The Regulation of Synthesis of Iron and Magnesium Tetrapyrroles

OBSERVATIONS WITH MUTANT STRAINS OF *RHODOPSEUDOMONAS SPHEROIDES*

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1. Cell suspensions of mutant strains of *Rhodopseudomonas spheroides*, which cannot form bacteriochlorophyll, have been examined for their ability to form other tetrapyrroles under conditions of low aeration. With the exception of strain L-57, the mutants could form carotenoids. 2. All strains, like the parent organism, formed iron protoporphyrin when incubated with δ -aminolaevulate, showing that the iron branch of the biosynthetic pathway operated. 3. Magnesium protoporphyrin or its monomethyl ester was also formed from δ -aminolaevulate by all strains with the exception of L-57. 4. Coproporphyrin and coproporphyrinogen were accumulated by the parent and by strains 2/73 and 2/21 when incubated with glycine and succinate in the presence of ethionine. Strain 2/33, which required methionine for growth, accumulated these compounds in the presence and absence of methionine. 5. Strain L-57 did not accumulate porphyrins from glycine and succinate under any conditions. However, the δ -aminolaevulate synthase of this mutant showed the same rise in activity in response to reduced aeration as did that of the parent organism. 6. Ethionine inhibited production of protoporphyrin and its derivatives from δ -aminolaevulate by the parent strain. 7. The accumulation of coproporphyrin(ogen) under conditions of methionine deficiency may reflect the presence of enzymes of the magnesium branch of the biosynthetic pathway. Strain L-57 may lack a genetic element which determines the development of the entire photosynthetic apparatus. Since this strain did not accumulate coproporphyrin(ogen), the possibility of a specific δ -aminolaevulate synthase, directed towards bacteriochlorophyll synthesis, should be considered.

Photosynthetic organisms make both haems and chlorophylls and the regulation of the synthesis of these tetrapyrrole derivatives raises several distinct problems. The formation of the iron and magnesium derivatives involves a branched pathway which stems from the common intermediate, protoporphyrin. Utilization of the latter is regulated in a way which results in the formation of about one hundred times more chlorophyll than haems. Also, each branch is apparently under independent control; in *Rhodopseudomonas spheroides* and related bacteria, for instance, synthesis of haems continues under conditions, such as high aeration, which almost totally repress bacteriochlorophyll synthesis (Porra & Lascelles, 1965).

The little information available on how such regulation is achieved has come mainly from work with *R. spheroides*. Partial repression of ALA*

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

synthase and ALA dehydratase occurs under conditions where bacteriochlorophyll synthesis is also repressed (Lascelles, 1959, 1960). It seems likely that enzymes on the magnesium branch of the pathway are also subject to repression since the enzyme responsible for the methylation of magnesium protoporphyrin is detectable only in pigmented cells (Gibson, Neuberger & Tait, 1963). This enzyme catalyses the reaction

Information about other enzymes on the magnesium branch is lacking since no other step has been demonstrated at the enzyme level.

Control of tetrapyrrole synthesis in *R. spheroides* may also be achieved by mechanisms of feedback inhibition. Iron protoporphyrin is a potent inhibitor of ALA synthase, though this action of the iron compound does not clarify the regulation of the

magnesium branch (Burnham & Lascelles, 1963). It has been suggested that failure of a normal feedback control mechanism may account for the accumulation of coproporphyrin that occurs when bacteriochlorophyll synthesis is blocked by interference with the methyltransferase step. This can be caused either by the analogue, ethionine (Gibson, Neuberger & Tait, 1962, 1963) or by limitation of methionine with an auxotroph (Lessie & Sistrom, 1964). The results observed by these workers are consistent with the possibility of regulation of an early step on the biosynthetic pathway by a compound coming after the methylation step.

In the present work accumulation of porphyrins, iron protoporphyrin and chlorophyll derivatives by wild-type and chlorophyll-less strains of *R. spheroides* have been examined with a view to obtaining more insight into the problem of the regulation of the branched biosynthetic pathway.

METHODS

Organisms. The parent strain was *R. spheroides* N.C.I.B. 8253 and all mutants were derived from it. The properties of the pigment mutants used in this work are summarized in Table 1. Strain L-57, previously described by Bull & Lascelles (1963), completely lacked the ability to form both bacteriochlorophyll and carotenoids under all conditions. Strains 2/73 and 2/21, described in the preceding paper (Lascelles, 1966), were blocked at different stages in bacteriochlorophyll synthesis but formed the normal complement of carotenoids when grown with low aeration. Strain 2/33 required methionine for growth and was also unable to synthesize bacteriochlorophyll even in the presence of high concentrations of methionine, though it could form carotenoids. It was isolated by treatment with mutagen as described in the previous paper. Strain 2/33 was apparently a single mutant since revertants could form bacteriochlorophyll and grew without added methionine.

Stock cultures of the parent organism were maintained photosynthetically as stab cultures on the yeast extract-malate-glutamate medium (Lascelles, 1956a, 1959); the pigment mutants were kept on slopes of the same medium (Lascelles, 1966).

Experiments with cell suspensions. Unless stated otherwise organisms were grown at 30° in the dark with low aeration in the M-G medium of Lascelles (1959), which was supplemented with 1 mM-L-methionine for growth of strain 2/33. Cultures to provide cells for suspension work were inoculated, grown and harvested as described in the preceding paper (Lascelles, 1966). The harvested cells were suspended in mixture A, modified as necessary, and incubated with low aeration as described in the previous paper.

Estimations. Protein and bacteriochlorophyll were determined as in the previous paper. P-631 and P-662 were measured spectrophotometrically in the supernatant medium as described previously. For comparative purposes the amounts of these pigments are expressed as μmoles ; these values were calculated from the extinction values in ether at the corresponding wavelength for magnesium vinylphaeocoporphyrin a_5 and chlorophyll a for P-631 and P-662 respectively (Falk, 1964). The figures are to be considered as approximate only since they are based on the assumption that the extinction coefficients for the pigments in ether are identical with their values in the medium.

P-590 was established as magnesium protoporphyrin monomethyl ester for the parent organism as described in the preceding paper. With the other organisms P-590 was assumed to be either magnesium protoporphyrin or its methyl ester. In reaction mixtures containing magnesium protoporphyrin or the ester, as well as coproporphyrin(ogen), each pigment was estimated by the method of Cooper (1963).

Coproporphyrinogen + coproporphyrin, referred to as coproporphyrin(ogen), were estimated in the supernatant fluids after they had been left in the dark in 1.4 N-HCl for at least 2 hr. to allow conversion of the porphyrinogen into coproporphyrin; the extinction at the Soret maximum (401 m μ) was then read. In some experiments coproporphyrin(ogen) and protoporphyrin were determined in the same reaction mixture by the method of Porra & Falk (1963).

Iron protoporphyrin in the supernatant fluids was estimated by measurement of the reduced minus oxidized spectrum (558–541 m μ) of the pyridine haemochromogen derivative (Falk, 1964). The Cary model 14 spectrophotometer with expanded scale accessory was used for these determinations.

δ -Aminolaevulate synthase was measured in cell-free extracts as described by Burnham & Lascelles (1963).

Table 1. *Pigments accumulated by strains of R. spheroides*

All strains were grown in the dark with low aeration in M-G medium, supplemented with 1 mM-L-methionine for strain 2/33. Tentative identification of extracellular pigments (Lascelles, 1966): P-590, magnesium protoporphyrin monomethyl ester; P-631, magnesium 2,4-divinylphaeocoporphyrin a_5 monomethyl ester; P-662, 2-devinyl-2-hydroxyethyl-chlorophyllide a .

Strain	Intracellular pigments		Extracellular pigments
	Bacteriochlorophyll	Carotenoids	
N.C.I.B. 8253	+	+	Traces of P-590
2/73	—	+	P-631
2/21	Trace	+	P-662
2/33*	Trace	+	Coproporphyrin(ogen)
L-57	—	—	—

* Requires methionine for growth.

RESULTS

Conversion of ALA into iron and magnesium derivatives. Synthesis of iron protoporphyrin from ALA by suspensions of the parent strain has been previously observed (Lascelles, 1956b). The production of this compound by the various mutants was examined in relation to the concurrent synthesis of magnesium derivatives (Table 2).

Iron protoporphyrin was not detectable in the supernatant fluids from any of the strains when they were incubated with glycine and succinate. Upon addition of ALA, however, the iron derivative was accumulated by all organisms and, except for strain L-57, there was concurrent synthesis of magnesium protoporphyrin or its methyl ester (Table 2). Strain 2/33 formed less of both metal derivatives

than did strains 2/73 and 2/21, but only strain L-57 appeared to lack the complete magnesium pathway. Strains 2/73 and 2/21, like the parent organism, accumulated three to four times more magnesium derivatives than iron protoporphyrin (Table 2). In all cases coproporphyrin(ogen) accompanied the metal derivatives in the supernatant fluids (see Table 4). The formation of iron protoporphyrin from ALA indicates the operation of the iron branch of the biosynthetic pathway in all organisms. The complete absence of magnesium derivatives from incubation systems with strain L-57 suggests that this strain may lack all enzymes of the magnesium branch. This is consistent with its complete inability to form bacteriochlorophyll.

Coproporphyrin production and methionine deficiency. The effect of ethionine on the production of

Table 2. *Conversion of ALA into iron and magnesium tetrapyrroles by various strains of R. spheroides*

Organisms were grown under low aeration and suspended to a density of 2 mg./ml. in mixture A supplemented with 1 mM-ALA. They were incubated with low aeration (2.5 ml. in 11 mm. tubes) for 16 hr. and the pigments in the supernatant fluids estimated as described in the text. The method for estimation of the magnesium derivative did not distinguish between magnesium protoporphyrin and the methyl ester. Formation of the ester was established only for the pigment synthesized by strain 8253 (Lascelles, 1966).

Strain	Compound formed (mμmoles/ml.)		
	Iron protoporphyrin	Magnesium protoporphyrin(ester)	P-631 or P-662
N.C.I.B. 8253	4.9	19	0.4 (P-662)
2/73	5.8	12	7 (P-631)
2/21	6.8	13	5 (P-662)
2/33	2.5	5	0
L-57	6.2	0	0

Table 3. *Accumulation of coproporphyrin(ogen) by various strains of R. spheroides*

Organisms were grown with low aeration and suspended (2 mg. dry wt./ml.) in mixture A without methionine and supplemented with ethionine or methionine as shown. Incubation was for 18 hr. with low aeration as in Table 2. Pigments were estimated as described in the Methods section. The final column gives concentrations of bacteriochlorophyll, P-631 and P-662 for strains 8253, 2/73 and 2/21 respectively.

Strain	Additions		Pigments (mμmoles/ml.)	
	DL-Ethionine (5 mM)	L-Methionine (0.1 mM)	Coproporphyrin(ogen)	Bacteriochlorophyll or precursor
N.C.I.B. 8253	—	—	1	40
	+	—	87	3
2/73	—	—	2	6.5
	+	—	35	2.3
2/21	—	—	6	8.2
	+	—	18	1.1
2/33	—	—	41	0
	—	+	46	0
L-57	—	—	1	0
	+	—	3	0

tetrapyrroles by the various organisms was examined (Table 3). Strains 2/73 and 2/21, like the parent strain, accumulated coproporphyrin(ogen) when incubated under low aeration with glycine and succinate in the presence of ethionine; at the same time synthesis of bacteriochlorophyll by the parent and of P-631 and P-662 by the mutants was inhibited. Strain L-57, however, which completely lacks the ability to form magnesium derivatives, did not accumulate porphyrins or porphyrinogens when incubated under the same conditions, either with or without the analogue. The methionine-requiring mutant 2/33 formed coproporphyrin(ogen) both in the presence and absence of methionine (Table 3). This organism makes only traces of bacteriochlorophyll even with optimum concentration of methionine and it behaves in the same way as the parent strain in the presence of ethionine.

Since ethionine interferes with the biosynthetic pathway at the methylation step it might be expected that protoporphyrin or the magnesium derivative would accumulate rather than coproporphyrin(ogen). The effect of ethionine on the parent strain was therefore examined with ALA as substrate under conditions that normally gave maximum production of magnesium protoporphyrin monomethyl ester. Spectroscopic examination of the supernatant fluids showed that ethionine inhibited formation of magnesium derivatives since the characteristic absorption band at $590\text{m}\mu$ was not detectable. The supernatants were analysed for coproporphyrin(ogen) and protoporphyrin by the method of Porra & Falk (1963); this method would convert any magnesium protoporphyrin into protoporphyrin. The analyses showed that ethionine inhibited protoporphyrin production from ALA and there was also inhibition of iron protoporphyrin formation (Table 4). It can be concluded that methionine deficiency has a secondary effect on the biosynthetic path between coproporphyrinogen and protoporphyrin and this may account for the accumulation of coproporphyrin(ogen) rather than protoporphyrin or magnesium protoporphyrin.

Formation of ALA synthase in response to low

aeration. It has been suggested that repression of ALA synthase accounts partly for the inhibitory effect of oxygen on bacteriochlorophyll synthesis by the parent organism (Lascelles, 1959, 1960). Suspensions of the parent grown under high aeration, and consequently devoid of pigment, adapt to form bacteriochlorophyll when incubated with low aeration, and pigmentation is accompanied by a rapid rise in ALA synthase activity. Such a rise in activity might possibly be attributable to the formation of an ALA synthase concerned specifically in bacteriochlorophyll synthesis and differing from the enzyme required for formation of iron porphyrins. If this were so, mutant L-57 might lack the ability to form the oxygen-repressible enzyme since it apparently lacks the magnesium branch of the biosynthetic pathway. This possibility was examined by assaying ALA synthase in suspensions of the mutant incubated under those conditions which cause synthesis of enzyme and pigment by the parent (Table 5). The mutant strain showed a rise in enzyme activity similar to that found with the parent organism, and this increase presumably represented new protein synthesis since it was inhibited by puromycin. This result therefore provides no support for the existence of an ALA

Table 5. *Development of ALA synthase by strain 8253 and L-57 in response to low aeration*

Organisms were grown with high aeration and then incubated as suspensions (2mg./ml. dry wt.) in M-G medium under low aeration as described by Lascelles (1959). Samples were removed at intervals and ALA synthase was determined on cell-free extracts (Burnham & Lascelles, 1963). Enzyme activity is expressed as $\text{m}\mu\text{moles}$ of ALA formed/hr./mg. of protein.

Strain	Puromycin (100 $\mu\text{g.}/\text{ml.}$)	ALA synthase after incubation		
		0	2hr.	4hr.
N.C.I.B. 8253	—	64	236	201
L-57	—	38	181	293
	+	38	17	15

Table 4. *Effect of ethionine on the production of tetrapyrroles from ALA by R. spheroides 8253*

The cells were grown with low aeration and suspended in mixture A without methionine but supplemented with 1mm-ALA. Incubation was for 20hr. as in Table 2. Copro- and proto-porphyrin were determined in the supernatant fluid, after dilution in 1.4N-HCl, by the method of Porra & Falk (1963).

DL-Ethionine (6mm)	Tetrapyrrole formed ($\text{m}\mu\text{moles}/\text{ml.}$)		
	Iron protoporphyrin	Protoporphyrin	Coproporphyrin(ogen)
—	5.0	18	23
+	1.25	6	59

synthase specifically concerned in bacteriochlorophyll synthesis, though it does not disprove this possibility.

DISCUSSION

Comparison of the behaviour of the mutants under similar conditions leads to the following tentative conclusions. (1) Strains 2/73 and 2/21 are blocked at single steps on the magnesium branch and these lesions do not interfere with the normal regulatory mechanisms since neither mutant accumulates excessive amounts of chlorophyll precursors. The formation of other components of the photosynthetic apparatus is apparently unaffected since these mutants can form carotenoids. (2) Strain L-57 has apparently lost the entire magnesium branch though the iron branch remains intact. The loss of all enzymes of the magnesium branch is assumed from the inability of this mutant to form magnesium protoporphyrin or other chlorophyll precursors from ALA. To establish unequivocally the absence of these enzymes requires assay of the individual steps. The inability of L-57 to form carotenoids as well as bacteriochlorophyll suggests the loss or dysfunction of a genetic element which governs the synthesis of the entire photosynthetic apparatus. The mutant is apparently analogous to bleached mutant strains of algae which cannot form the photosynthetic pigments or other components of the chloroplast (Gibor & Granick, 1964).

Strain 2/33 differs from the methionine auxotroph of *R. sphaeroides* used by Lessie & Siström (1964) in that it does not make bacteriochlorophyll, whereas it excretes coproporphyrin(ogen) even in the presence of methionine at concentrations sufficient to give maximum growth. The block in methionine synthesis in this strain occurs at the stage of homocysteine methylation, a reaction involving a coenzyme form of vitamin B₁₂ (S. E. Cauthen, J. R. Pattison & J. Lascelles, unpublished work). Extracts of the organism can form *S*-adenosyl-methionine from methionine and ATP. A possible reason for its inability to form bacteriochlorophyll is that it does not accumulate a sufficiently high internal concentration of methionine to promote a rate of *S*-adenosylmethionine synthesis necessary for pigment formation.

Accumulation of coproporphyrin(ogen) under conditions of methionine deficiency may result from failure of a feedback control mechanism involving an intermediate which comes after the methylation step (Gibson *et al.* 1963; Lessie & Siström, 1964). In strains 2/73 and 2/21 the lack of coproporphyrin(ogen) accumulation (unless methionine is present), coupled with the relatively small accumulation of chlorophyll precursors, suggests that both mutants form the substance(s) which may control an early

step in the biosynthetic path. Bacteriochlorophyll is not, apparently, the controlling agent since neither mutant makes this pigment. It is possible that the hypothetical control is exerted by an intermediate in the area between the methylation step and P-631. P-631, which is a protochlorophyll type of pigment, could be the key substance, possibly in a protein-bound form. In higher plants a bound form of protochlorophyll *a* (or its non-phytylated counterpart) may regulate the synthesis of chlorophyll (Granick, 1961). Thus etiolated plants contain the bound precursor but do not form chlorophyll, nor do they accumulate earlier intermediates on the biosynthetic pathway; upon illumination the protochlorophyll is converted into chlorophyll and the synthesis of this pigment then proceeds at the expense of glycine and succinyl-CoA.

Of the mechanisms for the regulation of the iron and magnesium branches of the biosynthetic pathway the possibility of two distinct ALA synthases should be considered. One of these might be directed towards chlorophyll synthesis and the other towards haems, each enzyme being regulated by the respective end product. Burnham & Lascelles (1963) could find no evidence of a particulate form of the enzyme, nor was there any apparent difference in the properties of the enzyme in non-pigmented and pigmented cells. Such negative evidence is not necessarily significant since the methods for extraction and assay may have revealed only one form of this labile enzyme. The pattern of coproporphyrin(ogen) accumulation from glycine and succinate by the various mutants does provide some support for two ALA synthases. Those mutants which are apparently blocked at a single step in the magnesium branch (2/33, 2/73 and 2/21) all accumulate the porphyrin under conditions of methionine deficiency. These mutants also formed carotenoids when grown with low aeration. In contrast, strain L-57 did not accumulate coproporphyrin(ogen) from glycine and succinate under any condition, nor did it make carotenoids. Coproporphyrin(ogen) accumulation might be a manifestation of the unregulated activity of the ALA synthase specifically associated with bacteriochlorophyll synthesis. If so, strain L-57 must be presumed to lack this enzyme, the synthesis of which may be under the control of the genetic element concerned in the elaboration of the entire photosynthetic apparatus.

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REFERENCES

- Bull, M. J. & Lascelles, J. (1963). *Biochem. J.* **87**, 15.
- Burnham, B. F. & Lascelles, J. (1963). *Biochem. J.* **87**, 462.
- Cooper, R. (1963). *Biochem. J.* **89**, 100.

- Falk, J. E. (1964). *Porphyrins and Metalloporphyrins*. Amsterdam: Elsevier Publishing Co.
- Gibor, A. & Granick, S. (1964). *Science*, **145**, 890.
- Gibson, K. D., Neuberger, A. & Tait, G. H. (1962). *Biochem. J.* **82**, 550.
- Gibson, K. D., Neuberger, A. & Tait, G. H. (1963). *Biochem. J.* **88**, 325.
- Granick, S. (1961). *Proc. 6th int. Congr. Biochem., Moscow*, vol. 6, p. 176. Oxford: Pergamon Press Ltd.
- Lascelles, J. (1956a). *Biochem. J.* **62**, 78.
- Lascelles, J. (1956b). *J. gen. Microbiol.* **15**, 404.
- Lascelles, J. (1959). *Biochem. J.* **72**, 508.
- Lascelles, J. (1960). *J. gen. Microbiol.* **23**, 487.
- Lascelles, J. (1966). *Biochem. J.* **100**, 175.
- Lessie, T. G. & Sistrom, W. R. (1964). *Biochim. biophys. Acta*, **86**, 250.
- Porra, R. J. & Falk, J. E. (1963). *Biochem. J.* **90**, 69.
- Porra, R. J. & Lascelles, J. (1965). *Biochem. J.* **94**, 120.