Magnesium Protoporphyrin Chelatase Activity in *Rhodopseudomonas spheroides*

STUDIES WITH WHOLE CELLS

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(Received 28 October 1971)

1. Whole cells of *Rhodopseudomonas spheroides* grown under semi-anaerobic conditions in the light incorporated magnesium into exogenous protoporphyrin when incubated with EDTA or the related chelators EGTA, *N*-(2-hydroxyethyl)-ethylenediamine-*NN'*-triacetate and *trans*-1,2-diaminocyclohexanetetra-acetate. 2. The reaction was demonstrated under anaerobic conditions in the light or at low oxygen partial pressure in the dark. Partial pressures of oxygen greater than 15% inhibited the reaction. 3. Cells grown under pure oxygen were completely inactive, but on adaptation to growth under low oxygen partial pressure (O_2+N_2 , 5:95) the development of activity paralleled the synthesis of bacteriochlorophyll. 4. The reaction with normal cells did not require protein synthesis, but cells that had lost their activity by being illuminated in Mg^{2+} -deficient medium did not recover it in the absence of protein synthesis. 5. The product of the reaction was magnesium protoporphyrin monomethyl ester. 6. Evidence is presented that insertion of magnesium is obligatorily coupled with methylation and it is concluded that the reaction is dependent on a multienzyme complex.

A general scheme for the biosynthesis of bacteriochlorophyll has been proposed: protoporphyrin + magnesium $\rightarrow$ magnesium protoporphyrin $\rightarrow$ magnesium protoporphyrin monomethyl ester $\rightarrow$ magnesium vinylphaeoporphyrin $a_5 \rightarrow$ chlorophyllide $a \rightarrow$ chlorophyll $a \rightarrow$ bacteriochlorophyll. Only one enzyme in this pathway has been demonstrated, *S*-adenosylmethionine-magnesium protoporphyrin methyltransferase (EC 2.1.1.11) (Gibson *et al.*, 1963). The information available on the other steps is based mainly on the excretion or accumulation of supposed precursors by mutants of photosynthetic bacteria blocked at different sites of the pathway (see, e.g., Lascelles, 1966).

The first reaction, the insertion of magnesium into protoporphyrin, is of importance since it is likely to be the rate-controlling step in the biosynthesis of chlorophyll. It has, however, not been demonstrated hitherto in spite of attempts in a number of laboratories (e.g. Neuberger & Tait, 1964; Porra & Lascelles, 1965; Jones & Jones, 1970).

This paper is the first report of the enzymic insertion of magnesium into exogenous protoporphyrin. The reaction in *Rhodopseudomonas spheroides* appears to be obligatorily coupled with methylation, so that the product formed is magnesium protoporphyrin monomethyl ester.

Materials and Methods

Chemicals

N-(2-Hydroxyethyl)-ethylenediamine-*NN'*-triacetate, ethanedioxybis(ethylamine)tetra-acetate (EGTA), *trans*-1,2-diaminocyclohexanetetra-acetate and EDTA were obtained from British Drug Houses Ltd., Poole, Dorset, U.K., or Sigma (London) Chemical Co., London S.W.6, U.K. *S*-Adenosylmethionine was obtained from Boehringer, Mannheim, Germany; DL-ethionine was from Roche Products Ltd., Welwyn Garden City, Herts., U.K.; L-[Me- ^{14}C]methionine (specific radioactivity 56.8 mCi/mmol) was from The Radiochemical Centre, Amersham, Bucks., U.K.

Porphyrins

Protoporphyrin was prepared from haem as described by Morell *et al.* (1961). Magnesium protoporphyrin was prepared as described by Baum *et al.* (1964); see also Ebbon & Tait (1969).

Organisms

Rhodopseudomonas spheroides N.C.I.B. 8253 was maintained in stab culture as described by Lascelles

(1956). The organisms were grown and harvested essentially as previously described (Gorchein *et al.*, 1968a) or as indicated in the text. The cells, however, were washed with ion-free water, in which they were resuspended at a concentration of 50 mg dry wt./ml.

Preparation of lipid sol and of lipid-protoporphyrin substrate

Lipids were extracted from cells of *Rps. spheroides* with chloroform-methanol by the method of Bligh & Dyer (1959) and were stored in chloroform under N_2 at $-20^\circ C$ until required. The lipid sol and the lipid-protoporphyrin were prepared as follows. Lipid extract (0.1 ml, containing $10 \mu g$ of lipid phosphorus) was pipetted into a glass test tube (16 mm $\times$ 40 mm) and the solvent was evaporated in a stream of N_2 . Then 2.5 ml of ion-free water, freed of O_2 by vigorous bubbling with N_2 , was added. Ultrasonic irradiation (MSE Ultrasonic Power Unit, 50 W) was applied at maximum power in bursts of 30 s, with 30 s rest periods, for a total time of 3 min. During the entire procedure the test tube was kept in an ice bath and N_2 was bubbled through the contents. To the water-clear lipid sol thus obtained was added 250–450 nmol of protoporphyrin suspended in the minimum volume of 5 M- NH_3 solution. Sonication was continued for a further 2 min under the conditions just described.

General methods

Glassware was freed of metals by treatment with $HNO_3-H_2SO_4$ as described by Thiers (1957). Buffer solutions were freed of zinc and certain other metals by extraction with dithizone (diphenylthiocarbazone) in carbon tetrachloride.

Electronic absorption spectra were obtained in a Unicam SP.700 recording spectrophotometer. Magnesium and zinc were determined by atomic absorption with a Unicam SP.90 spectrometer. Standards were made up in 1 M-HCl from Specpure metal salts obtained from Johnson, Matthey and Co., London E.C.1, U.K.

Preparation of samples for determination of magnesium

(a) *Whole cells and cell extracts.* Cells were ashed at $450^\circ C$ for 24 h and the residue was dissolved in 1 M-HCl. The 'water-soluble' magnesium was extracted by boiling the cells for 10 min in ion-free water. Samples of the clear supernatant were then ashed at $450^\circ C$. The 'ether-soluble' magnesium was obtained by extracting cells successively with acetone-water (4:1, v/v), ether, acetone, chloroform-methanol (1:1, v/v) and finally ether. The pooled organic phases were washed twice with ion-free water and evaporated to dryness in a stream of N_2 . The residue was dissolved in ether and portions were transferred

to crucibles; the ether was removed in a stream of N_2 and the residue was ashed at $450^\circ C$. Magnesium was then determined by atomic-absorption spectrometry after dissolving the residues in 1 M-HCl.

(b) *Metalloprotoporphyrins.* The ether solutions of the metalloprotoporphyrins were washed with ion-free water and evaporated to dryness in a stream of N_2 . The residues were treated with 1 M-HCl at $4^\circ C$ for 24 h. Magnesium and zinc were determined in the clear supernatant by atomic-absorption spectrometry.

Determination of magnesium protoporphyrin methyl ester in assays

The reaction was terminated and the porphyrins were extracted by procedures based on those of Granick (1961), modified to give a selective extraction of magnesium protoporphyrin monomethyl ester. To each conical tube there were added 4 ml of acetone-ammonia (9:1, v/v) and one drop of conc. NH_3 (sp.gr. 0.88) solution. After shaking, the mixture was centrifuged and the supernatant was removed and extracted twice with 3 ml of light petroleum (b.p. $60-80^\circ C$). To the aqueous phase there were then added 4 ml of ether, 1 ml of 0.5 M- KH_2PO_4 adjusted to pH 7.5 with 5 M-NaOH, and 0.75 ml of a saturated aqueous solution of NaCl. The mixture was shaken and the upper phase, containing magnesium protoporphyrin monomethyl ester, was removed and its spectrum was recorded. If a significant amount of protoporphyrin had been extracted, as shown by the spectrum, it could be removed by 'washing' the ether solution with 1–2 ml of ion-free water. Magnesium protoporphyrin monomethyl ester was determined from the extinction of the ether solution at 419 nm ($\epsilon = 3.08 \times 10^5$; Granick, 1948). Protoporphyrin and any residual metalloprotoporphyrin were then extracted, if required, by shaking the aqueous phase with a further 4 ml of ether and 2 ml of 0.5 M- KH_2PO_4 . The final pH in the aqueous phase under these latter conditions was about 6.1.

Chemical determinations

Dry weight and protein were determined as described previously (Gorchein *et al.*, 1968a). Bacteriochlorophyll was determined by the method of Cohen-Bazire *et al.* (1957) by using the extinction coefficient $75 \text{ mm}^{-1} \cdot \text{cm}^{-1}$ (Clayton, 1963).

Results

Conditions for the synthesis of metalloprotoporphyrin by whole cells of Rhodospseudomonas spheroides

Metalloprotoporphyrin was obtained in high yield when the cells were incubated anaerobically in the light as described in the legend to Table 1. Quantitatively

Table 1. Metalloprophyrin synthesis by whole cells of *Rhodospseudomonas spheroides*

To conical centrifuge tubes were added 50μmol of tris buffer, pH8.6, 10μmol of EGTA, 0.2ml of lipid-protoporphyrin sol, containing 40nmol of protoporphyrin, 10mg dry weight of semi-anaerobically grown cells, and ion-free water saturated with N₂ to a final volume of 0.6ml. The contents were mixed by vigorous flushing of N₂ over the surface and the tubes were immediately stoppered with rubber bungs. Incubation was in the light, provided by a bank of three 60W lamps at a distance of 25cm, at 35°C for 90min or as stated. Reactions were stopped and the magnesium protoporphyrin methyl ester was isolated and determined as described in the Materials and Methods section.

Tube no.	Omissions from reaction mixture	Other conditions	Total magnesium protoporphyrin methyl ester (nmol)
1	None	—	18.0
2	Lipid protoporphyrin	—	0.5
3	EGTA	—	1.8
4	Cells	—	0
5	None	Cells heated for 30s at 100°C	0.4
6	Lipid sol	Protoporphyrin added after sonication in water	2.5
7	None	Incubated in the dark	0.7

Fig. 1. Magnesium protoporphyrin chelatase activity during adaptation of *Rhodospseudomonas spheroides* from growth under oxygen to growth under O₂+N₂ (5:95) in the dark

At zero time, the culture, which was growing under O₂, was diluted with an equal volume of medium S (1.5 litres) and gassing was continued with O₂+N₂ (5:95). Samples were taken at intervals and determinations were made of dry weight (□), bacteriochlorophyll (Δ) and magnesium protoporphyrin chelatase activity (○). The long lag phase observed in this experiment (cf. Gorchein *et al.*, 1968b) is probably due to the addition of medium S at 4°C, not prewarmed to 32°C.

similar results were obtained when the cells were shaken under O₂+N₂ (5:95) in the dark. Incubations under anaerobic conditions in the dark gave no metalloprophyrin. Cells vigorously shaken in air or

in an atmosphere of O₂ of partial pressure greater than 15%, either in the light or in the dark, were inactive in metalloprophyrin formation.

No activity could be demonstrated with cells

grown under O_2 under any conditions of incubation, but cells grown up in air showed activity in proportion to the degree of pigmentation they had reached at the end of growth. The changes in the metal-inserting activity during adaptation from growth under O_2 to growth under O_2+N_2 (5:95) in the dark are shown in Fig. 1. The assays were set up under anaerobic conditions in the light and also under O_2+N_2 (5:95) in the dark. The activities obtained were similar under these two sets of conditions. The first demonstration of activity appeared to coincide with the start of bacteriochlorophyll synthesis, but its differential rate of increase was then more rapid than that of bacteriochlorophyll.

These results show that the conditions under which metalloporphyrin was produced were identical with those known to favour bacteriochlorophyll synthesis by *Rhodospseudomonas spheroides* *in vivo* (Cohen-Bazire *et al.*, 1957; Lascelles, 1959).

Definitive identification of the product of the reaction as magnesium protoporphyrin monomethyl ester

It was established that the metalloporphyrin produced was magnesium protoporphyrin by the following criteria. (1) The spectrum in ether was identical with that of authentic magnesium protoporphyrin: λ_{max} 419nm, β band 551nm and α band 589nm; the relative heights of these peaks were 100:16.1:16.8, in agreement with Granick (1948). (2) Direct demonstration that the metal was magnesium was by atomic-absorption spectrometry (Fig. 2), which shows that there is a 1:1 relation between nanomoles of metalloporphyrin (determined spectrophotometrically) and ng-atoms of magnesium (determined by atomic-absorption spectrometry). Analyses on the same samples were also performed for zinc, but the amounts detected were small and did not vary with the content of metalloporphyrin. It is likely that these small amounts of zinc are due to irremediable contamination and do not originate from zinc protoporphyrin formed enzymically.

The compound was shown to be the monomethyl ester of magnesium protoporphyrin by t.l.c. on cellulose MN300 (Macherey, Nagel and Co., Duren, Germany) in (1) 2,6-lutidine-ammonia (0.05M) (10:7, v/v) and (2) acetone-light petroleum (b.p. 60–80°C)–acetic acid (30:70:0.1, by vol.).

The chromatographic properties in solvent 1 were similar to those described by Granick (1961), but with solvent 2 (Fig. 3) protoporphyrin remained at the origin. This is considered to be due to differences in the physical properties of cellulose MN300 as compared with paper. Good separation of magnesium protoporphyrin and its esters and the protoporphyrin esters was obtained (Fig. 3). After treatment with diazomethane the R_F value of the product was identical with that of magnesium protoporphyrin

Fig. 2. Determination of magnesium and zinc in metalloporphyrin formed enzymically by *Rhodospseudomonas spheroides*

Metalloprotoporphyrin was obtained from a number of assay mixtures done essentially as described in the legend to Table 1, but incubated for different times and with various concentrations of substrate and of cells. In each case the ether solution was washed with ion-free water and its content of metalloprotoporphyrin was determined spectrophotometrically as described in the Materials and Methods section. The ether was then evaporated in a stream of N_2 and the residue was treated with 1M-HCl at 4°C for at least 24h. Magnesium and zinc were measured by atomic-absorption spectrometry in the clear supernatant. For each metalloporphyrin value (ordinate), there are two points on the abscissa, representing magnesium (○) and zinc (●) respectively. The line in the Figure indicates the expected position of points showing a 1:1 stoichiometric relationship between metalloporphyrin and metal content.

dimethyl ester. The properties of the material on treatment with HCl were as described by Granick (1961) for magnesium protoporphyrin monomethyl ester from *Chlorella*.

Some properties of the enzyme system

The rate of production of magnesium protoporphyrin monomethyl ester was linear for about 1h and was linearly proportional to the amount of cells used, up to 10mg dry weight (5mg of protein) per assay. Assays done with increasing amounts of lipid-protoporphyrin gave an S-shaped curve, but

Fig. 3. Identification of magnesium protoporphyrin monomethyl ester by t.l.c. and its conversion by methylation with diazomethane into magnesium protoporphyrin dimethyl ester and by removal of metal with 1M-HCl into protoporphyrin monomethyl ester

A tracing of the t.l.c. plate coated with MN300 cellulose and run in acetone–light petroleum (b.p. 60–80°C)–acetic acid (30:70:0.1, by vol.) for 10 min is shown. Spots: 1, protoporphyrin; 2, magnesium protoporphyrin; 3, magnesium protoporphyrin monomethyl ester isolated from the assay; 4, magnesium protoporphyrin dimethyl ester; 5, spot 3 after treatment with diazomethane; 6, protoporphyrin monomethyl ester obtained after treating spot 3 with 1M-HCl; 7, protoporphyrin dimethyl ester. The solvent front (SF) is 11 cm from the origin (O).

the kinetics were rendered 'normal' by further addition of lipid sol (Fig. 4). The optimum concentration of protoporphyrin was about 80 μ M. The pH optimum was 8.6.

In the absence of chelating agent, slight activity only was found (Table 1). The maximum activating effect was at 20 mm, although 80–90% of the activity

Fig. 4. Effect of concentration of lipid–protoporphyrin on the synthesis of magnesium protoporphyrin monomethyl ester by *Rhodospseudomonas spheroides*

The assays were done as described in the legend to Table 1, but with 0.1 ml of cell suspension (5 mg dry weight) only. Increasing volumes of lipid–protoporphyrin sol, containing 5–100 nmol of protoporphyrin, were added to different assay mixtures up to a maximum of 0.5 ml. The reaction mixtures were then made up to 0.75 ml by addition of lipid sol (O) or ion-free water (Δ).

was obtained at 1 mM (Fig. 5). At higher concentrations of *trans*-1,2-diaminocyclohexanetetra-acetate the reaction was inhibited.

Maximum activity was usually found in cells allowed to reach the early-to-middle exponential phase of growth under semi-anaerobic conditions in the light in 3-litre flasks of medium S (Lascelles, 1956). Further growth was usually associated with less activity. The activity was unstable to freezing even when immediately followed by thawing, or to storage of the cells at 0°C or at –20°C; any of these procedures resulted in a decrease in the activity, to about 30%. Only a slight decrease took place, however, when the cells were kept at 4°C for 72 h at a concentration of 50 mg dry weight/ml. The activity of frozen cells could be restored to the original value by illumination in medium S at a concentration of 2 mg dry wt./ml for 4–6 h.

Cofactor requirement

A requirement for the addition of methionine and ATP or of *S*-adenosylmethionine was not demonstrable under the assay conditions above. However, cells dialysed for 16 h against ion-free water lost about half their activity, and this was restored to the

Fig. 5. Activation of the synthesis of magnesium protoporphyrin monomethyl ester by chelating agents

The assays were done as described in the legend to Table 1, but with *trans*-1,2-diaminocyclohexanetetraacetate (CDTA). Similar results were obtained with EDTA, EGTA or *N*-(2-hydroxyethyl)-ethylenediamine-*NN'*-triacetate.

original by addition of *S*-adenosylmethionine or methionine and ATP to the assay. Measurement of methionine with a Locarte amino acid analyser in extracts of cells boiled with water for 10 min gave values of 3–6 nmol/10 mg dry wt. Although the concentration of *S*-adenosylmethionine was not measured, it seemed likely that during the incubations synthesis of both methionine and *S*-adenosylmethionine was taking place from endogenous substrates, especially since a net synthesis of more than 20 nmol of magnesium protoporphyrin monomethyl ester was obtained in some assays. To determine how the availability of methionine or of *S*-adenosylmethionine was related to magnesium chelation the following experiments were done.

After addition of a tracer amount of L-[Me-¹⁴C]-methionine to assay tubes, the specific radioactivity of the magnesium protoporphyrin monomethyl ester fell markedly as the amount synthesized increased (Fig. 6). This is consistent with dilution of the labelled pool by newly synthesized methionine. On the other hand, with substrate amounts of labelled methionine, added to cells partly depleted of endogenous substrates by dialysis, the specific radioactivity of the product was constant (Fig. 7).

Inhibition of the reaction by ethionine and PP_i

Inhibition of the overall reaction was shown with sodium pyrophosphate and with DL-ethionine (Table 2), both of which prevent methylation, as discussed

Fig. 6. Changes in the specific radioactivity of magnesium protoporphyrin mono[¹⁴C]methyl ester synthesized by *Rhodospseudomonas spheroides* in assays with tracer amounts of L-[Me-¹⁴C]methionine

The assays were done as described above but to each tube was added L-[Me-¹⁴C]methionine (0.05 μ Ci, 0.88 nmol). The ether solutions of the metalloprotoporphyrin were washed three times with ion-free water. Samples were dried in glass vials under a stream of N₂ and the radioactivity was determined with an efficiency of about 80% after addition of 10 ml of a dioxan-based scintillator containing naphthalene (50 g), 2,5-diphenyloxazole (7 g), 1,4-bis-(5-phenyloxazol-2-yl)benzene (0.05 g) and dioxan to 1000 ml, in a Nuclear-Chicago mark I liquid-scintillation spectrometer. ○, Magnesium protoporphyrin monomethyl ester; △, sp. radioactivity of the ester.

below. Inhibition by ethionine was slight if added to the assay medium, but it was much greater after illumination of the cells in medium S containing 2 mM DL-ethionine. Up to 90% inhibition was obtained after illumination for 20 h, and this was associated with a decrease in free methionine in the cells (determined with the amino acid analyser) to undetectable values. Magnesium protoporphyrin did not accumulate under these conditions. Thus the presence of methionine or of *S*-adenosylmethionine appears essential for the chelation of magnesium by protoporphyrin. This seems to be due to a requirement for methylation.

Effect of adding Mg²⁺ to the assay system

The reaction did not require addition of Mg²⁺, consistent with the high concentrations found in the cells (Table 3), and indeed, addition of Mg²⁺ to the assay system resulted in inhibition. After illumination

of the cells for 20h in Mg^{2+} -free medium S, activity became almost undetectable. Under these conditions the 'water-soluble' magnesium was decreased by about 60%, and the magnesium that was not extractable either by boiling water or with organic solvents, and probably includes the magnesium bound to nucleic acids, decreased to about 20% of its original value. The 'ether-soluble' magnesium was

equal to the bacteriochlorophyll content on a molar basis in all circumstances (Table 3).

Effect of inhibitors of protein synthesis

The activity of the magnesium-depleted cells could not be restored by addition of Mg^{2+} to the assay system, nor by prolonged illumination in either magnesium-containing buffer or in medium S lacking a nitrogen source. Activity, however, was readily demonstrated again after illumination in medium S for 1–2h and reached a maximum after about 4h. This restoration of activity could be completely prevented by addition of puromycin (0.02mM) or of chloramphenicol (0.2mM) to the medium. Addition of these inhibitors of protein synthesis to assays done with active cells either had no effect, or, in some experiments, resulted in markedly increased activity.

Studies with other photosynthetic organisms

Synthesis of magnesium protoporphyrin monomethyl ester as described above could not be shown in two other photosynthetic bacteria tested, *Rhodospirillum rubrum* and *Chromatium* sp., nor in *Euglena gracilis* strain Z or in etiolated barley shoots. The conditions employed allow the demonstration of enzyme activity only in *Rhodopseudomonas spheroides*.

Discussion

It has been shown that whole cells of *Rps. spheroides* synthesize magnesium protoporphyrin monomethyl ester in high yield from exogenous protoporphyrin when incubated with EDTA or the closely related chelating agents EGTA, *trans*-1,2-diaminocyclohexanetetra-acetate and *N*-(2-hydroxyethyl)-ethylenediamine-*NN'*-triacetate. It should be noted, however, that slight but definite activity

Fig. 7. Specific radioactivity of magnesium protoporphyrin mono[^{14}C]methyl ester synthesized by *Rhodopseudomonas spheroides* in assays with substrate amounts of L-[Me- ^{14}C]methionine

The procedures were as described in the legends to Table 1 and Fig. 6. The assays were done with cells dialysed for 24h against ion-free water. To each incubation mixture were added L-methionine (200nmol) and L-[Me- ^{14}C]methionine (1.76nmol, 0.1 μ Ci). o, Magnesium protoporphyrin monomethyl ester; Δ , sp. radioactivity of the ester.

Table 2. Effect of inhibiting methylation on the formation of magnesium protoporphyrin monomethyl ester by *Rhodopseudomonas spheroides*

Assays were done as described in the legend to Table 1. The cells used for Expt. 1 were resuspended at a concentration of 2mg dry weight/ml and illuminated in medium S containing 2mM-DL-ethionine for 4h. The control was similarly illuminated in ordinary medium S. In Expt. 2, PP_i was added directly to the assay system as a solution adjusted to pH8.6 with dilute HCl.

Expt. no.	Inhibitor	Other additives	Magnesium protoporphyrin monomethyl ester (nmol)
1	None	None	10.5
	DL-Ethionine (2mM)	None	5.7
	DL-Ethionine (2mM)	S-Adenosylmethionine (1 mM)	7.6
2	None	None	8.0
	PP _i (2mM)	None	5.2
	PP _i (2mM)	S-Adenosylmethionine (1 mM)	9.72

Table 3. *Distribution of magnesium in Rhodopseudomonas spheroides grown in ordinary and in magnesium-deficient medium S*

The cells used for the different experiments were obtained as follows. Expt. 1, grown semi-anaerobically in the light in medium S for 20h, harvested and washed with ion-free water as described in the Materials and Methods section. Expt. 2, a sample of 50mg dry weight of preparation (1) in 1 ml of ion-free water was inoculated into 1 litre of medium S in which MgSO_4 was replaced by an equimolar amount of Na_2SO_4 ; illumination was for 20h, after which the cells were harvested and washed as for preparation (1) above. Expt. 3, a sample (50mg dry weight) of preparation (2) in 1 ml of ion-free water was illuminated in 1 litre of normal medium S for 4h and then treated as the other samples.

Expt. no.	Magnesium (ng-atoms/mg dry weight of cells)				Bacteriochlorophyll (nmol/mg dry weight)
	Total (a)	'Water-soluble' (b)	Ether-soluble (c)	$a-(b+c)$	
1	152.0	21.3	11.5	119.2	11.4
2	41.3	8.4	5.6	27.3	5.7
3	86.4	14.1	5.6	66.7	5.8

occurs in the absence of chelators (Table 1); their requirement in the formation of magnesium protoporphyrin monomethyl ester is thus not absolute.

Possible functions of the chelating agents

Chelators were used initially in these experiments to decrease Zn^{2+} in the system to very low concentrations. Previous unsuccessful attempts to demonstrate chelation of magnesium in this laboratory (Neuberger & Tait, 1964) had shown that Zn^{2+} and some other cations could be readily incorporated into protoporphyrin by cell-free extracts of *Rps. spheroides*, probably as a result of the low specificity for the metal of the haem-forming enzyme ferrochelatase (Mazanowska *et al.*, 1966). Since the group of chelating agents used binds Mg^{2+} much less strongly than Zn^{2+} and a number of other metals (Sillen & Martell, 1964), it seemed a useful way to remove possible interference by these cations. It is known, however, that EDTA has a number of other effects on bacterial cells (Leive, 1965, 1968; Neu & Heppel, 1965); its exact mode of action in the present experiments cannot yet be stated.

Role of lipid

Slight chelatase activity was found when protoporphyrin was added after dispersion in water (Table 1), but protoporphyrin is poorly soluble under these conditions, and much greater chelatase activity was obtained when the substrate was added in a lipid sol. This is almost certainly due to increasing the effective concentration of protoporphyrin, and this view is supported by the finding that further addition of lipid sol to assay mixtures set up with small quantities of lipid-protoporphyrin increased the amount of product formed. It seems unlikely that

the lipid extracted from this micro-organism has any specific activating properties not related to its 'solubilizing' the protoporphyrin, since lipid sols prepared with mixed phospholipids from ox heart have a similar effect on the activity. The lipid sol may, in addition, have a direct effect on the enzyme system, perhaps by making the environment more hydrophobic and thus facilitating the removal of the large hydration shell of the magnesium cation (Williams, 1959).

A number of detergents and organic solvents could not replace the lipid since, although they dissolved the protoporphyrin, they completely inhibited the reaction even at low concentration.

Dependence on magnesium

The concentration of magnesium in the cells was high (Table 3), but the nature and the relative amounts of the complexed and ionic magnesium in the different fractions examined is not known. It is therefore not possible to decide whether the loss of activity associated with the lower magnesium concentrations found when the cells were allowed to grow in magnesium-deficient medium was due to preferential loss of a specific form of magnesium, or to an effect on the stability of the enzyme. It appears unlikely, however, that the magnesium required for chelation is in an organic form, since all the ether-soluble magnesium was accounted for by the bacteriochlorophyll content. This contrasts with the conclusions that may be drawn from the work of Smith (1947) with etiolated barley, that magnesium for chlorophyll synthesis is likely to be in an organic form.

Role of protein synthesis

It is a long-standing observation that protein synthesis is required for bacteriochlorophyll syn-

thesis (see e.g., Sistrom, 1962*a,b*; Bull & Lascelles, 1963), but the site or sites of coupling are unknown. The results obtained here indicate that protein synthesis is not required for the biosynthesis of magnesium protoporphyrin monomethyl ester by normal cells. However, cells that have lost their activity by being illuminated in magnesium-free medium do not recover it in the absence of protein synthesis. This raises the possibility that the enzyme is unstable under conditions of magnesium deficiency and has to be resynthesized; alternatively, protein synthesis may be necessary for the formation of a magnesium-carrier protein.

The markedly elevated activity observed with some batches of cells when assayed in the presence of inhibitors of protein synthesis is probably due to the increase in methionine available for *S*-adenosylmethionine formation when available supplies are not involved in protein synthesis.

Multienzyme complex synthesizing magnesium protoporphyrin monomethyl ester

The finding that the product of the enzymic reaction was magnesium protoporphyrin monomethyl ester was unexpected, but it is consistent with a number of observations that this compound, and not magnesium protoporphyrin, is excreted into the culture medium of photosynthetic bacteria (Cooper, 1963; Jones, 1963; Lascelles, 1966).

Methylation was prevented by inhibiting the synthesis of *S*-adenosylmethionine with PP_i (Mudd, 1963) and also by incubating the cells with ethionine. This latter compound is known to become converted into *S*-adenosylethionine, which is a competitive inhibitor of *S*-adenosylmethionine-magnesium protoporphyrin methyltransferase (Gibson *et al.*, 1963). Another, perhaps more important, effect of ethionine observed here was to decrease the intracellular pool of methionine to very low values. Methionine is known to inhibit its own biosynthesis in bacterial cells by repressing the formation of 5,10-methylenetetrahydrofolate reductase (Katzen & Buchanan, 1965). It seems likely that ethionine acts in the same way. Under these different conditions magnesium incorporation was inhibited, suggesting that this reaction and the methylation step are closely coupled, probably owing to the existence of a multi-enzyme complex responsible for the overall reaction.

It is noteworthy therefore that during adaptation from growth under O₂ to growth under O₂+N₂ (5:95) the formation of magnesium protoporphyrin monomethyl ester parallels the synthesis of bacteriochlorophyll, whereas the activity of the methylating enzyme can be demonstrated several hours before the onset of pigment synthesis (Gorchein *et al.*, 1968*b*). This difference may be due to changes in the permeability of the cells to the protoporphyrin substrate,

since activity of the methylating enzyme was determined in a cell-free extract and represents only a part of the overall reaction measured in the present experiments. It seems unlikely, however, that lack of penetration of the substrate accounts for the failure to demonstrate insertion of magnesium by cells grown under O₂, since extracts from such cells have extremely low activity of the methylating enzyme (Gorchein *et al.*, 1968*b*). Another possibility, however, was that the generally accepted order of the pathway, in which insertion of magnesium into protoporphyrin precedes methylation, might not be correct. In view of this, 'natural' protoporphyrin monomethyl ester (prepared by removing magnesium under mild conditions with 1M-HCl from biosynthetically produced magnesium protoporphyrin monomethyl ester) was tested as a substrate for insertion of magnesium. It was found to be neither a substrate nor an inhibitor; synthetic magnesium protoporphyrin, however, was readily methylated when tested under similar conditions. These findings support the generally accepted order of the reactions.

A number of questions arise, however, about the control mechanisms. Is the magnesium-inserting part of the complex simply latent at the early stage when the methyltransferase can be demonstrated, or does the activity of the whole complex depend on the synthesis of an enzyme apparently preceding the methyltransferase in the pathway? There is at this time no means of distinguishing between these alternatives.

Control of magnesium tetrapyrrole synthesis

Support for the view that the system described here may be the one operating *in vivo* comes from the observations that the conditions of illumination and of O₂ partial pressure required to demonstrate activity are the same as those that have been found necessary for bacteriochlorophyll synthesis in growing cells. In addition, it has been calculated that the activity of the magnesium-inserting enzyme is close to that required to account for the bacteriochlorophyll content of cells in the exponential phase. The experiments described provide direct evidence that environmental factors operate at the magnesium-insertion step. Thus, as postulated by Lascelles & Altshuler (1969) on the basis of experiments with mutants, a direct inhibitory effect of O₂ has been shown on magnesium insertion. Also, under anaerobic conditions, a requirement for light has been demonstrated.

These observations do not yet explain the mechanisms by which these controls operate at the molecular level, but it seems likely that the magnesium-inserting portion of the complex is directly influenced by the electron-transport chain or by phosphorylation.

I thank Professor A. Neuberger, C.B.E., F.R.S., and Dr. G. H. Tait for continued help and encouragement.

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