The Accumulation of Bacteriochlorophyll Precursors by Mutant and Wild-Type Strains of *Rhodospseudomonas spheroides*

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1. Two mutant strains of *Rhodospseudomonas spheroides*, which are blocked in the synthesis of bacteriochlorophyll, accumulate pigments. These have been tentatively identified as magnesium 2,4-divinylphaeoporphyrin a_5 monomethyl ester and the magnesium derivative of 2-devinyl-2-hydroxyethyl-pheophorbide a , formed by mutant 2/73 and 2/21 respectively. 2. Maximum extracellular production of these pigments occurs when suspensions of the organisms are incubated with low aeration in a growth medium containing iron and supplemented with glycine, succinate, methionine and Tween 80. 3. Concomitant protein synthesis is required for pigment production by the mutants from glycine and succinate but this requirement is less marked when δ -aminolaevulinic acid is the substrate. 4. In the absence of Tween 80, a considerable proportion of the total pigment is retained within the cells and appears in the particulate fraction of cell-free extracts. 5. Suspensions of the parent strain containing δ -aminolaevulinic acid can be made to accumulate extracellular pigments which are tentatively identified as magnesium protoporphyrin monomethyl ester and the magnesium derivative of 2-devinyl-2-hydroxyethyl-pheophorbide a . 6. Maximum production occurs with cells incubated photosynthetically after a period of oxygen repression of bacteriochlorophyll synthesis. Formation of the pheophorbide derivative is enhanced by 8-azaguanine or 5-fluorouracil, or by adenine deficiency in a nutritional mutant; Tween 80 is also needed and iron is essential. 7. Synthesis of bacteriochlorophyll might possibly involve the participation of lipoprotein-bound intermediates, which may be formed at the initial stage of condensation between glycine and succinyl-CoA to give δ -aminolaevulinic acid.

In photosynthetic organisms the synthesis of haems and chlorophylls follows a common path which diverges at the stage of protoporphyrin into the iron and magnesium branches (Scheme 1; Lascelles, 1965). Knowledge of the steps from protoporphyrin to the chlorophylls is derived largely from the accumulation of possible precursors by photosynthetic bacteria and algae when chlorophyll synthesis is blocked by mutation or by inhibitors. The pathway of bacteriochlorophyll synthesis outlined in Scheme 1 has been proposed by Jones (1963*a,b,c*, 1964) as a result of studies of compounds found in cultures of *Rhodospseudomonas spheroides* grown in the presence of 8-hydroxyquinoline. Only one of the steps in this pathway has been studied at the enzyme level, this being the methyl transferase which catalyses the formation of magnesium protoporphyrin monomethyl ester from magnesium protoporphyrin and *S*-adenosylmethionine (Gibson, Neuberger & Tait, 1963). The enzyme is found in the particulate fraction of photosynthetically grown *R. spheroides*.

As a preliminary to work at the enzyme level

it is helpful to have information about the conditions which promote maximum accumulation of chlorophyll precursors by whole cells and the present studies were undertaken with this object. Two mutants of *R. spheroides*, strains 2/73 and 2/21, have been used; the nature of the pigments accumulated by them suggests that they are blocked in bacteriochlorophyll synthesis at the stages shown in Scheme 1. These pigments accumulate when the organisms are incubated in the dark under low aeration, conditions previously shown to permit bacteriochlorophyll synthesis by the parent organism (Lascelles, 1959). The present work also defines conditions which cause the parent organism to form bacteriochlorophyll precursors from δ -aminolaevulinic acid.

METHODS

Organisms

Cultures and maintenance. *Rhodospseudomonas spheroides* N.C.I.B. 8253 was the parent strain and all the mutants were derived from it. Stock cultures of the parent were

Scheme 1. Suggested scheme for iron and magnesium branches of the tetrapyrrole biosynthetic pathway.

maintained as described previously (Lascelles, 1956). The pigment mutants 2/21 and 2/73 made little or no bacteriochlorophyll and consequently grew only under aerobic conditions. Stock cultures were maintained on slopes of malate-glutamate agar containing 0.2% (w/v) of Difco yeast extract (Lascelles, 1959); they were incubated at 30° for 24 hr. and stored at 0°. Subcultures of the pigment mutants were made fortnightly since they tended to lose viability when stored for longer periods.

Some experiments were with strain 6A5, which requires adenine and histidine for growth (Bull & Lascelles, 1963); this organism forms the photosynthetic pigments normally.

Isolation of pigment mutants. Strains 2/21 and 2/73 were isolated by treatment of *R. spheroides* 8253 with the mutagen *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine. The cells from 10 ml. of a photosynthetic culture in malate-glutamate medium plus 0.2% (w/v) of Difco yeast extract were centrifuged and the pellet (about 10 mg. dry wt. of cells, 2×10^{10} organisms) was suspended in 0.9 ml. of the same medium. The mutagen (0.1 ml., 4 mg./ml.) was added, the mixture incubated at 37° for 30 min. and then diluted with 5 ml. of the M-G* medium with yeast extract. The treated mixture was incubated aerobically for 6 hr. at 30° and dilutions were spread over plates of the yeast-extract medium; these were incubated for 3–5 days at 30°. Usually 0.1 ml. of 1/100 or 1/1000 dilution gave a workable number of colonies. Pigment mutants were readily discernible on such plates as the colonies were only faintly pigmented and sometimes showed a greenish tinge, whereas the wild-type colonies were red. Potential mutants were replated to establish their purity and then checked for inability to grow anaerobically in the light in the yeast-extract medium.

Experiments with cell suspensions

Growth of organisms. Strains 8253, 2/21 and 2/73 were grown in the M-G medium of Lascelles (1959); this was supplemented with L-histidine and adenine (1 mM and 0.2 mM respectively) for mutant 6A5. Cultures of the pigment mutants were incubated at 30° with low aeration. Such conditions were achieved empirically by shaking well-filled flasks; usually 250 ml. Erlenmeyer flasks containing 180 ml. of medium were shaken on a reciprocating shaker operating at 120 strokes/min. The inoculum (0.5%,

v/v) was grown aerobically on M-G medium plus 0.2% (w/v) of yeast extract.

Strains 8253 and 6A5 were incubated photosynthetically or with high aeration as described by Lascelles (1959).

Preparation and incubation of cell suspensions of mutants 2/21 and 2/73. Cultures were harvested after 14–20 hr. when the extinction at 680 m μ (1 cm. light-path) was between 0.8 and 1.6; this was equivalent to 0.5–1 mg. dry wt. of cells/ml. The cells were centrifuged for 15 min. at 5000g and resuspended to a density of 2 mg./ml. in mixture A (M-G medium supplemented with: glycine and succinate, 10 mM each; L-methionine, 0.1 mM; Tween 80, 0.2%, w/v). Incubation was at 30° under low aeration. The suspensions were shaken on the shaker (see above) either in 11 mm. internal diam. tubes held at an angle of about 45° or in 25 ml. Erlenmeyer flasks; 2.5 ml. volumes were used in the tubes and 15 ml. in the flasks.

Preparation and incubation of strains 8253 and 6A5. Cultures were harvested at the same stage as described for the pigment mutants. The harvested cells were resuspended in M-G medium to a density of 2 mg. dry wt./ml. and pretreated by aeration as described in the text. They were finally suspended to about the same density in mixture B (M-G medium supplemented with: glycine and succinate, 10 mM; ALA, mM; 8-azaguanine, 0.4 mM; L-methionine, 0.1 mM; Tween 80, 0.2%, w/v). Incubation was at 34° in 11 mm. tubes containing 4–10 ml. of suspension and held in a glass-sided bath; the tubes were illuminated with tungsten lamps at an intensity of 150 ft. candles. The air above the suspensions was displaced by nitrogen and the tubes were sealed with Parafilm.

Identification of pigments

The excreted pigments were not rigorously identified and are therefore designated in the text as P-590, P-631 and P-662 according to the position of their red absorption maximum in the supernatant medium. Tentative identification was made as described below.

P-590. This pigment was formed maximally only in the presence of ALA and was always accompanied by coproporphyrin and coproporphyrinogen. The sharp band at 590 m μ could, however, be clearly distinguished with a hand spectroscope and in the spectrophotometer. The responsible pigment was probably magnesium protoporphyrin monomethyl ester, which has been previously found in the culture fluids of some photosynthetic bacteria

* Abbreviations: M-G, malate-glutamate; ALA, δ -aminolaevulinic acid.

(Jones, 1963a; Cooper, 1963); the methods used to identify the pigment were as described by these workers and by Granick (1961a). The absorption spectrum of ether extracts of supernatants containing P-590 (1 ml. of supernatant extracted with 5 ml. of ether) was identical with that found by Granick (1961a) with the authentic compound, and the pigment was converted upon extraction of the ether solution with 2.8N-HCl into a porphyrin with an absorption spectrum identical with that of protoporphyrin (Granick, 1961a). This porphyrin was re-extracted from the acid solution with ether after adjustment of the pH to 4.5-5.0 with saturated sodium acetate. It was established as the monomethyl ester by the following criteria. (1) Ascending chromatography in 2,6-lutidine-aq. 0.05N-NH₃ soln. (10:7, v/v); the R_F of the unknown was 0.88, that of authentic protoporphyrin 0.78. (2) Radioactivity was found in this porphyrin when it had been derived from an incubation system containing methyl-labelled methionine.

This experiment was with *R. spheroides* 8253 under the conditions described in Table 4, Expt. 1, except that L-[Me-¹⁴C]methionine was used (0.1 μ C/ml.; 0.05 mm).

P-631. The greenish-yellow supernatant fluid from cultures of strain 2/73 exhibited pronounced maxima at 631, 574 and 445 m μ (Fig. 1a). The main pigment was probably the protochlorophyll-like compound isolated by Jones (1963c) from 8-hydroxyquinoline-treated cultures of *R. spheroides* and identified as magnesium 2,4-divinylphaeoporphyrin *a*₅ monomethyl ester.

The pigment was extracted into ether (1 ml. of supernatant shaken with 5 ml. of ether), when the spectrum showed maxima at 624, 573 and 438 m μ (Fig. 2a). Upon shaking the ethereal extract with 2.8N-HCl for 10 min. there was a change in the spectrum consistent with loss of magnesium from the molecule (Fig. 2a). The spectrum of the untreated and acid-treated ethereal solutions of P-631 were similar to those reported by Jones (1963b,c). Further tests were made on the acid-treated pigment by his methods. The material gave a positive phase test indicating the

Fig. 1. Spectra of supernatant fluids from suspensions of (a) mutant 2/73, and (b) mutant 2/21. The fluids were diluted 1 in 8 and 1 in 4 respectively for readings below 500 m μ .

Fig. 2. Spectra of ethereal solutions of (a) P-631, and (b) P-662; —, before acid treatment; ---, after acid treatment. The solutions were diluted 1 in 10 for readings below 500 m μ .

presence of an isocyclic ring substituted with a carboxylic group. Absence of an esterified long-chain hydrocarbon (e.g. phytol) was indicated by its ready extractability from ether with 4.2N-HCl (but not with 2.8N). The presence of two vinyl groups was suggested (1) by the marked shift in absorption maxima towards the blue end of the spectrum upon hydrogenation and (2) by the shift in absorption spectrum upon formation of the oxime derivative. In all these respects the acid-treated material derived from P-631 behaved in a fashion identical with that described by Jones (1963b,c) for the protochlorophyll-type pigment.

P-662. The supernatant fluid from cultures of strain 2/21 was bright green and showed pronounced absorption maxima at 662, 624, 430 and 418 m μ (Fig. 1b). The pigment was unstable to light, which caused a rapid decline in the extinction at 662 m μ , with a broadening of the peak. The pigment was extracted with ether (1 ml. of supernatant shaken with 5 ml. of ether) and the fresh extract exhibited maxima at 660, 620 and 427 m μ . Upon shaking with 1.4N-HCl, the band at 660 m μ did not change its position but declined in intensity, and bands appeared at 532 and 502 m μ while the Soret maximum shifted to 405 m μ (Fig. 2b). The spectrum of the acid-treated material resembled closely that of 'compound 2', isolated from 8-hydroxy-quinoline-treated cultures of *R. spheroides* and tentatively identified as 2-devinyl-2-hydroxyethyl-phaeophorbide *a* (Jones, 1964). The acid-treated material from mutant 2/21 gave a positive phase test and was readily extracted from ether with 4.2N-HCl. Hydrogenation, which resulted in a marked shift in the absorption spectrum of acid-treated P-631, caused no alteration to the spectrum of the material derived from P-662. The behaviour of the pigment accumulated by strain 2/21 is consistent with its being the magnesium derivative of 2-devinyl-2-hydroxyethyl-phaeophorbide *a*.

Estimation of pigments

After incubation, cells were removed by centrifuging (5000g for 15 min.) and extracellular pigments in the supernatant fluid were measured with the Cary model 14 spectrophotometer, with the expanded scale slide wire,

scanning from 720 to 580 m μ . Results for P-590, P-631 and P-662 are expressed as extinction values (1 cm. light-path) given by the supernatants at the appropriate absorption maximum.

Bacteriochlorophyll was estimated by the method of Cohen-Bazire, Sistrom & Stanier (1957).

Chemicals

Tween 80 was from Koch-Light Laboratories Ltd. (Colnbrook, Bucks.), 8-azaguanine and puromycin from Sigma Chemical Co. (London, S.W. 6), δ -aminolaevulinic acid from Calbiochem (Lucerne, Switzerland), and *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine from Aldrich Chemical Co. (London, S.E. 1). 5-Fluorouracil was kindly provided by Dr R. Morrison of Roche Products Ltd. (Welwyn Garden City, Herts.).

RESULTS

Accumulation of pigments by mutants 2/21 and 2/73

Conditions for pigment excretion. Both strains formed extracellular pigment when incubated in the M-G growth medium, but greater quantities were excreted when the medium was supplemented with glycine, succinate, Tween 80 and methionine (Table 1). Of these, Tween 80 had the greatest effect. In the complete system mutant 2/21 and 2/73 accumulated only P-662 and P-631 respectively and pigment production occurred at a linear rate for at least 8 hr. (Fig. 3). With ALA in place of glycine plus succinate, different kinetics were observed, and P-590 was also accumulated by both strains (Fig. 3). Pigment production by both mutants was considerably repressed by high aeration (Table 1) and thus exhibited the same response to oxygen as did bacteriochlorophyll synthesis by the parent strain (Lascelles, 1959).

Effect of iron. Synthesis of P-631 and P-662 was

Table 1. *Effect of incubation conditions on pigment excretion by R. spheroides 2/21 and 2/73*

The complete reaction mixture contained cells (2 mg. dry wt./ml.) suspended in mixture A. Incubation was for 7 hr. in 11 mm. (int. diam.) tubes, containing 2.5 ml. or 1.0 ml. of suspension for conditions of low and high aeration respectively.

Expt. no.	Incubation conditions	Reaction mixture	Extinction of extracellular fluid	
			From 2/21 $E_{662}^{1\text{cm.}}$	From 2/73 $E_{631}^{1\text{cm.}}$
1	Low aeration	Complete (mixture A)	0.52	0.14
		Without glycine and succinate	0.44	0.10
		Without Tween 80	0.26	0.08
		Without methionine	0.50	*
		M-G medium only	0.13	0.05
2	Low aeration	Mixture A	0.58	0.14
	High aeration	Mixture A	0.24	0.02

* Not determined.

Fig. 3. Kinetics of pigment synthesis by (a) mutant 2/73, and (b) mutant 2/21. Cells were incubated with low aeration in mixture A (—) or in this mixture modified to contain ALA (1mm) in place of glycine and succinate (---). ●, P-631 or P-662 for (a) and (b) respectively; ○, P-590.

Fig. 4. Effect of iron citrate on formation of pigments by (a) mutant 2/73 and (b) mutant 2/21. Cells were incubated with low aeration in mixture A with iron citrate at the concentrations shown. ●, P-631 or 662 for (a) and (b) respectively; ○, P-590.

dependent upon iron; suspensions of cells grown on iron-deficient medium responded to iron concentrations within the range 1–10 μM (Fig. 4). This is the same range that influences bacteriochlorophyll synthesis by iron-deficient cells of the parent strain (Lascelles, 1956). Some P-590 was formed by both strains when iron was limiting (Fig. 4).

Effect of inhibitors. Bacteriochlorophyll formation by *R. spheroides* is linked to protein synthesis; it is prevented by inhibitors of protein synthesis and is dependent upon added amino acids and nucleic acid derivatives in the nutritional mutants (Lascelles, 1959; Sistrom, 1962; Bull & Lascelles, 1963).

Pigment synthesis from glycine+succinate by strains 2/21 and 2/73 was drastically curtailed by puromycin and chloramphenicol and to a lesser extent by amino acid and nucleic acid analogues (Table 2). A sensitive step in the biosynthetic path seemed to be the ALA synthase since pigment formation with ALA as substrate was far less affected by these inhibitors (Table 2).

Bound pigments. The increase in pigment excretion in the presence of Tween 80 suggested that the pigments might normally be bound to lipid or protein or to both within the cells. Evidence for this was obtained by examining cell-free extracts of organisms that had been incubated with and without the detergent (Table 3). With strain 2/21, over 80% of P-662 was bound within the cells, when incubation was without Tween 80, and with strain 2/73, about 50% of P-651 was bound. No intracellular pigment was detected in either organism incubated with the detergent.

The intracellular pigments in both mutants were

apparently largely bound to particulate material. Centrifugation (100 000g, 1 hr.) of crude extracts, prepared as described in Table 3, showed that 75–80% of the total pigment formed without Tween was in the particulate fraction. Treatment of the particles with Tween 80 released some of the bound pigment. In these experiments the pigmented particles (1mg. of protein/ml. in 0.04M-tris buffer, pH 7.5) were kept with 0.2% Tween 80 for 1 hr. at 0° and then centrifuged at 100 000g for 1 hr. About 40% of the pigment was in the supernatant fraction. Similar treatment with Triton-X100, which solubilizes most of the bacteriochlorophyll from chromatophores of *R. spheroides* (Bril, 1958), released 86% of P-662 from particles of mutant 2/21. The absorption spectra of both pigments, whether in the cell-free extracts or in the extracellular fluids, were identical. The protochlorophyll in plant tissues exhibits marked changes in absorption maxima according to whether it is in the bound or free form (Granick, 1961b).

Formation of P-662 and P-590 by the parent strain of R. spheroides

8-Azaguanine inhibits bacteriochlorophyll synthesis by *R. spheroides* under conditions of adaptation from the non-pigmented to the pigmented state (Lascelles, 1959; Bull & Lascelles, 1963). The extracellular accumulation of a green pigment with absorption maxima similar to those of P-662 has been frequently observed in experiments with this analogue and conditions for its formation have now been examined in more detail.

Requirements for synthesis. The previous history of the organisms was important in determining their subsequent ability to accumulate P-662. It

Table 2. *Effect of inhibitors of protein and nucleic acid synthesis on pigment synthesis by mutants 2/21 and 2/73*

Cells were incubated under low aeration in mixture A or in this system containing ALA (1mM) in place of glycine and succinate. The incubation times were 7.25 and 14 hr. respectively in Expts. 1 and 2. Control values: strain 2/21 (P-662), glycine + succinate 0.33, ALA 0.28; strain 2/73 (P-631), glycine + succinate 0.24, ALA 0.22.

Expt. no.	Strain	Inhibitor	Concn.	Pigment formed (% of uninhibited control)	
				With glycine + succinate	With ALA
1	2/21	Puromycin	60 µg./ml.	14	70
		Chloramphenicol	0.1 mM	23	90
		<i>p</i> -Fluorophenylalanine	3.0 mM	62	100
		8-Azaguanine	0.4 mM	60	96
		5-Fluorouracil	0.1 mM	66	80
2	2/73	Puromycin	60 µg./ml.	27	80
		Chloramphenicol	0.1 mM	25	100

Table 3. *Bound pigments in mutants 2/21 and 2/73*

The cells (15 ml. in 25 ml. flasks) were incubated for 7 hr. in mixture A without Tween 80 and with ALA as shown. They were harvested, washed once in 30 ml. of 0.04M-tris buffer, pH 7.5, and resuspended in 15 ml. of the same buffer. They were disrupted by sonic oscillation for 10 min. and clarified by centrifugation for 10 min. at 10000g. The extinctions at the absorption maxima were determined in the extracts and in the suspension media.

Expt. no.	Strain	Additions		Pigment formed	
		Tween 80 (0.2%, w/v)	ALA (1 mM)	Medium ($E_{662}^{1cm.}$)	Extract ($E_{662}^{1cm.}$)
1	2/21	—	—	0.06	0.39
		—	+	0.05	0.20
		+	+	0.20	0
				($E_{631}^{1cm.}$)	($E_{631}^{1cm.}$)
2	2/73	—	+	0.15	0.13
		+	+	0.27	0

Table 4. *Conditions for P-662 and P-590 synthesis by R. spheroides 8253*

Cells, harvested after growth as shown, were resuspended in M-G medium to a density of 2 mg./ml. Pre-treatment was for 3 hr. under high aeration, followed by harvesting and resuspending to 2 mg./ml. in mixture B; in Expt. 3 this mixture was modified by omission of components as shown. The final incubation was for 6 hr.

Expt. no.	History of cells		Final incubation	Pigments in extracellular fluid	
	Growth conditions	Pretreatment of suspensions		($E_{662}^{1cm.}$)	($E_{590}^{1cm.}$)
1	Anaerobic-light	Nil	Anaerobic-light, mixture B	0.08	0.28
		High aeration	Anaerobic-light, mixture B	0.42	0.50
		High aeration	Low aeration-dark, mixture B	0.19	0.72
2	High aeration-dark	Nil	Low aeration-dark, mixture B	0.09	0.15
3	Anaerobic-light	High aeration	Mixture B	0.47	0.45
			Without ALA	0.09	0
			Without Tween	0.15	0.21
			Without azaguanine	0.33	0.63
			Without methionine	0.45	0.46

was formed most actively by cells recovering from oxygen repression of bacteriochlorophyll synthesis (Table 4). Maximum yields of extracellular pigment were obtained as follows. Cells, grown anaerobically in the light, were harvested, suspended in fresh M-G medium to 2mg. dry wt./ml. and incubated for 3hr. under high aeration; no bacteriochlorophyll was formed in this period and there was about a 25% increase in cell density. The aerated cells were harvested, resuspended in the same volume of mixture B and incubated anaerobically in the light, of intensity about 150ft. candles at the surface of the reaction vessel. This was the standard experimental procedure. Cells which had been grown with high aeration and were therefore devoid of photosynthetic pigments also formed P-662 when incubated in mixture B under low aeration in the dark. Such organisms were, however, less active than those grown and treated as above (Table 4).

Under the standard conditions, the amount of pigment accumulated in the medium reached a maximum within 5-6hr. and then the extinction at 662 declined with a broadening of the peak. These changes could be attributed to light-instability and to loss of magnesium to form a phaeophorbid.

For maximum production of P-662, ALA, Tween 80 and 8-azaguanine were all needed (Table 4). P-590 was formed simultaneously but its production, unlike that of P-662, was depressed by azaguanine (Table 5, Expt. 1).

Effect of nucleic acid derivatives. The stimulation

by 8-azaguanine was not specific; fluorouracil had a similar action with the parent strain (Table 5). More direct evidence for a connexion between limitation of RNA synthesis and accumulation of P-662 was provided by experiments with the mutant strain 6A5, which needs adenine and histidine for growth. Maximum production of P-662 occurred with low concentrations of adenine insufficient to promote maximum bacteriochlorophyll synthesis (Table 5). Limitation of protein synthesis either by inhibitors in the parent strain or by deprivation of histidine in the mutant decreased synthesis of P-662 (Table 5).

Effect of iron. Bacteriochlorophyll synthesis from glycine requires iron and there is evidence that one of its functions is in the conversion of coproporphyrinogen into protoporphyrin (Lascelles, 1956). Recent work with *Euglena gracilis* has now indicated that iron also acts at another point on the biosynthetic pathway to chlorophyll. Thus a degree of iron deficiency which drastically curtails chlorophyll synthesis has little effect on the conversion of porphobilinogen into protoporphyrin (Carell & Price, 1965). The present work with *R. spheroides* supports the idea of two (at least) iron-requiring steps, one before magnesium protoporphyrin monomethyl ester (P-590) and the other between this compound and P-662.

To demonstrate the effect of iron, cells were grown in M-G medium without added iron and then aerated in the same medium supplemented with various concentrations of iron. They were finally suspended in mixture B, without and with

Table 5. *Effect of limitation of nucleic acid and protein synthesis on pigment formation by R. spheroides 8253 and 6A5*

Cells were grown and pretreated as in Expt. 1 in Table 4, except that L-histidine (mm) and adenine (0.2mm) were added to M-G medium with mutant 6A5. Final incubation was for 6hr. anaerobically in the light in mixture B without azaguanine and with other additions as shown. Initial bacteriochlorophyll in Expt. 2, 15.6 mμmoles/ml. —, Not determined.

Expt. no.	Strain	Additions to incubation system	Pigments in extracellular fluid		Bacteriochlorophyll synthesized (mμmoles/ml.)
			($E_{662}^{1cm.}$)	($E_{590}^{1cm.}$)	
1	8253	{ Nil	0.31	0.40	—
		{ Azaguanine (0.4 mm)	0.40	0.36	—
		{ Azaguanine (1 mm)	0.38	0.33	—
		{ Fluorouracil (0.25 mm)	0.47	0.38	—
		{ Puromycin (100 μg./ml.)	0.13	0	—
2	6A5	{	Histidine Adenine		
		{ 0 0.02 mm	0.03	—	0.9
		{ 0.1 mm 0	0.28	—	7.7
		{ 0.1 mm 0.01 mm	0.34	—	8.8
		{ 0.1 mm 0.03 mm	0.29	—	11.1
		{ 0.1 mm 0.1 mm	0.20	—	11.1
		{ 0.1 mm 0.2 mm	0.15	—	10.8
		{			

Table 6. *Effect of iron on pigment synthesis by R. spheroides 8253*

Organisms were grown anaerobically in the light in M-G medium without added iron. They were suspended in the same medium, supplemented as shown with iron citrate, and aerated for 3 hr. They were finally suspended in mixture B without and with iron as shown, and incubated anaerobically in the light for 4 hr.

Concn. of iron (μM)		Pigments in extracellular fluid	
First incubation	Second incubation	($E_{662}^{1\text{cm}}$)	($E_{590}^{1\text{cm}}$)
0	0	0	0
	10	0.31	0.25
2	0	0.16	0.50
	10	0.32	0.33
4	0	0.22	0.51
	10	0.27	0.36
10	0	0.17	0.65
	10	0.29	0.44

Table 7. *Bacteriochlorophyll and P-662 synthesis by R. spheroides 8253*

Cells were grown and pretreated under the standard conditions. Final incubation was anaerobically in the light in mixture B without azaguanine and ALA, which were added as shown. Initial bacteriochlorophyll, 18 μm -moles/ml.

Additions	Bacteriochlorophyll synthesized (μm moles/ml.)		Pigment formed in 6 hr. ($E_{662}^{1\text{cm}}$)
	In 3 hr.	In 6 hr.	
Nil	11	28	0
ALA (1 mM)	4	10	0.17
Azaguanine (0.4 mM)	7	19	0.09
ALA (1 mM) + azaguanine (0.4 mM)	3	6	0.41

iron, and their ability to form P-662 and P-590 was tested under standard conditions (Table 6). For maximum production of P-662, iron was a necessary component of the final incubation system, irrespective of its concentration during the first incubation. With P-590, added iron was not required in the final reaction mixture and maximum synthesis occurred in its absence with cells previously incubated with iron at concentrations as low as 2 μM . Neither pigment was formed by cells which had not received iron throughout, but considerable amounts of coproporphyrinogen and coproporphyrin accumulated.

Synthesis of bacteriochlorophyll and P-662. Though ALA was required for maximum synthesis of P-662 by the parent strain of *R. spheroides*, it strongly

inhibited bacteriochlorophyll synthesis; the concentration of azaguanine used in the system for P-662 production also inhibited, though less markedly (Table 7). Thus production of P-662 occurred under conditions where bacteriochlorophyll synthesis was limited, both by inhibition by ALA (or a product) and by azaguanine. A similar observation with respect to adenine deficiency was made with mutant 6A5 (Table 5).

Inhibition of growth of *R. spheroides* by ALA has been frequently observed by the author. Such effects occur with concentrations as low as 0.1 mM, and photosynthetic growth is more susceptible than is aerobic growth. Since the organism depends on bacteriochlorophyll under the former but not the latter conditions, inhibition of synthesis of this pigment may account for its effect on growth.

DISCUSSION

Pigments similar to P-631 and P-662 as well as magnesium protoporphyrin monomethyl ester have been previously observed in cultures of normal and mutant strains of *R. spheroides* (Sistrom, Griffiths & Stanier, 1956; Stanier & Smith, 1959; Griffiths, 1962; Jones, 1963a,b,c, 1964). Such pigments may exist as lipoprotein complexes in the normal biosynthetic pathway to bacteriochlorophyll. The fact that their extracellular accumulation is enhanced by the detergent Tween 80 supports this and the bound pigments found in extracts of strains 2/73 and 2/21 may represent the true intermediates.

Synthesis of P-631 and P-662 from glycine and succinate is apparently linked obligatorily to protein synthesis just as bacteriochlorophyll formation is. With ALA as substrate, however, synthesis of the bacteriochlorophyll precursors is far less sensitive to inhibitors of protein synthesis. This suggests that, in the normal pathway to bacteriochlorophyll involvement with protein, synthesis occurs at the stage of the initial condensation catalysed by ALA synthase. Protein synthesis may be needed to maintain an adequate activity of ALA synthase, which is rapidly destroyed or inactivated when *R. spheroides* is incubated under conditions which prevent protein synthesis (Bull & Lascelles, 1963). Lability of the enzyme under similar conditions has also been noted by Higuchi, Goto, Fujimoto, Namiki & Kikuchi (1965). The synthase may therefore be subject to rapid turnover in *R. spheroides*. This may also apply to ALA synthase in erythrocytes, since this activity is lost upon maturation of the erythrocyte, though ability to convert ALA into porphyrins is maintained (Falk & Dresel, 1960).

Another possibility is that in the normal path to bacteriochlorophyll the intermediates, including ALA, are bound to a specific protein(s) whose

formation is linked with the functioning of ALA synthase. The inability of the parent strain to form bacteriochlorophyll from ALA would support this possibility if it is assumed that exogenous ALA does not become attached to the 'carrier protein'. An analogous situation occurs in higher plants, where ALA is converted in the dark into free magnesium vinyl phaeoporphyrin a_5 and not to the bound form, found normally in etiolated plants. Upon illumination chlorophyll is synthesized from endogenous precursors and not from the pigment accumulated from ALA (Granick, 1961a,b). The obligatory linkage between protein and chlorophyll synthesis in *Euglena* adapting from dark to light also supports the possibility of protein-bound intermediates which occur at the earliest stages of pigment formation (Kirk & Allen, 1965).

There is no simple explanation obvious to the author which accounts for the curious conditions required for the accumulation of P-662 by *R. spheroides* 8253. What is particularly difficult to understand is the apparently rather specific effect of limitation of nucleic acid metabolism on accumulation of the pigment. The fact that fluorouracil and azaguanine, as well as deficiency of adenine, all stimulated its production suggest that the effect involves some form of RNA rather than a mononucleotide.

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