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The use of *N*-methylprotoporphyrin dimethyl ester to inhibit ferrochelatase in *Rhodopseudomonas sphaeroides* and its effect in promoting biosynthesis of magnesium tetrapyrroles

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N-Methylprotoporphyrin dimethyl ester inhibits ferrochelatase in isolated membranes of *Rhodopseudomonas sphaeroides* at low concentrations (around 10nm). Full inhibition developed after a short lag phase. The inhibition was non-competitive with porphyrin substrate. Addition of inhibitor to growing cultures of *Rps. sphaeroides* caused a decrease (near 40%) in cytochrome content and a severe inhibition of ferrochelatase; the excretion of haem into the medium by cell suspensions was also severely inhibited. The addition of *N*-methylprotoporphyrin dimethyl ester to suspensions of photosynthetically competent *Rps. sphaeroides* Ga caused excretion of Mg-protoporphyrin monomethyl ester. When added to mutants V3 and O1, magnesium divinylphaeoporphyrin a_5 monomethyl ester and 2-devinyl-2-hydroxyethylphaeophorbide *a* were excreted, with maximum effect at around 3μM-inhibitor in the medium. The results are interpreted to suggest that the inhibitor decreases concentration of intracellular haem, which normally controls the activity of 5-aminolaevulinate synthetase. Unregulated activity of this enzyme leads to overproduction of protoporphyrin, which is diverted to the bacteriochlorophyll pathway. Further control operates at magnesium protoporphyrin ester conversion in normal cells.

In the photosynthetic bacterium *Rhodopseudomonas sphaeroides* the major light-harvesting and reaction-centre component is bacteriochlorophyll *a*, the structure of which closely resembles that of green-plant chlorophyll *a*. The synthesis of bacteriochlorophyll *a* is regulated by oxygen concentration and by light intensity; either high oxygen or high light suppress pigment synthesis (see Cohen-Bazire *et al.*, 1957). Under conditions of high aeration, *Rps. sphaeroides* obtains its energy from the oxidation of substrates supplied in the medium and does not assemble the extensive pigment-rich deeply invaginated membrane characteristic of the photosynthetic form. *Rps. sphaeroides* synthesizes the iron tetrapyrrole prosthetic groups of the cytochromes involved in either photosynthetic or respiration-dependent electron transport (Connelly *et al.*, 1973), but synthesizes magnesium tetrapyrroles only for photosynthetic growth, and so careful regulation of the flux of tetrapyrroles along the pathways of haem and bacteriochlorophyll synthesis is necessary. These pathways, from the common precursor 5-aminolaevulinate, branch at protoporphyrin [see Jones (1978) for a review]. Either ferrous ions are inserted into protoporphyrin by the enzyme ferrochelatase to make protohaem, or Mg^{2+} is inserted by a reaction as yet uncharacterized to yield magnesium protoporphyrin (Scheme 1). Control is regulated so that 30 or 40 times as much bacteriochlorophyll *a* is present as haem in photosynthetic cells and no bacteriochlorophyll is present in highly oxygenated cells.

Iron-deficient cultures of *Rps. sphaeroides* accumulate a huge excess of porphyrin, very largely coproporphyrinogen (Lascelles, 1956), and it was shown by Burnham & Lascelles (1963) that haem was a potent inhibitor of 5-aminolaevulinate synthetase, the rate-limiting enzyme in protoporphyrin synthesis. It was suggested that, in iron deficiency, haem could not be synthesized and that conse-

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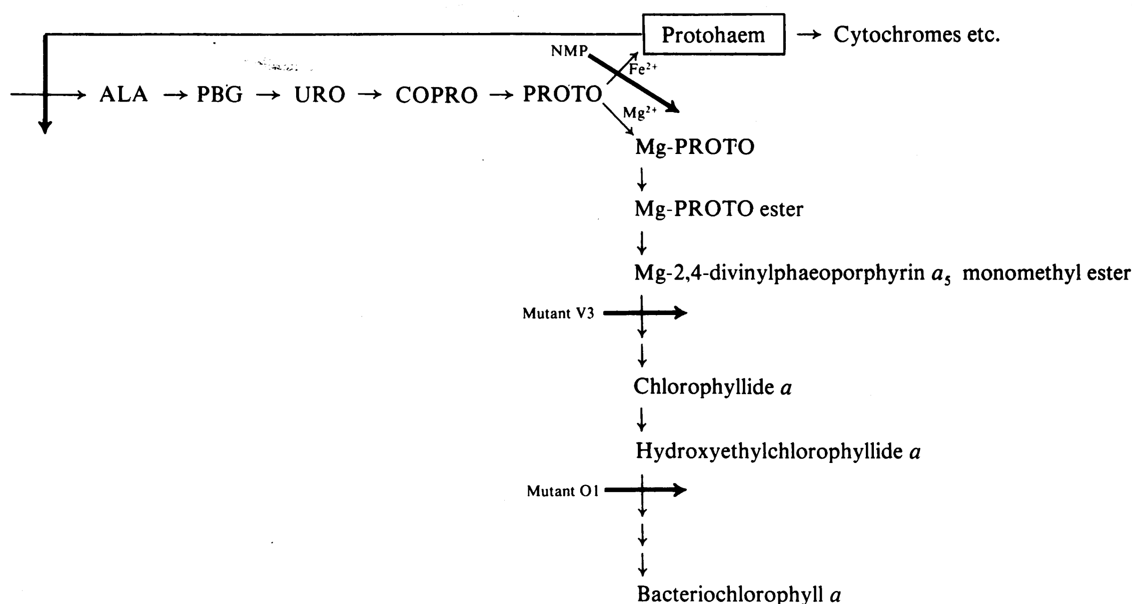

Scheme 1. *Pathway of bacteriochlorophyll and haem biosynthesis in Rps. sphaeroides, showing location of blocks in pigment synthesis in mutants V3 and O1 and site of action of N-methylprotoporphyrin dimethyl ester*
Abbreviations used: ALA, 5-aminolaevulinate; PBG, porphobilinogen; URO, uroporphyrinogen; COPRO, coproporphyrinogen; PROTO, protoporphyrin; NMP, *N*-methylprotoporphyrin

quently 5-aminolaevulinate synthetase was unregulated, leading to excess tetrapyrrole-pigment synthesis. It was further proposed that, since Mg^{2+} insertion into protoporphyrin was apparently inhibited by oxygen (Gorchein, 1972, 1973), the magnesium pathway would be blocked on aeration and protoporphyrin would be diverted to haem synthesis. Haem produced in excess of requirements would inhibit 5-aminolaevulinate synthetase and the pathway would be closed down (Lascelles & Hatch, 1969). Some features of this regulatory scheme leave questions unanswered. Particularly, why is it that, in iron deficiency, coproporphyrinogen accumulates, not magnesium pigments?

We have investigated the relationship of the iron and the magnesium tetrapyrrole pathways using *N*-methylprotoporphyrin dimethyl ester to inhibit ferrochelatase. The free acid, *N*-methylprotoporphyrin, is a very potent inhibitor of ferrochelatase in rat liver mitochondria (De Matteis *et al.*, 1980). We show that the methyl ester blocks ferrochelatase non-competitively with protoporphyrin in *Rps. sphaeroides* and affects the magnesium pathway in a fashion quite distinct from iron deficiency.

We have examined also the effects of *N*-methylprotoporphyrin treatment of photosynthetically competent *Rps. sphaeroides* and of mutants V3 and O1 blocked at specific sites in bacteriochlorophyll synthesis. The mutants accumulate respectively magnesium 2,4-divinyl phaeoporphyrin a_5 monomethyl ester (M. C. Quirke, W. T. Griffiths & O. T. G. Jones, unpublished work) and 2-desvinyl-2-hydroxyethylchlorophyllide *a* (Jones, 1964). The addition of the inhibitor of ferrochelatase is shown to increase production of the pigment characteristic of the mutant.

Materials and methods

Organisms

Three strains of *Rps. sphaeroides* were used: Ga is carotenoid-deficient but photosynthetically competent; V3, obtained by mutagenesis of wild-type cells with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine accumulates magnesium 2,4-divinylphaeoporphyrin a_5 monomethyl ester; O1 accumulates 2-desvinyl-2-hydroxyethylchlorophyllide *a*. All three were grown in the dark at 30°C in the aerobic culture medium of Sistrom (1960). Cells were treated with *N*-methylprotoporphyrin dimethyl ester either by growth in medium containing the indicated concentration of the inhibitor or cells were harvested, resuspended in 8 ml portions in fresh medium supplemented with 10 mM-glycine at an apparent A_{680} of 2.0 to which inhibitor was added and the cells incubated with limited aeration (Lascelles, 1966) for 16 h, when A_{680} was again recorded.

Assay of pigment production

Cells were centrifuged out of medium and spectra of the supernatants were recorded. The cell pellets

were made up in 6ml of 20mM-Mops (4-morpholinepropanesulphonic acid)/100mM-KCl, pH 7.0, and their absorption spectra measured by using a suitable dilution of milk as a light-scattering reference. In some experiments the pigments in the supernatants were transferred to diethyl ether by shaking in a separating funnel and the pigments in the cells were extracted in acetone/methanol (3:1, v/v) and transferred to diethyl ether in a separating funnel by the addition of water to make a biphasic system. Spectra in ether were recorded. The following absorption coefficients were used in calculating concentrations: magnesium protoporphyrin monomethyl ester, $\varepsilon_{mM}^{590} = 18.2\,mM\cdot cm^{-1}$ (Granick, 1961); magnesium 2,4-divinylphaeoporphyrin a_5, $\varepsilon_{mM}^{623} = 35.6\,mM\cdot cm^{-1}$; 2-desvinyl-2-hydroxyethyl phaeophorbide *a*, $\varepsilon_{mM}^{659} = 80\,mM\cdot cm^{-1}$.

Ferrochelatase assay

The enzyme was assayed in a dual-wavelength spectrophotometer at 30°C with deuteroporphyrin as porphyrin and Co^{2+} as metal substrates (Jones & Jones, 1969, 1970).

Haems and cytochromes

Haem in the supernatants after removal of whole cells by centrifugation was assayed in dithionite-reduced-minus-ferricyanide-oxidized difference spectra after the addition of 1 ml of pyridine and 1 ml of 1M-KOH to 4ml of supernatant. A $\Delta\varepsilon_{mM}^{550-540}$ value of $20\,mM\cdot cm^{-1}$ was assumed. Cytochromes were assayed in either whole cells or membrane preparations in dithionite-reduced-minus-aerated difference spectra.

A $\Delta\varepsilon_{mM}^{550-540}$ value of 20 was assumed for cytochrome *c* and a $\Delta\varepsilon_{mM}^{560-570}$ value of $20\,mM\cdot cm^{-1}$ was assumed for cytochrome *b*. No attempt was made to resolve different types of *c* and *b* cytochromes.

Potentiometric titrations

Mixtures of cytochromes in isolated membranes were resolved by potentiometric titration in buffer at pH 7.0 in an anaerobic cuvette, with constant scanning of spectra (Cross *et al.*, 1981).

Preparation of membranes from Rps. sphaeroides

Cells suspended in 20mM-Mops/100mM-KCl, pH 7.0, were passed twice through a French pressure cell at 96.6–110.4 MPa (14000–16000lbf/sq.in.). The suspension was centrifuged at 14000***g*** for 15min to remove unbroken cells and the supernatant re-centrifuged at 120000***g*** for 120min. The membrane pellet was made up in the Mops/KCl buffer at approx. 30mg of protein/ml.

Protein determination

The method of Lowry *et al.* (1951) was used.

Chromatography of porphyrins

To determine the number of free aerobic groups, porphyrins extracted from medium or cells were separated on t.l.c. plates of cellulose (Whatman, CC41) in a solvent system containing 80ml of 2,6-lutidine and 30ml of water. The atmosphere was saturated with NH_3 vapour from two small beakers fitted with paper wicks. Deuteroporphyrin IX, deuteroporphyrin IX dimethyl ester, protochlorophyllide and coproporphyrin III were used as standard materials.

Chemicals

Deuteroporphyrin IX dimethyl ester was purchased from Koch–Light Laboratories, Colnbrook, Slough, Berks. SL3 0BZ, U.K. Protochlorophyllide was a gift from Dr. W. T. Griffiths, Department of Biochemistry, University of Bristol, and *N*-methylprotoporphyrin dimethyl ester (a mixture of isomers) was prepared as described in Tephly *et al.* (1981). Free porphyrins were prepared from esters by hydrolysis in 25% (w/v) HCl (Falk, 1964).

Results

Effect of N-methylprotoporphyrin dimethyl ester upon haem pigments in Rps. sphaeroides

The addition of *N*-methylprotoporphyrin dimethyl ester to *Rps. sphaeroides* membranes inhibited ferrochelatase activity. It appears that the ferrochelatase of *Rps. sphaeroides* has a sensitivity to *N*-methylprotoporphyrin ester similar to that of rat liver ferrochelatase to *N*-methylporphyrins (De Matteis *et al.*, 1980). Full inhibition developed only after some seconds (Fig. 1). In separate experiments it was found that the free acid form, *N*-methylprotoporphyrin, was not a more potent inhibitor (results not shown). The concentration of *N*-methylprotoporphyrin dimethyl ester required to produce inhibition of ferrochelatase was extremely low; 30% inhibition was measured at 1.5nM (see Fig. 2). The inhibition was non-competitive with the porphyrin substrate (Fig. 2).

The effect of *N*-methylprotoporphyrin dimethyl ester upon whole cells of *Rps. sphaeroides* was next investigated. Cultures of mutant O1 were grown in the presence of 0.8μM-inhibitor. The cells were harvested towards the end of the exponential phase of growth and the cytochrome content measured, using difference spectra of whole cell suspensions (Table 1). The content of cytochromes was diminished by growth in the presence of *N*-methylprotoporphyrin ester; both cytochromes *b* and *c* were present at about 60% of the concentration found in untreated cells. In separate experiments (results not shown) it was found by potentiometric titration that the same mixture of cytochromes of the

b-type and the *c*-type was present in both control and inhibitor-treated cells.

The ferrochelatase activity of membranes prepared from inhibitor-grown cells was less than 10% of that of the control membranes (Table 1). When whole cell suspensions were incubated for 16h in the glycine-supplemented medium, *N*-methylprotoporphyrin dimethyl ester did not affect the growth of the cells (which usually doubled under these conditions) and had little effect upon their cytochrome content, although ferrochelatase was inhibited.

The effects of the inhibitor upon ferrochelatase activity measured in membranes isolated after disruption of the cells grown in *N*-methylprotoporphyrin ester could arise during the act of disruption. The membranes could be exposed to precipitated *N*-methylprotoporphyrin ester on the outer envelope of the bacteria. However, the effects upon cytochrome content make this appear an unlikely explanation, and additional experiments

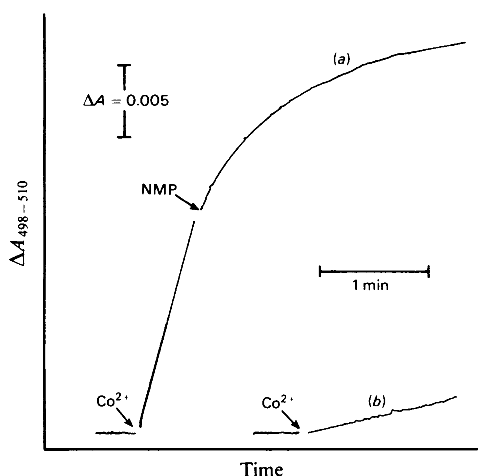

Fig. 1. *Effect of addition of N-methylprotoporphyrin dimethyl ester, with and without pre-incubation, upon ferrochelatase activity of Rps. sphaeroides membranes*

A cuvette containing 2.5ml of 100mM-Tris/HCl, pH7.2, 0.1% (w/v) Tween 80, 0.8mg of membranes from *Rps. sphaeroides* O1 and deuteroporphyrin (50μM) was incubated at 30°C in a dual-wavelength spectrophotometer; Co^{2+} (40μM) was added to start the reaction. In (*a*) 40nM-*N*-methylprotoporphyrin was added where indicated on the trace by NMP; (*b*) 40nM-*N*-methylprotoporphyrin was added 1min before Co^{2+} addition.

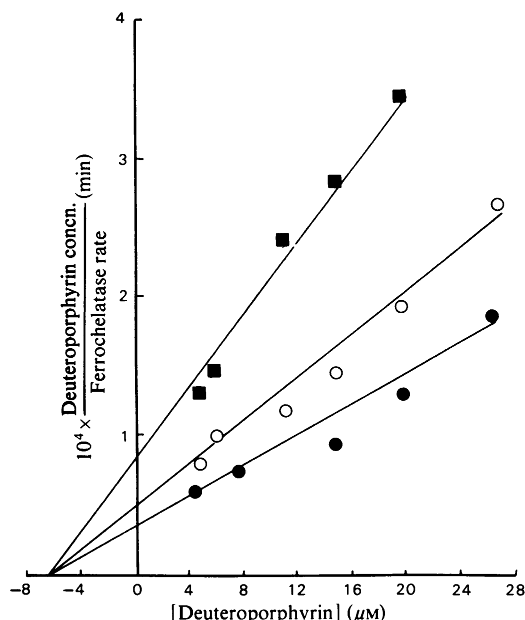

Fig. 2. *Kinetic analysis of the effect of N-methylprotoporphyrin dimethyl ester upon ferrochelatase activity of Rps. sphaeroides O1*

Experimental details are as given in the legend to Fig. 1(*b*). The inhibitor was preincubated with the membranes. The Co^{2+} concentration (40μM) was fixed and the deuteroporphyrin concentration was varied as shown. ●, No inhibitor; ○, 1.5nM-inhibitor; ■, 7.5nM-inhibitor. The K_m for deuteroporphyrin, calculated from the intercept, was 6.5μM.

Table 1. *Effect of growth of Rps. sphaeroides O1 in medium containing N-methylprotoporphyrin dimethyl ester upon cytochrome content and ferrochelatase activity*

Cells were grown in the dark, with shaking, in medium supplemented with *N*-methylprotoporphyrin dimethyl ester (0.8μM). Cytochrome concentrations were determined by dithionite-reduced-minus-air-oxidized difference spectroscopy of whole cell suspensions (see the Materials and methods section). Samples of cells were washed twice, disrupted and a membrane fraction prepared by centrifugation (see the Materials and methods section) for assay of ferrochelatase activity.

Cell sample	Cytochrome *b* (nmol/mg of protein)	Cytochrome *c* (nmol/mg of protein)	Ferrochelatase activity (nmol of Co-porphyrin/ min per mg of protein)
Control	0.28	0.33	2.4
+*N*-Methylprotoporphyrin	0.16	0.18	0.2

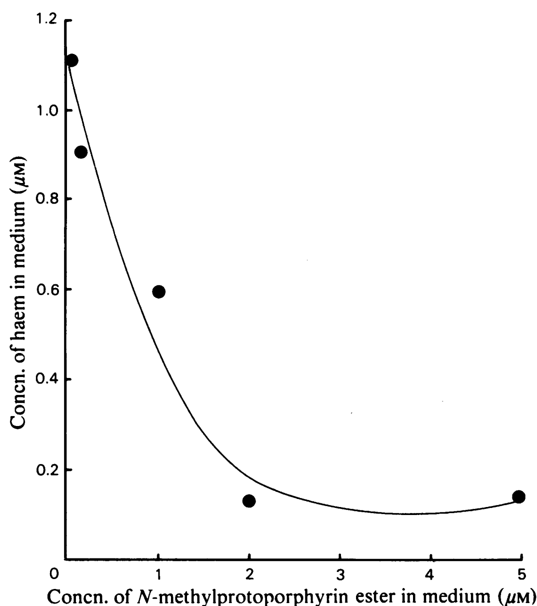

Fig. 3. *Effect of addition of N-methylprotoporphyrin dimethyl ester on the excretion of haem by Rps. sphaeroides O1*

Freshly harvested cells were resuspended, at $A_{680} = 2.0$, in fresh medium supplemented with 10mM-glycine (see the Materials and methods section) and incubated for 16h with gentle shaking with added inhibitor. Cells were removed by centrifugation and the concentration of haem in the supernatant determined by the pyridine haemochrome assay (see the Materials amd methods section).

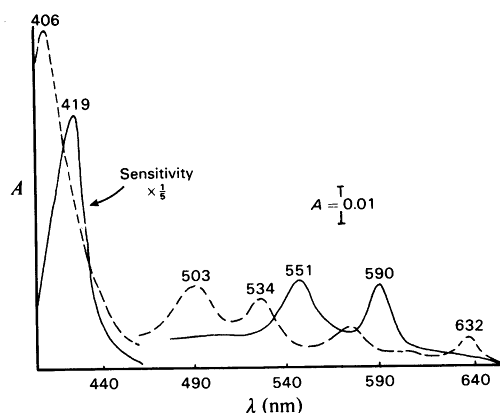

Fig. 4. *Spectrum, in diethyl ether, of pigment excreted in medium by Rps. sphaeroides Ga on treatment with N-methylprotoporphyrin dimethyl ester*

Freshly harvested dark-grown (but photosynthetically competent) cells were resuspended in glycine-enriched medium as described in the legend to Fig. 3. One group of cells was treated with *N*-methylprotoporphyrin dimethyl ester (6μM). Spectroscopic examination of cells and media after 16h incubation showed a pigment resembling Mg-protoporphyrin (absorption bands at 552 and 592nm) produced in response to inhibitor in both cells and medium. The medium was extracted with an equal volume of diethyl ether and spectra recorded before (——) and after (– – – –) shaking diethyl ether with 5M-HCl to remove Mg^{2+}.

with whole cell suspensions support this view. Whole cells of *Rps. sphaeroides* when placed in a medium containing succinate and glycine excrete haem into the supernatant medium. Lascelles (1966) noted this surprising phenomenon, but found that the addition of 1mM-5-aminolaevulinate was necessary for haem excretion. We have found that excretion of haem takes place without added 5-aminolaevulinate and was very sensitive to inhibition by *N*-methylprotoporphyrin ester (Fig. 3), even when little change was apparent in the cytochrome content.

Effect of N-methylprotoporphyrin dimethyl ester upon production of magnesium tetrapyrrole pigments by Rps. sphaeroides

When cell suspensions of aerobically grown *Rps. sphaeroides* Ga were shaken gently for 16h in the presence of 5μM-*N*-methylprotoporphyrin ester, the development of bacteriochlorophyll in the suspension was unaffected at <4nmol·ml⁻¹. However, a pigment was excreted into the medium. This was extracted into diethyl ether and also treated with dilute HCl before spectra were recorded in ether solution (Fig. 4). The absorption spectra of the first diethyl ether extract ($\lambda_{max.}$ 419, 551 and 590nm) and of the acid-treated (i.e. Mg^{2+}-free) form (406, 503, 534, 575 and 632nm) coincided with those of magnesium protoporphyrin and protoporphyrin respectively. Chromatography on thin-layer cellulose in lutidine solvent indicated that the magnesium protoporphyrin was present as a monoester, presumably monomethyl ester. Spectroscopic examination of the whole cells showed that they contained a component with two strong absorption bands at 554 and 592nm which, on extraction in acetone/methanol (3:1, v/v) and transfer to diethyl ether had the spectroscopic properties of magnesium protoporphyrin. The total content of magnesium protoporphyrin (monomethyl ester) in cells and medium was calculated to be approx. 4.4nmol·ml^{-1}.

Similar experiments were carried out with mutants V3 and O1. In each case the addition of *N*-methylprotoporphyrin ester to the medium led to a great increase in pigment excretion (Figs. 5*a*, 5*b*, 5*c*, 6*a*, 6*b* and 6*c*). In both cases substantial amounts of pigment were retained within the cells. The position of the absorption maxima of the pigments in

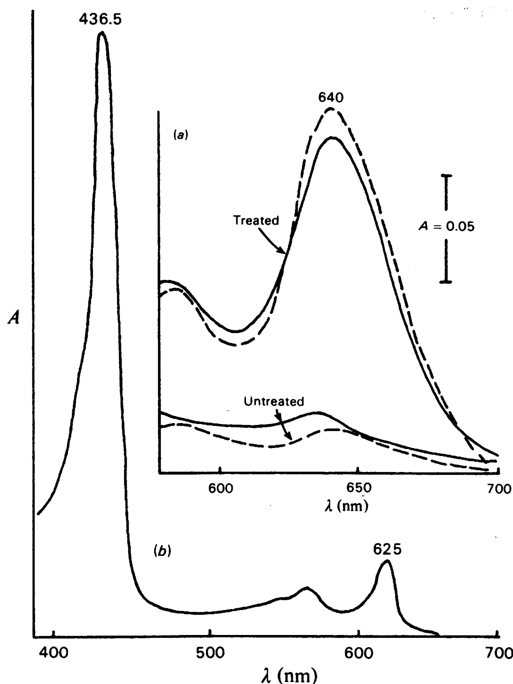

Fig. 5. *Effect of N-methylprotoporphyrin dimethyl ester upon pigment production by Rps. sphaeroides V3*
Experimental conditions were as described in the legend to Fig. 4, except that cells of mutant V3, which accumulates Mg-2,4-divinylphaeoporphyrin a_5 ester, were used. (*a*) Spectra of control and inhibitor-treated cells (----) and supernatants (——). (*b*) Spectrum of diethyl ether extract of supernatant of inhibitor-treated suspension.

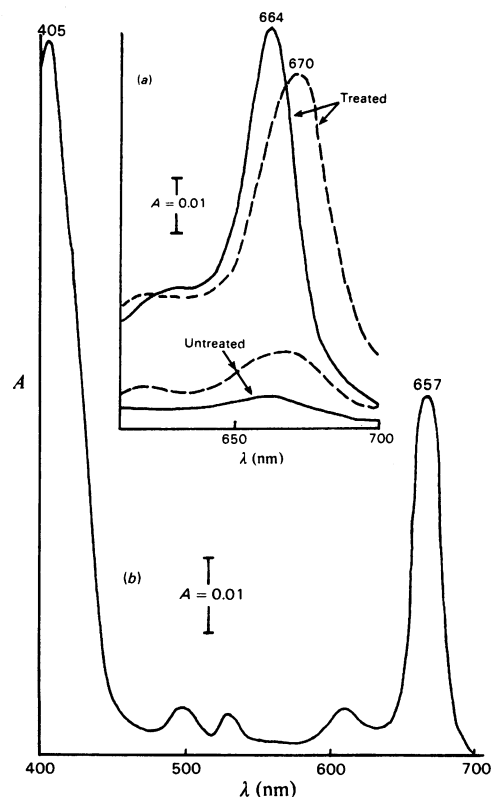

Fig. 6. *Effect of N-methylprotoporphyrin dimethyl ester upon pigment production by Rps. sphaeroides O1*
Experimental conditions were as described in the legend to Fig. 4, except that cells of mutant O1, which accumulates the Mg^{2+}-free derivative of hydroxyethylchlorophyllide *a* were used. (*a*) Spectra of control and inhibitor-treated cells (----) and supernatants (——). (*b*) Spectrum of diethyl ether extract of inhibitor-treated suspension.

ether clearly indicated that each mutant was producing unusual amounts of its characteristic pigment. In the case of V3 this is magnesium 2,4-divinylphaeoporphyrin a_5 monomethyl ester, in the case of O1 this is 2-desvinyl-2-hydroxyethyl-phaeophorbide *a*, a magnesium-free pigment (almost certainly derived from the metal complex 2-desvinyl-2-hydroxyethylchlorophyllide by loss of Mg^{2+} on standing). The effects of varying the concentration of inhibitor upon pigment production are shown in Fig. 7, mutant O1 being used. Both excreted and retained pigment increase as the inhibitor concentration is increased to about 3 μM.

Discussion

Our results clearly show that *N*-methylprotoporphyrin dimethyl ester is a potent non-competitive inhibitor of ferrochelatase in isolated membranes of *Rps. sphaeroides*, although protoporphyrin but not protoporphyrin dimethyl ester is a substrate for ferrochelatase in *Rps. sphaeroides* (Jones & Jones, 1970). The inhibitor can act on whole cells to diminish the concentrations of haem proteins (the cytochromes *b* and *c*) and, more dramatically, of free haem. The role of the free haem is obscure, but its sensitivity to inhibition suggests that it may act as a guide to the concentration of the postulated pool of 'regulatory haem' which may control the activity of 5-aminolaevulinate synthetase in this organism (Lascelles & Hatch, 1969) and also in mammalian cells (Granick & Sassa, 1971). In *Rps. sphaeroides* the specific activity of ferrochelatase when assayed under ideal conditions *in vitro* is far greater than appear necessary for the synthesis of its haem pigments. Rates of insertion of Fe^{2+} into protoporphyrin are close to 0.1 nmol/min per mg of membrane protein, and the total of cytochromes *c* and of cytochromes *b* are each about 0.5 nmol/mg of membrane protein. This excess capacity appears sufficient to ensure that some

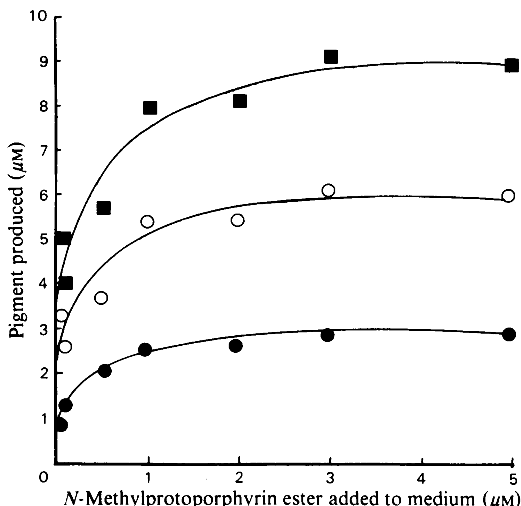

Fig. 7. *Effect of different concentrations of N-methylpro--toporphyrin dimethyl ester upon pigment production by Rps. sphaeroides O1*

Conditions were as described in legend to Fig. 6, except that the concentrations of inhibitor added to cell suspensions was varied as shown. Results for pigment in cells (●), in medium (○) and total pigment (■) are shown.

haem-protein synthesis occurs even when ferrochelatase is strongly inhibited *in vivo*.

The inhibitor-induced decrease in concentration of free haem in the medium coincides with the production of magnesium tetrapyrrole pigments by whole cells. Clearly the inhibitor must be specific to enzymic iron chelation and does not affect Mg^{2+} chelation, strongly suggesting that two separate enzymes are involved in these reactions, although each has protoporphyrin as substrate.

The effect of N-methylprotoporphyrin ester upon the production of magnesium pigments by *Rps. sphaeroides* is a little puzzling. In cells competent to make bacteriochlorophyll (strain Ga) there is greatly increased accumulation of magnesium protoporphyrin monomethyl ester. This pigment is also produced by *Rps. sphaeroides* in conditions of iron limitation (Jones, 1963; Cooper, 1963), and it is not accompanied by other magnesium pigments (except for bacteriochlorophyll). Thus the effects of iron deprivation and of inhibition of ferrochelatase appear similar. In iron deficiency, or inhibitor treatment, regulatory haem levels diminish and protoporphyrin is diverted to the magnesium pathway (see Scheme 1). In the mutants V3 and O1, however, inhibition of ferrochelatase and lowered haem concentration promotes production of pigments in the biosynthetic pathway later than magnesium protoporphyrin monomethyl ester (see Scheme 1). This effect could be explained if bacteriochlorophyll itself, or some product of a competent photosynthetic membrane, acted as an inhibitor of the conversion of magnesium protoporphyrin ester. It is noteworthy that in mutants the effects of iron deficiency and ferrochelatase inhibition are different. Lascelles (1966) found that in iron deficiency the production of pigments by mutants similar to V3 and O1 was greatly decreased. Possibly iron itself is required for the enzymic conversion of magnesium protoporphyrin monomethyl ester into magnesium 2,4-divinylphaeoporphyrin a_5 monomethyl ester. In green plants, too, iron deficiency promotes the accumulation of magnesium protoporphyrin and its ester (Spiller *et al.*, 1982), suggesting very similar mechanisms in bacteria and higher-plant chlorophyll synthesis.

By the use of N-methylprotoporphyrin monomethyl ester to block ferrochelatase, the effect of iron deficiency in causing accumulation of coproporphyrin(ogen) (Lascelles, 1956) and inhibiting the onward conversion of magnesium protoporphyrin monomethyl ester can be clearly distinguished, from the role of iron salts in the synthesis of haem to regulate 5-aminolaevulinate synthetase. It appears that when intracellular haem concentrations are low, 5-aminolaevulinate synthesis is uninhibited, leading to increased protoporphyrin production, which in turn is diverted to the magnesium tetrapyrrole pathway. Some regulatory mechanism, possibly involving iron and a functional photosynthetic membrane, operates at the level of magnesium protoporphyrin monomethyl ester conversion. This regulatory site cannot function in the mutants V3 and O1, which cannot assemble bacteriochlorophyll-containing membranes.

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