·2
Red carotenoid	μg.	1	8·4	10·5	4·1	5·7
		2	9·1	6·1	7·2	4·3
Carbohydrate	μg.	1	108	102	69	44
		2	79	77	39	72
Total P	μmoles	2	—*	—	0·62	0·56
Lipid P	μmoles	2	0·52	0·35	0·40	0·34
Haemin	μm-moles	1	1·1	1·4	3·1	2·4
		2	3·5	2·6	2·9	6·0

* Not determined.

Table 7. *Enzyme activities in cell-free extracts from normal and biotin-deficient organisms*

Organisms were grown anaerobically in the light in medium S containing the concentrations of biotin stated. Cell-free extracts were prepared and enzymes were assayed as described in the text. All activities are expressed as μmoles/mg. of protein/hr.

Expt.	Concn. of biotin in growth medium (μmM)	Aminolaevulate synthetase	Aminolaevulate dehydratase	Alanine–dioxovalerate aminotransferase	Threonine dehydrogenase	β-Hydroxy-butyrate dehydrogenase
1	40	0·29	0·05	—*	—	—
	0·8	0·19	0·05	—	—	—
	0·4	0·15	0·05	—	—	—
2	40	0·17	0·12	—	0·09	8·52
	0·8	0·09	0·10	—	0·12	9·72
	0·2	0·07	0·08	—	0·17	13·50
3	40	0·27	—	0·50	0·10	10·26
	0·6	0·12	—	0·56	0·10	12·66

* Not determined.

Table 8. *Enzyme activities in cell-free extracts from organisms deficient in nicotinic acid or thiamine and organisms grown in acetate*

Organisms were grown anaerobically in the light in medium S except where otherwise stated. The concentrations of vitamins are as in standard medium S except where otherwise stated. Cell-free extracts were made and enzymes were assayed as described in the text. Activities are expressed as μmoles/mg. of protein/hr.

Expt.	Vitamin (concn.)	Amino-laevulate synthetase	Amino-laevulate dehydratase	Alanine–dioxovalerate aminotransferase	Threonine dehydrogenase	β-Hydroxy-butyrate dehydrogenase
1	Nicotinic acid (8 μM)	0·35	—*	—	0·07	7·08
	Nicotinic acid (0·45 μM)	0·14	—	—	0·05	5·46
	Nicotinic acid (0·15 μM)	0·18	—	—	0·05	5·46
	Nicotinic acid (0·05 μM)	0·05	—	—	0·07	4·44
2	Thiamine (3 μM)	0·33	—	—	0·07	7·62
	Thiamine (10 μmM)	0·22	—	—	0·09	8·16
	Thiamine (5 μmM)	0·14	—	—	0·09	7·92
	Thiamine (2·5 μmM)	0·16	—	—	0·08	8·10
	Growth substrate					
3	Malate	0·33	0·10	0·41	0·09	11·28
	Acetate	0·04	0·02	0·42	0·09	9·60

* Not determined.

dehydrogenase and D-β-hydroxybutyrate dehydrogenase. In a large number of experiments with biotin-deficient organisms, aminolaevulate synthetase was the only one of these enzymes which was consistently decreased (Table 7). In Expt. 2 of Table 7 there was also a significant decrease in aminolaevulate dehydratase. The activity of aminolaevulate synthetase in extracts of biotin-deficient cells could not be increased by addition of biotin, biotin plus ATP, or boiled extracts of normal organisms. On illumination of biotin-deficient organisms in mixture I plus biotin there was no increase in the activity of aminolaevulate synthetase as assayed in extracts, although the organisms grew and formed pigments. Thus in an experiment where organisms grown with 0·6 μmM-biotin were subsequently illuminated in mixture I plus 40 μmM-biotin, the dry weight doubled in 20 hr. but the enzyme activity fell from 0·21 to 0·11 μmole/mg. of protein/hr. A marked decrease in aminolaevulate-synthetase activity can be demonstrated in organisms grown in a medium deficient in nicotinic acid or thiamine (Table 8). There is an even greater decrease in aminolaevulate-synthetase activity and also one in aminolaevulate-dehydratase activity in organisms grown in acetate. The activities of the other enzymes measured showed some minor variations of an irregular nature.

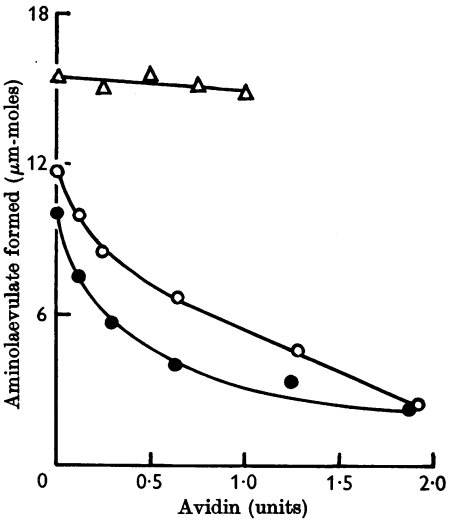

Fig. 1. Effect of avidin on aminolaevulate-synthetase activity. The assay for aminolaevulate synthetase is as described in the text. Three different samples of avidin in the quantity shown were preincubated with the enzyme extract, containing approx. 0·3 mg. of protein in all three experiments, for 10 min. at 0°. The reaction was started by addition of succinyl-CoA. ●, Commercial avidin (2·5 units/mg. of protein); ○, avidin (4·9 units/mg. of protein); △, avidin (9·6 units/mg. of protein).

Effect of avidin on aminolaevulate-synthetase activity

A commercial preparation of avidin, containing 2·5 units/mg. of protein, inhibited aminolaevulate-synthetase activity markedly (Fig. 1). Avidin pre-incubated with excess of biotin still inhibited the enzyme activity. On the other hand, the effect of avidin appeared to be specific, since ovalbumin, ovomucoid and lysozyme did not inhibit when used at the same concentration as avidin. The inhibition by avidin was not reversed by excess of succinyl-CoA or pyridoxal phosphate in the assay system, and avidin did not interfere with the colorimetric estimation of aminolaevulate.

This apparent contradiction was resolved when purer avidin became available. A sample containing 4·9 units/mg. still inhibited enzyme activity, but the quantity required for 50 % inhibition was twice that of the commercial avidin. A sample with 9·6 units/mg. did not inhibit at all. Avidin containing 9·6 units/mg. is approximately 80 % pure, as estimated by comparison with the purest preparation of avidin obtained by Dr N. M. Green (unpublished work).

DISCUSSION

Conditions affecting the synthesis of bacteriochlorophyll

The work reported here strongly suggests that, although *Rps. spheroides* can grow without forming bacteriochlorophyll, it is unable to synthesize bacteriochlorophyll unless it can also grow. The criterion for growth was an increase in turbidity which is an indication that some increase in cell mass or number has taken place. The nature of the mechanisms which couple the synthesis of bacteriochlorophyll to growth is at present obscure. The bacteriochlorophyll is confined to the chromatophores (Schachman, Pardee & Stanier, 1952), which also contain carotenoids, proteins, phospholipid and other components (Newton & Newton, 1957). It could be imagined that inhibition of the synthesis of one of these components, such as might occur if the general anabolic processes of the cell were suspended, would prevent the formation of new chromatophores and hence of any substance contained in them. A mechanism which prevents the synthesis of bacteriochlorophyll in organisms which are unable to grow or divide would explain why adaptation of aerobically grown organisms to form bacteriochlorophyll when illuminated is inhibited by inhibitors of growth (Lascelles, 1959). However, it is unlikely that the rate of growth of the cell or any of its components is the only factor regulating the concentration of bacteriochlorophyll in the chromatophores.

The effect on the synthesis of bacteriochlorophyll of a deficiency in biotin, nicotinic acid or thiamine can be partly explained by the coupling of the formation of this pigment to growth, as such deficiencies lead to reduced growth; but it is clear that these conditions exert some additional effect on pigment synthesis, since there is a very marked reduction in the actual concentration of bacteriochlorophyll in the cell. With biotin deficiency at least, bacteriochlorophyll and the carotenoids appear to be the only components of the chromatophores which are greatly decreased, indicating that the reduction in the rate of synthesis of these pigments is not simply due to a diminished rate of formation of the chromatophores as a whole. It seems that these conditions of vitamin deficiency must be added to the ones already mentioned above, namely aerobiosis and strong illumination, in which the differential rate of synthesis of bacteriochlorophyll is decreased. Illumination in a medium containing acetate instead of malate is another condition unfavourable to the formation of bacteriochlorophyll. There is at present no direct evidence to explain why any of these conditions should be associated with a reduction in the concentration of bacteriochlorophyll in the cell; however, the suggestion of Cohen-Bazire *et al.* (1957) mentioned above offers a possible explanation for the effects of oxygen and strong illumination.

Concentrations of bacteriochlorophyll, carotenoid and aminolaevulate synthetase

Any hypothesis which seeks to explain the reduction in concentration of bacteriochlorophyll in these organisms associated with the conditions of growth enumerated above must take account of the fact that in all cases which have been investigated so far the concentrations of the carotenoids and of aminolaevulate synthetase in the cell follow closely that of bacteriochlorophyll. A correlation between the concentration of carotenoid and of bacteriochlorophyll in the chromatophores or in whole cells has been observed under illumination of various intensities (Cohen-Bazire *et al.* 1957; Cohen-Bazire & Kunisawa, 1960), exposure to oxygen (Cohen-Bazire *et al.* 1957), biotin deficiency (Tables 5 and 6) and growth on acetate. Carotenoids are probably synthesized from geranylgeranyl pyrophosphate (Grob, Kirscher & Lynen, 1961), and the close structural resemblance between geranylgeraniol and the phytol side chain of bacteriochlorophyll suggests that the latter compound is derived from the same precursor as carotenoids (Lynen & Henning, 1960). Possibly some of the growth conditions which specifically reduce the concentrations of the pigments do so by interfering primarily with the biosynthesis of 'allyl pyrophosphates'. Alternatively, a mechanism can be envisaged whereby the accumulation of a precursor of phytol would lead to inhibition of an early step in the synthesis of other isoprenoid compounds including the carotenoids. In this way any condition which leads to a reduction in the concentration of bacteriochlorophyll would secondarily reduce those of the carotenoids.

Reductions in the concentration of aminolaevulate synthetase and sometimes of aminolaevulate dehydratase, roughly parallel to the reductions in that of bacteriochlorophyll, have been observed in organisms grown in the presence of oxygen (Lascelles, 1959) and in cells deficient in biotin (Lascelles, 1960*b*), nicotinic acid or thiamine, or grown in

acetate (Tables 7 and 8). The decrease in enzyme activity is almost certainly not enough to account completely for the reduction in the concentration of the pigment. It may be that the activities are reduced by some mechanism of feed-back repression. The only substance known to repress aminolaevulate synthetase is haemin (Lascelles, 1960*a*), but the evidence is insufficient to indicate whether this is a possible physiological repressor, nor is it obvious how a reduction in the concentration of bacteriochlorophyll could lead to a rise in the concentration of haemin in the cell. The question of the mechanism of repression must still be regarded as open.

Regulation of porphyrin synthesis

The porphyrins produced when cultures of *Rps. spheroides* are illuminated in a medium containing α-oxoglutarate or succinate and glycine consist mainly of coproporphyrin III with some uroporphyrin III (Lascelles, 1956). These are oxidation products of actual intermediates in the normal biosynthetic pathway leading to protoporphyrin and bacteriochlorophyll (Bogorad, 1960). Their production is probably connected with the fact that both the growth medium and the illumination medium are deficient in iron, since addition of iron to either of these media prevents their accumulation (Lascelles, 1956). In the experiments reported above a correlation was observed between the formation of porphyrins and of bacteriochlorophyll from α-oxoglutarate or succinate and glycine in that neither was synthesized under conditions which prevented growth. On the other hand, there was no correlation when aminolaevulate was used as substrate. Porphyrins are formed readily from aminolaevulate even though the organisms do not synthesize bacteriochlorophyll (Lascelles, 1956) or grow (Table 1). These results suggest that the formation of the photosynthetic pigment may be regulated by mechanisms which exert their effect on the first step in this pathway, namely the formation of aminolaevulate. Any conditions of growth which tend to reduce the synthesis of bacteriochlorophyll from α-oxoglutarate and glycine would then also reduce the production of porphyrins. On the other hand there are certain conditions under which porphyrin synthesis is dissociated from the formation of bacteriochlorophyll. The use of aminolaevulate as substrate and sole carbon source has already been mentioned. The formation of porphyrins from aminolaevulate does not require any other compound; however, bacteriochlorophyll is not synthesized, perhaps because this medium contains no precursors of the methyl ester and phytol groups. The action of ethionine and threonine in stimulating porphyrin formation by organisms illuminated with α-oxoglutarate and glycine also falls into this category. These compounds inhibit one of the steps in bacteriochlorophyll synthesis (Gibson *et al.* 1962).

There is at present little evidence to indicate how the synthesis of aminolaevulate is regulated. As with bacteriochlorophyll, the reduction in the concentration of aminolaevulate synthetase which occurs under various conditions is almost certainly insufficient to account for the total inhibition of porphyrin synthesis which often occurs. The synthesis of aminolaevulate could be reduced by a decrease in the amount of a precursor, by non-availability of a cofactor or by some mechanism of inhibition. When *Rhodospirillum rubrum*, whose metabolism is very similar to that of *Rps. spheroides*, is grown under aerobic conditions, the oxidation of many substrates is mediated by the Krebs cycle, but under anaerobic conditions this is not so (Elsden & Ormerod, 1956). A mechanism can be envisaged whereby succinyl-CoA is deacylated rapidly (as part of the normal operation of the cycle) under aerobic conditions, but much more slowly under anaerobic conditions. This could alter the concentration of succinyl-CoA sufficiently to account for the lack of synthesis of porphyrin in aerobically grown organisms. However, it cannot account entirely for the effect of deficiency in biotin, nicotinic acid or thiamine. The last two conditions might reduce the synthesis of aminolaevulate from α-oxoglutarate and glycine, as pointed out by Lascelles (1956), since these vitamins are precursors of cofactors in the reactions which convert α-oxoglutarate into succinyl-CoA. However, the effect of biotin on porphyrin synthesis cannot at present be explained in terms of a simple direct interference in the formation of succinyl-CoA. The possible effect of the growth conditions on the synthesis of pyridoxal phosphate has not been explored, but this may be significant, particularly as aminolaevulate synthetase in extracts of organisms requires to be activated by exogenous pyridoxal phosphate (Gibson, 1958; Kikuchi *et al.* 1958). A third possible explanation of the findings in some or all of the conditions which inhibit the accumulation of porphyrins is that the synthesis of aminolaevulate is regulated by the concentration of some inhibitor present in the cell. Tentative evidence for the existence of such a substance was provided by Kikuchi *et al.* (1958). We found that haemin, besides repressing the formation of aminolaevulate synthetase (Lascelles, 1960*a*), is a potent inhibitor of its activity (Gibson *et al.* 1961). However, as discussed above, the physiological significance of this observation is uncertain as no connexion has been established between the rate of formation of haemin and that of bacteriochlorophyll. It must be concluded that, though several possible mechanisms exist for the regulation of aminolaevulate synthesis,

any one of which might be important under some particular environmental conditions, there is at present insufficient evidence to enable any of these to be chosen in any one case, or even to state whether all such mechanisms have yet been recognized.

Biotin and aminolaevulate synthetase

Lascelles (1956) observed that biotin-deficient organisms do not form porphyrins when illuminated in the presence of α-oxoglutarate and glycine unless biotin is added to the illumination mixture, but that no such addition is required when they are illuminated in the presence of aminolaevulate. The addition of biotin is also necessary if such cells are to form porphyrin when illuminated in the presence of succinate and glycine (Table 1). Since α-oxoglutarate and succinate form succinyl-CoA by independent pathways, neither of which is thought to involve biotin, these results suggested at first that aminolaevulate synthetase might contain biotin. The reduction in the concentration of aminolaevulate synthetase brought about by biotin deficiency also appeared to support this view, as did the finding that commercial avidin inhibited the activity in a cell-free extract (see also Neuberger, 1961).

However, further investigation has shown that alternative and more likely explanations exist for all these observations. As discussed above, the failure of biotin-deficient organisms to produce porphyrins unless supplemented with biotin is probably due to the fact that they cannot grow or form bacteriochlorophyll. The reductions in the amounts of aminolaevulate synthetase are not confined to biotin-deficient cells, having been observed also with cells deficient in nicotinic acid or thiamine or grown under certain other conditions. Further, the inhibition observed with commercial avidin did not occur when this relatively impure preparation was replaced by one which was approximately 80% pure. The fact that preincubation of commercial avidin with biotin did not affect its inhibitory activity is consistent with this last finding. In this connexion Hatch & Stumpf (1961) observed that inhibition of acetyl-coenzyme A carboxylase by commercial avidin is only partially reversed by preincubation with biotin. It is possible that the irreversible inhibition is due to an impurity in the preparation of avidin which may or may not be the same as the one which inhibits aminolaevulate synthetase.

Consideration of all the evidence discussed above favours the conclusion that aminolaevulate synthetase does not contain biotin. The nature of the reaction catalysed is consistent with this view. All enzymes known to contain biotin catalyse reactions in which carbon dioxide is activated or transferred, but the reaction in which aminolaevulate is synthesized involves a decarboxylation which is facilitated by the intrinsic instability of α-amino-β-oxoadipic acid (Laver, Neuberger & Scott, 1959) and particularly of its pyridoxal phosphate complex.

SUMMARY

1. *Rhodopseudomonas spheroides* was grown either aerobically in the dark or anaerobically in the light with suboptimum amounts of biotin. These cultures formed porphyrins and bacteriochlorophyll when illuminated in the presence of α-oxoglutarate and glycine, but only when biotin was added at this stage. The same results were obtained when succinate was substituted for α-oxoglutarate.

2. In these experiments there was a close association between growth, as determined by dry weight, and the formation of porphyrins and bacteriochlorophyll.

3. Deficiency of nicotinic acid or thiamine affected dry weight and porphyrin and bacteriochlorophyll formation in the same way as deficiency of biotin.

4. Organisms deficient in biotin, nicotinic acid or thiamine had decreased amounts of bacteriochlorophyll/unit dry weight. The concentration of carotenoids was determined in biotin-deficient organisms and was found to be reduced. Chromatophores from these organisms also showed a reduced concentration of bacteriochlorophyll and of the yellow carotenoid but little change in other components measured.

5. Extracts of organisms deficient in any of the three vitamins or grown with acetate in place of malate showed a marked reduction in the activity of aminolaevulate synthetase and in some cases of aminolaevulate dehydratase. There was no significant change in L-threonine dehydrogenase, D-β-hydroxybutyric dehydrogenase or L-alanine–γδ-dioxovalerate aminotransferase where these were determined.

6. A preparation of commercial avidin inhibited aminolaevulate synthetase. Preincubation with biotin did not abolish this inhibition. On further purification the inhibitory power of avidin decreased and finally disappeared. It is therefore believed that biotin is not directly concerned in the synthesis of aminolaevulate.

7. It is concluded that the synthesis of bacteriochlorophyll and growth are linked in some unknown manner, but that additional mechanisms exist to regulate the synthesis of porphyrins and bacteriochlorophyll, including inhibition and repression of aminolaevulate synthetase.

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