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

4. *S*-ADENOSYLMETHIONINE–MAGNESIUM PROTOPORPHYRIN METHYLTRANSFERASE*

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Gibson, Neuberger & Tait (1962*b*) have shown that ethionine inhibits the biosynthesis of bacteriochlorophyll and stimulates the excretion of coproporphyrin by illuminated suspensions of *Rhodopseudomonas spheroides*, and that this effect can be reversed by methionine. It was suggested that methionine might be concerned in a specific manner in the formation of bacteriochlorophyll, and evidence was presented to show that the methyl group of methionine is a direct precursor of the methyl ester group of bacteriochlorophyll. Tait & Gibson (1961) reported that chromatophores from *Rps. spheroides* catalyse the transfer of the methyl group of *S*-adenosylmethionine to magnesium protoporphyrin to form a compound that was tentatively identified as magnesium protoporphyrin monomethyl ester. The present paper describes the further characterization of the enzyme, *S*-adenosylmethionine–magnesium protoporphyrin methyltransferase, and the identification of the product formed.

MATERIALS AND METHODS

Chemicals

The reineckates of *S*-adenosyl-L-methionine, *S*-adenosyl-L-[*Me*-^{14}C]methionine and *S*-adenosyl-L-ethionine were prepared enzymically (Cantoni, 1957) and stored at −20°. They were decomposed by Cantoni's (1957) method before use. Crystalline *S*-adenosyl-L-homocysteine was prepared by the method of de la Haba & Cantoni (1959).

Porphyrins

The various porphyrins used were synthesized as described by the authors indicated: protoporphyrin IX dimethyl ester (Grinstein, 1947); mesoporphyrin IX dimethyl ester (Grinstein & Watson, 1943); deuteroporphyrin IX dimethyl ester (Chu & Chu, 1952); haematoporphyrin IX dimethyl ester (Fischer & Orth, 1937). The spectra of these compounds in chloroform solution were determined with a recording spectrophotometer (Unicam SP. 700). In all cases they were identical with the spectra reported by the authors quoted above. It should be noted that the haematoporphyrin dimethyl ester is probably not absolutely pure. By using countercurrent distribution Granick, Bogorad & Jaffe (1953) detected a number of other porphyrins in crystalline haematoporphyrin dimethyl ester prepared in this way.

The free porphyrins were prepared from the esters, as required, by hydrolysis with 7N-HCl for 5 hr. at room temperature in the dark. The acid was removed *in vacuo* and the porphyrins were dissolved in water, with a few drops of dilute NH_3, to give solutions with a concentration of 5 mg./ml. which were stored at −20°.

Coproporphyrin III was purified by Dr J. M. Turner in this Department from culture filtrates of *Rps. spheroides* illuminated under suitable conditions (cf. Neilands & Garibaldi, 1959).

Protoporphyrinogen was prepared immediately before use by heating a solution of protoporphyrin with sodium amalgam (Sano & Granick, 1961).

Metalloporphyrins

Haemin (ferrihaem) was prepared by the method of Fischer (1955) and manganese, zinc and copper protoporphyrin dimethyl esters were prepared, all in identical fashion by the reaction of protoporphyrin with the appropriate acetate in pyridine, as described by Fischer & Pützer (1926). Manganese protoporphyrin obtained by alkaline hydrolysis of the dimethyl ester is the manganic complex (Dr J. N. Phillips, personal communication). In 0·01N-NaOH it has absorption maxima at 370, 466 and 554 mμ (relative extinction coefficients 1·0:0·32:0·12). After treatment of such a solution with a few milligrams of sodium dithionite the spectrum changed. The maximum at 466 mμ disappeared whilst that at 554 mμ remained unchanged. This treatment is known to convert ferrihaem into ferrohaem (Smith, 1959) and it was assumed that sodium dithionite reduces manganic protoporphyrin to the manganous complex. Ferrohaem and manganous protoporphyrin were therefore prepared immediately before use by treating solutions of ferrihaem or manganic protoporphyrin in 0·01N-NaOH with sodium dithionite (Smith, 1959).

Magnesium protoporphyrin dimethyl ester. The method of Granick (1948*a*) for preparing this compound, which involves the reaction of protoporphyrin dimethyl ester with a decomposed Grignard reagent, worked satisfactorily once, but failed completely on a number of other occasions. The reasons for these failures could not be determined and another method was therefore devised for inserting magnesium into the porphyrin molecule. This method did not give as high a yield as that of Granick (1948*a*) but was more reproducible.

Magnesium turnings (1·0 g.), anhydrous methanol (70 ml.) and resublimed iodine (a few crystals) were allowed

* Part 3: Gibson, Neuberger & Tait (1962*c*).

to react together under reflux to give a clear solution. After cooling, protoporphyrin dimethyl ester (100 mg.) dissolved in anhydrous pyridine (15 ml.) was added and the resulting solution was refluxed for 16–48 hr. The reaction was followed by removing samples, adding them to a mixture of ether and water, and determining the spectra of the resulting ether solutions with a recording spectrophotometer. The reaction was allowed to proceed until most or all of the protoporphyrin dimethyl ester had been converted, judged by the disappearance of the peak at 630 mμ. The solution was then mixed with water (300 ml.) and ether (300 ml.). The ether layer was removed, washed three times with 100 ml. of water and evaporated to dryness under reduced pressure. The solid residue was extracted with small volumes of ether. As judged from the spectra the first few ether extracts contained mainly magnesium protoporphyrin dimethyl ester. In experiments where the reaction did not go to completion the later extracts contained increasing amounts of protoporphyrin dimethyl ester. The fractions richest in magnesium protoporphyrin dimethyl ester were combined and evaporated to a small volume. Magnesium protoporphyrin dimethyl ester crystallized on adding light petroleum (b.p. 40–60°) in the cold. The yields varied from 10 to 48 mg. (Found: Mg, 3·85. $C_{36}H_{36}MgN_4O_4$ requires Mg, 3·97%).

The spectrum in ether of the magnesium protoporphyrin dimethyl ester is identical with that reported by Granick (1948*a*). Magnesium protoporphyrin, prepared from the dimethyl ester by alkaline hydrolysis as described below, was extracted from ether solution into aq. HCl, in the process losing its magnesium. The solution was neutralized and the product, after extraction into ether, gave a spectrum identical with that of an authentic sample of protoporphyrin.

Magnesium deuteroporphyrin dimethyl ester. This was prepared from deuteroporphyrin dimethyl ester by allowing it to react with magnesium methoxide exactly as described above (Found: Mg, 4·21. $C_{32}H_{32}MgN_4O_4$ requires Mg, 4·34%). The spectrum in ether of the crystalline product had two maxima of equal extinction at 539 and 576 mμ and in addition small maxima at 494 and 617 mμ. These last two are probably due to traces of deuteroporphyrin dimethyl ester. This spectrum is similar in type to that given by magnesium protoporphyrin dimethyl ester (cf. Granick, 1948*a*, 1961). Extraction of an ether solution of magnesium deuteroporphyrin with N-HCl, followed by neutralization and re-extraction into ether, gave a compound having a spectrum identical with that of deuteroporphyrin.

Magnesium mesoporphyrin dimethyl ester. This compound was prepared from mesoporphyrin dimethyl ester as described above (Found: Mg, 3·27. $C_{36}H_{40}MgN_4O_4$ requires Mg, 3·94%). The spectrum in ether of the crystalline product showed maxima of equal extinction at 541 and 578 mμ and in addition small maxima at 500 and 618 mμ. The last two were probably due to the presence of mesoporphyrin dimethyl ester. This is also indicated by the low analytical value for Mg. The spectrum could be changed to one identical with that of mesoporphyrin by treating an ether solution of magnesium mesoporphyrin with dilute HCl as described above.

Calcium protoporphyrin dimethyl ester. This compound was prepared in exactly the same way as magnesium protoporphyrin dimethyl ester by using calcium instead of magnesium. The reaction was done twice; both times the yield was 8 mg. from 100 mg. of protoporphyrin dimethyl ester. (Found: Ca, 5·98. $C_{36}H_{36}CaN_4O_4$ requires Ca, 6·38%). The spectrum in ether was identical with that of magnesium protoporphyrin dimethyl ester, showing maxima of equal extinction at 547 and 586 mμ. The calcium can be readily removed from calcium protoporphyrin by treating an ether solution with dilute HCl to give a compound having a spectrum identical with that of protoporphyrin.

The magnesium porphyrins and the other metal–protoporphyrin complexes were prepared from their dimethyl esters by alkaline hydrolysis as described by Granick (1948*a*). In each case the product was suspended in 0·5M-potassium phosphate buffer, pH 6·7, and kept at −20°.

All other chemicals were obtained or prepared as described by Gibson, Neuberger & Tait (1962*a*, *b*).

Organisms

The strain of *Rps. spheroides* used and the conditions of maintenance, and of anaerobic growth in the light, were as described by Gibson *et al.* (1962*a*). A spontaneous green mutant of *Rps. spheroides* was isolated and grown in the same way as the wild-type organism. *Rhodospirillum rubrum* (strain S_1) was obtained from Dr J. Lascelles, Microbiology Unit, Department of Biochemistry, University of Oxford. The conditions of maintenance and growth were as for *Rps. spheroides*.

Rps. spheroides was also grown aerobically in the dark as described by Lascelles (1959).

Preparation of chromatophores

Suspensions (80 mg. dry wt./ml.) of organisms were disrupted in the Hughes (1951) press, diluted with 2 vol. of 0·05M-potassium phosphate buffer, pH 7·4, and centrifuged for 7 min. at 25 000 *g*. The supernatant (crude extract) was removed and centrifuged for 90 min. at 105 000 *g*. The clear orange supernatant was discarded and the red pellet was resuspended, with the aid of a glass homogenizer, in 0·05M-potassium phosphate buffer, pH 7·4, and recentrifuged. The supernatant was again discarded and the pellet suspended in buffer to give a final concentration of 5–15 mg. of protein/ml. The suspension was kept frozen until required. This chromatophore preparation consists of both 'light' and 'heavy' particles described by Newton & Newton (1957) and Cohen-Bazire & Kunisawa (1960). All the experiments described below were performed with chromatophores prepared in this way from light-grown *Rps. spheroides* unless specifically stated otherwise. 'Unwashed' chromatophores were prepared as above except that the preparations were only centrifuged once at 105 000 *g*. For some experiments chromatophores were separated into 'heavy' and 'light' particles. The supernatant obtained after centrifuging the crushed homogenate for 7 min. at 25 000 *g* was centrifuged for 30 min. at 25 000 *g*. The precipitated 'heavy' particles were resuspended in buffer. The supernatant was centrifuged for 90 min. at 105 000 *g* to give a precipitate of 'light' particles which was resuspended in fresh buffer. Both preparations were kept frozen until required.

Determinations

Protein. The protein content of chromatophore preparations was determined by the method of Lowry, Rosebrough, Farr & Randall (1951).

Porphyrins. The concentration of porphyrins and of metalloporphyrins was determined by measuring the extinctions of solutions in N-HCl or in ether at the absorption maximum in the Soret band, and using the appropriate molar extinction coefficients. The assumption was made that porphyrin monomethyl esters have the same molar extinction coefficients as the porphyrins themselves.

Magnesium and calcium. The magnesium porphyrins and calcium protoporphyrin dimethyl ester were 'ashed' at 500° for 2 hr. Magnesium in the residue was determined either by flame photometry or by the colorimetric method of Bradfield (1961); calcium was determined by the method of Kuttner & Cohen (1927).

Radioactivity. Solutions were plated on 6·25 cm.^{2} aluminium planchets, dried *in vacuo*, and counted at infinite thinness in a Nuclear–Chicago gas-flow counter with a Micromil end-window and operated at the centre of the plateau; 1 μc of [*Me*-^{14}C]methionine gave 420 000 counts/min. under these conditions.

Assay of enzyme activity

Enzyme activity was assayed by following the incorporation of radioactivity from *S*-adenosyl[*Me*-^{14}C]methionine into the total porphyrin fraction. To incubation mixtures (total volume 1 ml.) 5 ml. of ethyl acetate–acetic acid (3:1, v/v) was added. The mixture was centrifuged and the precipitate was washed with a further 5 ml. of ethyl acetate–acetic acid. The combined supernatants were neutralized by shaking with 6 ml. of saturated sodium acetate. The aqueous phase was discarded and the ethyl acetate solution was washed twice with 10 ml. of water. Porphyrins were extracted from this solution with 5 ml. plus 3 ml. of 3 N-HCl. The combined HCl extracts were washed with 6 ml. of ethyl acetate. Then 4 ml. of fresh ethyl acetate was added and the mixture was neutralized by adding just sufficient solid $NaHCO_3$ to displace all the porphyrins into the ethyl acetate. The aqueous layer was discarded and the ethyl acetate layer was washed twice with 8 ml. of water. A portion of the ethyl acetate solution, usually 1·0 ml., was plated and its radioactivity measured. Another portion of the ethyl acetate solution, usually 0·1 ml., was added to 3·0 ml. of N-HCl, and, after thorough mixing, the extinction of the solution was measured at the appropriate wavelength in the Soret region. The molar specific radioactivity of the porphyrins isolated was taken as a measure of the enzyme activity.

This procedure extracts all the metal-free porphyrins used in this work with a recovery of about 30–50%, losses being due to the number of manipulations involved rather than to differences in distribution coefficients. It was assumed that the porphyrins and their monomethyl esters behave in identical fashion during the extraction and that the relative amounts are the same in the final ethyl acetate solution as they are at the end of incubation. The acidic conditions used convert magnesium, calcium and zinc porphyrins into free porphyrins. With manganese protoporphyrin the unchanged complex appears in the final ethyl acetate solution. Copper protoporphyrin is not extracted by this procedure. Haemin is not extracted from ethyl acetate solution by 3 N-HCl, but can be recovered with aq. N-NH_3. On neutralizing the NH_3 solution the haemin can be extracted into fresh ethyl acetate.

In experiments where porphyrins were tested for their ability to inhibit the methylation of magnesium protoporphyrin the final ethyl acetate solution contained protoporphyrin plus the added inhibitor porphyrin. The concentration of each component was calculated in a manner similar to that described by Dresel & Falk (1956) after measuring the extinction of a portion of the solution diluted in N-HCl at 401 and 408 mμ.

Paper chromatography of porphyrins

This was carried out on Whatman no. 1 paper with the solvents described by Granick (1961), either the ascending or descending technique being used. Amounts of 10 μg. or less were applied to the paper to minimize trailing. The porphyrins were detected with ultraviolet light.

RESULTS

Identification of magnesium protoporphyrin monomethyl ester. A mixture containing tris buffer, pH 8·4 (8 m-moles), magnesium protoporphyrin (11·2 μ-moles), *S*-adenosyl[*Me*-^{14}C]methionine (13·2 μ-moles; 60 400 counts/min./μmole at infinite thinness) and chromatophores (205 mg. of protein) in a total volume of 40 ml. was incubated for 4 hr. at 37°. The reaction was stopped by adding 150 ml. of acetone–0·1 N-ammonia (9:1, v/v) and after centrifuging to remove protein the supernatant was treated by the method used by Granick (1961) to purify magnesium protoporphyrin monomethyl ester. The final ether solution contained 1·65 μ-moles of porphyrin and the spectrum was identical with that of magnesium protoporphyrin. The radioactivity was found to be 40 040 counts/min./μmole of porphyrin, indicating that the material was composed of 66% of monomethyl ester and 34% of free magnesium protoporphyrin. This was confirmed by paper chromatography in lutidine–ammonia when two spots were obtained, one running at the same R_F as magnesium protoporphyrin, and the other, a larger spot, having an R_F intermediate between that of magnesium protoporphyrin and its dimethyl ester. A sample of the material containing 330 μm-moles of porphyrin, as determined spectrophotometrically, was found to contain 320 μmg. atoms of magnesium. The magnesium porphyrins were then converted into the metal-free porphyrins by extracting them from ether into 3 N-hydrochloric acid. After being neutralized and re-extracted into ether the material had a spectrum in the visible region identical with that of protoporphyrin and its radioactivity was 40 000 counts/min./μmole of porphyrin.

A sample of this material (112 μm-moles of porphyrin) in a small volume of anhydrous chloroform was mixed with 6 mg. of powdered potassium chloride and the suspension evaporated to dryness. The resulting powder was pressed to form a micro-disk. A similar micro-disk was prepared with 105 μmoles of authentic protoporphyrin dimethyl ester. The infrared-absorption spectra of these

disks were measured on a Unicam SP. 200 spectrophotometer (Fig. 1). The spectra are very similar to those reported by Granick (1961) for protoporphyrin dimethyl ester and protoporphyrin monomethyl ester respectively.

Another sample was analysed for its content of ester groups. For this purpose the hydrolysis and distillation procedure of Granick (1961) was adapted to allow examination of the distillate by gas chromatography. The reaction was carried out in a smaller version of the apparatus described by Granick (1961) in which 'bulb B' was replaced by a capillary tube. A solution of the material in chloroform (0·22 ml.; 88 μm-moles of porphyrin) was introduced into 'bulb A' and the solvent was removed under reduced pressure. The apparatus was cooled in ice, 0·03 ml. of ice-cold 5N-sodium hydroxide was pipetted into 'bulb A' and the apparatus was rapidly evacuated and sealed. It was then immersed in a boiling-water bath for 1 hr., after which it was cooled to room temperature. The 'capillary tube B' was cooled in ice–water, and 'bulb A' was warmed to about 50° until 17 μl. of distillate had collected in 'tube B'. A sample (10 μl.) of this distillate was injected into a column (200 cm. × 2·5 mm.) of Carbowax 200 (10 %, by wt.) on Celite (100–120 mesh), operated at 50°, in which the carrier gas was hydrogen (inlet pressure 10 lb./in.^{2}; flow rate 40 ml./min.). The flame ionization detector, amplifier and integrator were supplied by Gas Chromatography Ltd., Maidenhead, Berks. The chromatogram of the distillate is shown in Fig. 2, together with a chromatogram of a mixture of methanol and ethanol. Higher alcohols have longer retention times. Apart from a small peak of chloroform near the origin the chromatogram shows only methanol and some ethanol. Integration of this and a similar chromatogram showed that the sample contained 60 μm-moles of methanol and 17 μm-moles of ethanol. The values obtained in a duplicate experiment were the same within experimental error. This shows that 68 % of the porphyrin was present as methyl ester, which agrees well with the value of 66 % obtained from radioactivity measurements. The origin of the ethanol is unknown, but the most probable explanation is that it was introduced as a trace contaminant of the chloroform. When a sample of authentic protoporphyrin dimethyl ester was hydrolysed and assayed in the same manner it yielded approximately the theoretical amount of methanol, contaminated with ethanol to about the same extent as was found above.

Properties of S-*adenosylmethionine–magnesium protoporphyrin methyltransferase.* In the preliminary communication Tait & Gibson (1961) showed that the porphyrins isolated after incubating magnesium protoporphyrin and *S*-adenosyl[*Me*-^{14}C]-methionine with a chromatophore preparation were strongly radioactive. The amount of radioactivity incorporated increased with the amount of chromatophores added, and also with the time of incubation. No radioactivity appeared in the final ethyl acetate solution in the absence of added porphyrin. When chromatophores were heated to 100° before incubation no radioactivity was incorporated into

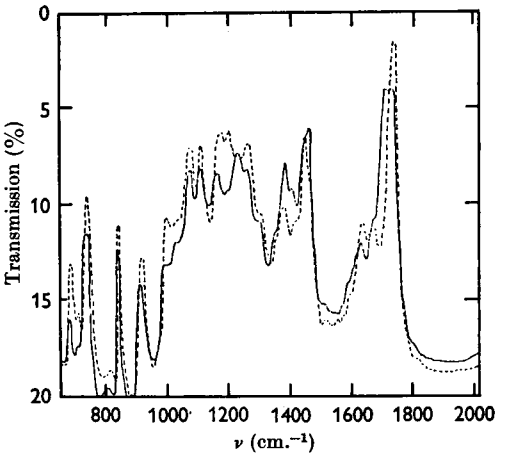

Fig. 1. Infrared-absorption spectra of protoporphyrin dimethyl ester (broken line) (105 μm-moles/6 mg. of KCl) and of the isolated porphyrin (continuous line) (112 μm-moles/6 mg. of KCl) in the Unicam SP. 200 spectrophotometer fitted with a scale-expansion accessory.

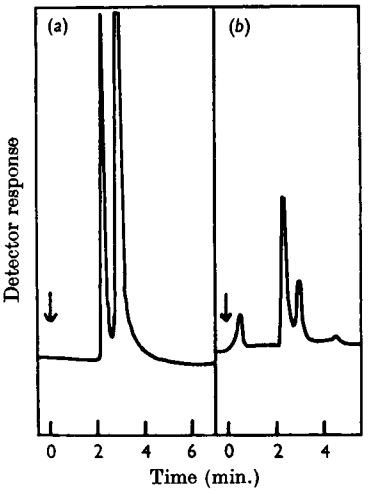

Fig. 2. Identification of the esterifying alcohol in the isolated porphyrin by gas chromatography. The experimental details are given in the text. The points at which the samples were injected are shown by arrows. (*a*) A mixture of methanol and ethanol; (*b*) the hydrolysate of the isolated porphyrin.

porphyrins. This enzymic activity was present only in chromatophores, the supernatant fraction obtained after centrifuging crushed organisms at 105 000 *g* for 90 min. being inactive.

It was subsequently found that glutathione, Mg^{2+} ions and Mn^{2+} ions did not enhance enzymic activity, either singly or in combination. They have therefore been omitted in the present experiments.

The progress of methylation with time and the rate of the reaction in the presence of different amounts of chromatophores are shown in Fig. 3. When the incubation time is 60 min. or less the radioactivity incorporated into porphyrin is proportional to the amount of chromatophores up to 5 mg. of protein. With unwashed chromatophores the amount of radioactivity incorporated into the porphyrins does not increase linearly either with time or concentration of chromatophores. The reasons for these deviations from linearity are unknown.

The optimum activity of the enzyme is at pH 8·4 in tris buffer (Fig. 4). The enzyme is equally active in phosphate buffer, but in carbonate–hydrogen carbonate the activity is only about one-tenth of that in tris at a similar pH.

The enzyme is stable to freeze-drying, but an acetone-dried powder has only one-third of the activity of fresh chromatophores. No activity could be extracted from either fresh chromatophores or from an acetone-dried powder by a variety of methods including the use of butanol and deoxycholate. The latter reagent was found to be a strong inhibitor of the enzyme.

A similar enzyme has been detected in chromatophores prepared from the related micro-organism *Rsp. rubrum* and in a spontaneous green mutant of *Rps. spheroides*. The activity/mg. of protein in chromatophores from the green mutant was the same as in those of the wild-type organism and in *Rsp. rubrum* it was slightly lower. 'Heavy' and 'light' chromatophores from *Rps. spheroides* were equally active on a protein basis.

When *Rps. spheroides* is grown aerobically in the dark the extent of pigmentation depends on the degree of aerobiosis. Organisms grown under strongly aerobic conditions contain almost no bacteriochlorophyll, whereas organisms grown under low aeration have about the same amount of bacteriochlorophyll as organisms grown anaerobically in the light (Lascelles, 1959). In addition no chromatophores are present in unpigmented organisms (Schachman, Pardee & Stanier, 1952). Extracts from unpigmented and pigmented aerobically-grown organisms were tested for their ability to methylate magnesium protoporphyrin. The activity was absent from crude extracts of unpigmented organisms, but was present in crude extracts from pigmented organisms in about the same amount as in extracts from organisms grown anaerobically in the light.

The rate of the reaction was determined in the presence of different amounts of magnesium protoporphyrin and *S*-adenosylmethionine, and K_m values for both these substrates were calculated.

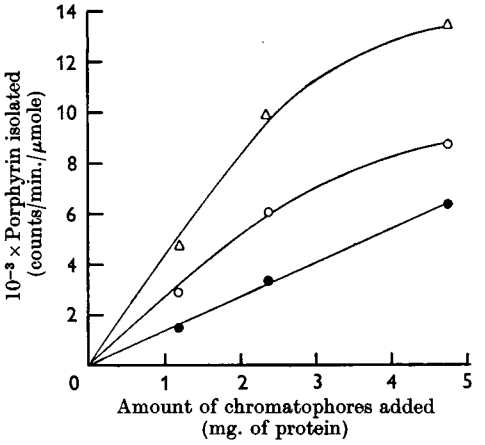

Fig. 3. Progress of enzymic methylation with time and in the presence of different amounts of chromatophores. Tris buffer, pH 7·5 (100 μmoles), magnesium protoporphyrin (250 μm-moles), *S*-adenosyl[*Me*-^{14}C]methionine (150 μm-moles; 105 000 counts/min./μmole) and chromatophores (as indicated), in a total volume of 1 ml., were incubated at 37° for 30 min. (●), 60 min. (○) or 120 min. (△). Porphyrins were isolated and their radioactivity was determined as described in the Materials and Methods section.

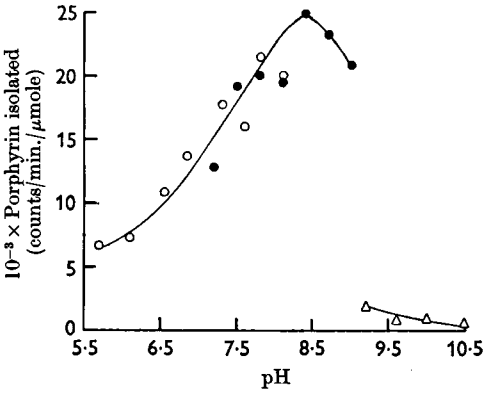

Fig. 4. *S*-Adenosylmethionine–magnesium protoporphyrin methyltransferase: pH–activity curve. Buffer (200 μmoles), magnesium protoporphyrin (125 μm-moles), *S*-adenosyl-[*Me*-^{14}C]methionine (90 μm-moles; 210 000 counts/min./μmole) and chromatophores (1·4 mg. of protein), in a total volume of 1 ml., were incubated at 37° for 1 hr. The porphyrins were isolated and counted as described in the Materials and Methods section. Buffers: potassium phosphate (○), tris (●) and carbonate–hydrogen carbonate (△).

The results were plotted in a number of ways (Dixon & Webb, 1958), one of which is shown in Fig. 5 for magnesium protoporphyrin. At pH 7·5 and 37° the K_m for magnesium protoporphyrin was 40 μM and that for *S*-adenosylmethionine was 55 μM.

Both *S*-adenosylethionine and *S*-adenosylhomocysteine are strong inhibitors of the reaction. The rates of the reaction were measured in the presence of different amounts of *S*-adenosylmethionine and of each inhibitor. The results were plotted in a number of ways (Dawes, 1956), one of which is shown in Fig. 6 for *S*-adenosylethionine. In this way the inhibition exerted by both *S*-adenosylethionine (K_i, 110 μM) and *S*-adenosylhomocysteine (K_i, 170 μM) was found to be competitive with respect to *S*-adenosylmethionine.

Chromatography of the porphyrin fraction isolated from an enzymic incubation performed with magnesium protoporphyrin and *S*-adenosylethionine failed to show any evidence for the formation of a monoethyl ester. In a parallel experiment with *S*-adenosyl[*Me*-^{14}C]methionine there was a 20 % conversion of magnesium protoporphyrin into the monomethyl ester.

Specificity of the enzyme for porphyrins. A number of different porphyrins were tested as possible substrates for methylation (Table 1). Of the metal-free porphyrins protoporphyrin is the best substrate, having about one-tenth of the activity of magnesium protoporphyrin (but see the Discussion section). The other porphyrins with two carboxyl groups/molecule are much less active, and coproporphyrin is completely inactive. Protoporphyrinogen is also inactive. The magnesium complexes of deuteroporphyrin and mesoporphyrin have about one-fifth of the activity of magnesium protoporphyrin. Among the other metal ion complexes of protoporphyrin only the calcium and zinc ion complexes are substrates for methylation, having about 75 and 55 % of the activity of magnesium protoporphyrin respectively.

These porphyrins were also tested for their ability to inhibit the methylation of magnesium protoporphyrin (Table 2). Of the metal-free porphyrins haematoporphyrin appears to be the most potent inhibitor; however, the haematoporphyrin

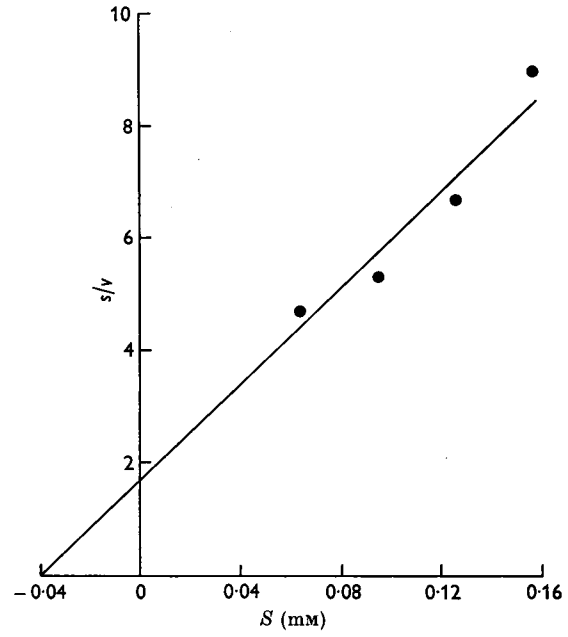

Fig. 5. *S*-Adenosylmethionine–magnesium protoporphyrin methyltransferase: Michaelis constant for magnesium protoporphyrin. Tris buffer, pH 7·5 (100 μmoles), magnesium protoporphyrin (as indicated; *s* is expressed as mM), *S*-adenosyl[*Me*-^{14}C]methionine (150 μm-moles; 210 000 counts/min./μmole) and chromatophores (2·36 mg. of protein), in a total volume of 1 ml., were incubated at 37° for 90 min. The porphyrins were isolated and counted as described in the Materials and Methods section. The amount of porphyrin monomethyl ester formed was calculated from the specific radioactivities of the porphyrin isolated and of the *S*-adenosyl[*Me*-^{14}C]methionine added. The initial velocity, *v*, is expressed as μmoles of magnesium protoporphyrin monomethyl ester formed/ml. of reaction mixture/90 min.

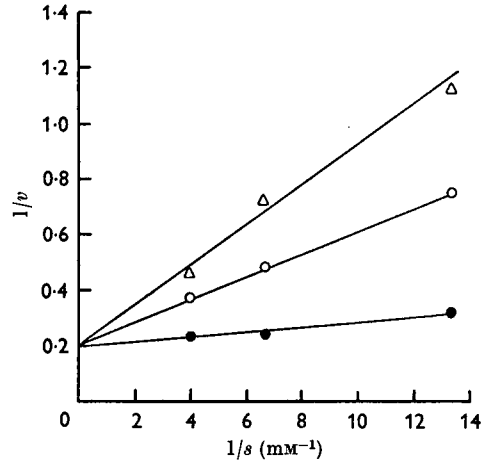

Fig. 6. *S*-Adenosylmethionine–magnesium protoporphyrin methyltransferase: inhibition by *S*-adenosylethionine. Tris buffer, pH 7·5 (100 μmoles), magnesium protoporphyrin (250 μm-moles), *S*-adenosyl[*Me*-^{14}C]methionine (420 000 counts/min./μmole) (as indicated; *s* is expressed as mM), *S*-adenosylethionine (●, none; ○, 0·3 mM; △, 0·6 mM) and chromatophores (1·7 mg. of protein), in a total volume of 1 ml., were incubated at 37° for 1 hr. Porphyrins were isolated and counted as described in the Materials and Methods section. The initial velocity, *v*, is expressed as the percentage of protoporphyrin monomethyl ester present in the isolated porphyrin mixture.

used is known to contain a number of other porphyrins as impurities (Granick *et al.* 1953). Deuteroporphyrin and mesoporphyrin also inhibit, but protoporphyrin and coproporphyrin have little or no action at the concentrations tested. Of the metal protoporphyrins tested the ferric, ferrous, manganic and manganous complexes are strong inhibitors whereas copper protoporphyrin is not.

Table 1. *Specificity of the methylating enzyme for porphyrins*

Tris buffer, pH 8·4 (200 μmoles), porphyrin (amounts stated), *S*-adenosyl[*Me*-^{14}C]methionine (88 μm-moles) and chromatophores, in a total volume of 1 ml., were incubated for 90 min. at 37°. The amounts of chromatophores (1·0–1·4 mg. of protein) varied from experiment to experiment. The porphyrins were isolated and counted as described in the Materials and Methods section. The amount of porphyrin monomethyl ester formed was calculated from the specific radioactivities of the porphyrin isolated and of the *S*-adenosyl[*Me*-^{14}C]methionine added.

Expt. no.	Substrate	Amount of substrate added (μm-moles)	Amount of monomethyl ester formed (μm-moles)
1	Magnesium protoporphyrin	125	14·2
	Protoporphyrin	170	1·9
	Protoporphyrinogen	170	0·0
	Mesoporphyrin	210	0·2
	Deuteroporphyrin	210	0·2
	Haematoporphyrin	170	0·3
	Coproporphyrin	450	0·0
2	Magnesium protoporphyrin	125	15·8
	Magnesium deuteroporphyrin	130	3·2
	Magnesium mesoporphyrin	120	3·6
3	Magnesium protoporphyrin	125	19·4
	Calcium protoporphyrin	120	14·2
	Zinc protoporphyrin	160	11·0
	Ferric protoporphyrin	240	0·0
	Ferrous protoporphyrin	240	0·0
	Manganic protoporphyrin	160	0·0
	Manganous protoporphyrin	160	0·0

Table 2. *Porphyrins as inhibitors of the methylation of magnesium protoporphyrin*

Tris buffer, pH 8·4 (200 μmoles), magnesium protoporphyrin plus other porphyrins (as stated), *S*-adenosyl-[*Me*-^{14}C]methionine and chromatophores, in a total volume of 1 ml., were incubated for 90 min. at 37°. The amounts of *S*-adenosylmethionine (80–150 μm-moles) and chromatophores (1·0–1·5 mg. of protein) varied from experiment to experiment. The porphyrins were isolated and counted as described in the Materials and Methods section. The amount of porphyrin monomethyl ester formed was calculated from the specific radioactivities of the porphyrin isolated and of the *S*-adenosyl[*Me*-^{14}C]methionine added.

Expt. no.	Amount of magnesium protoporphyrin added (μm-moles)	Other porphyrin	Amount of other porphyrin added (μm-moles)	Total amount of monomethyl ester formed (μm-moles)
1 (*a*)	125	—	—	7·3
	125	Protoporphyrin	170	6·9
	125	Mesoporphyrin	160	5·1
	125	Deuteroporphyrin	180	3·5
	125	Coproporphyrin	160	7·0
1 (*b*)	125	—	—	29·4
	125	Haematoporphyrin	170	8·8
2	125	—	—	16·1
	125	Magnesium deuteroporphyrin	130	4·4
	125	Magnesium mesoporphyrin	130	8·0
3	125	—	—	11·2
	125	Zinc protoporphyrin	160	13·3
	125	Calcium protoporphyrin	110	6·3
	125	Ferrous protoporphyrin	240	6·1
	125	Ferric protoporphyrin	240	3·2
	125	Manganous protoporphyrin	170	6·3
	125	Manganic protoporphyrin	170	3·5
	125	Copper protoporphyrin	170	10·2
	220	—	—	10·4

Table 3. *Effect of various compounds on* S-*adenosylmethionine–magnesium protoporphyrin methyltransferase*

Tris buffer, pH 8·4 (200 μmoles), magnesium protoporphyrin (125 μm-moles), *S*-adenosyl[*Me*-^{14}C]methionine (64 μm-moles; 1 μc/μmole), chromatophores (2·3 mg. of protein in Expt. 1; 0·9 mg. of protein in Expt. 2) and other compounds as stated, in a total volume of 1·0 ml., were incubated at 37° for 90 min. in Expt. 1 and 120 min. in Expt. 2. Porphyrins were isolated and counted as described in the Materials and Methods section. The amount of porphyrin monomethyl ester formed was calculated from the specific radioactivities of the porphyrin isolated and of the *S*-adenosyl[*Me*-^{14}C]methionine added.

Expt. no.	Compound	Concn. of added compound (mM)	Amount of monomethyl ester formed (μm-moles)
1	—	—	18·5
	p-Chloromercuribenzoate	0·05	1·2
	L-Methionine	1·0	20·2
	L-Ethionine	1·0	16·2
	Iodoacetamide	2·0	19·1
	EDTA	5·0	17·9
2	—	—	11·2
	Diphenylthiocarbazone	3·0	4·0
	1,10-Phenanthroline	3·0	2·4
	8-Hydroxyquinoline	3·0	6·0
	Sodium diethyldithiocarbamate	3·0	3·2
	$AgNO_3$	1·0	0·3
	$ZnSO_4$	1·0	5·1
	$MnCl_2$	1·0	7·3
	Iodoacetic acid	1·0	12·5
	Sodium azide	1·0	12·3

The amount of sodium dithionite used to reduce ferric protoporphyrin and manganic protoporphyrin was shown in a control experiment not to inhibit the enzymic reaction. Haemin at a concentration of 120 μM inhibited the reaction by about 50 %, and at 200 μM by about 75 %, in the presence of concentrations of magnesium protoporphyrin from 63 μM to 190 μM. This shows that haemin is a non-competitive inhibitor. The results with the porphyrins just discussed are clear-cut in that none of them is a substrate, and, further, some of them do not even appear in the purified porphyrin solution whose radioactivity is measured. With zinc protoporphyrin the interpretation is complicated by the fact that it is a substrate for enzymic methylation. The total amount of monomethyl ester formed is not decreased; in fact it is slightly increased. However, it is not known how much of the product is the magnesium complex and how much the zinc complex. The same arguments can be applied to the results with calcium protoporphyrin, magnesium deuteroporphyrin and magnesium mesoporphyrin, but in these cases it is clear that the amounts of monomethyl esters formed are markedly decreased.

Effect of other inhibitors of the methylation of magnesium protoporphyrin. A number of compounds were tested for their effect on the enzyme activity (Table 3). Although *S*-adenosylethionine is a competitive inhibitor (cf. Fig. 6) ethionine is inactive. Methionine does not dilute the radioactivity of the porphyrins isolated, showing that it does not donate its methyl group directly. The marked inhibition exerted by *p*-chloromercuribenzoate and by Ag^+ ions suggests that thiol groups are essential for activity. However, iodoacetic acid and iodoacetamide are without effect. Although the rate of the reaction is hardly affected by the presence of 5 mM-EDTA, it is inhibited to different extents by the other metal ion-complexing reagents. The effect of some of these reagents may be on the substrate itself rather than on the enzyme.

DISCUSSION

Granick (1948*a*, *b*, 1950*a*, 1961) isolated and identified a number of porphyrins that are accumulated by chlorophyll-less mutants of *Chlorella* and by barley seedlings incubated with δ-aminolaevulic acid. On the basis of these findings Granick (1950*b*, 1960, 1961) proposed the following pathway for the biosynthesis of chlorophyll and bacteriochlorophyll from protoporphyrin: Protoporphyrin → protoporphyrin monomethyl ester or magnesium protoporphyrin → magnesium protoporphyrin monomethyl ester → magnesium vinyl phaeoporphyrin a_5 → magnesium vinyl phaeoporphyrin a_5 phytyl ester → chlorophyll *a* → bacteriochlorophyll. The order in which the magnesium and the ester group are added to protoporphyrin cannot be deduced from Granick's results.

In the present work an enzyme has been detected in chromatophores isolated from *Rps. spheroides*

that catalyses the transfer of a methyl group from *S*-adenosylmethionine to magnesium protoporphyrin. The identification of the product of the reaction rests on observations reported above and in a preliminary communication (Tait & Gibson, 1961).

(1) When incubations were carried out under standard conditions with *S*-adenosyl[*Me*-^{14}C]-methionine and magnesium protoporphyrin, porphyrins of high radioactivity were isolated. In these experiments the porphyrins were extracted under conditions sufficiently acid to remove magnesium and certain other metals from their complexes. On paper chromatography radioactivity was confined to one spot having the mobility expected for a porphyrin with one free carboxyl group/molecule. The radioactivity was also readily removed by acid hydrolysis.

(2) In a large-scale incubation the product was isolated under conditions that do not degrade magnesium complexes. The material had a spectrum identical with that of synthetic magnesium protoporphyrin and analysis showed a magnesium:protoporphyrin molar ratio of 0·97:1·0. Its molar radioactivity was 66% of that of the donor *S*-adenosyl[*Me*-^{14}C]methionine. Behaviour during extraction and on chromatography showed that it had at least one free carboxyl group/molecule.

(3) When the metal was removed from this product porphyrin was obtained which had an infrared-absorption spectrum almost identical with that reported by Granick (1961) for protoporphyrin monomethyl ester and which yielded methanol on acid hydrolysis in an amount that indicated that 68% of the material was present as methyl ester.

From all this evidence it is concluded that the product of the enzyme reaction is magnesium protoporphyrin monomethyl ester. It is presumed that the methyl ester group is on ring C since this is its position in chlorophyll and bacteriochlorophyll. The methyltransferase is contained exclusively in the chromatophores, or pigment-containing particles. Extracts from unpigmented organisms, which are known not to contain chromatophores, have no activity. The activity of chromatophores from the spontaneous green mutant is the same as that of those from the wild-type organism. This might be expected since the lesion in the green mutant is known to involve one of the later steps in carotenoid synthesis and does not affect the production of bacteriochlorophyll (Jensen, Cohen-Bazire & Stanier, 1961). From the relatively small amount of work done on the specificity of this enzyme it appears that the complexes of transition metals with protoporphyrin, e.g. manganous, manganic, ferrous and ferric complexes, are not substrates but are strong inhibitors. On the other hand the complexes of protoporphyrin with calcium and zinc, whose atomic structures are very similar to that of magnesium, are substrates. With regard to the porphyrin moiety of the substrate it is clear that modification of the vinyl side chains of the magnesium complex does not completely abolish the activity (Table 1: Expt. 2).

Protoporphyrin, mesoporphyrin, deuteroporphyrin and haematoporphyrin were methylated by the chromatophore preparation to a small but significant extent, but coproporphyrin was not acted on at all. More recent work has shown that with protoporphyrin as substrate there is no methylation in the presence of EDTA. This suggests that protoporphyrin is converted into an acid-labile metal complex before being methylated. A zinc–protoporphyrin chelatase has been detected in chromatophores (A. Neuberger and G. H. Tait, unpublished work), and it therefore seems likely that in these experiments protoporphyrin and possibly also the other porphyrins were converted into their zinc complexes, before they were methylated, traces of zinc present in the chromatophores or in the other reagents being used.

S-Adenosylmethionine has previously been shown to be the methyl donor in cell-free systems for the formation of *N*-methyl groups (Cantoni & Vignos, 1954), *C*-methyl groups (Parks, 1958*a*) and methoxyl groups (Axelrod & Tomchick, 1958). This is the first time to our knowledge that the formation of a methyl ester group has been studied in a cell-free system and here also *S*-adenosylmethionine is the donor of the methyl group. This finding was expected in view of the previous studies on the incorporation of the methyl group of L-[*Me*-^{14}C]-methionine into bacteriochlorophyll in whole cells (Gibson *et al.* 1962*b*). It is known that the methyl ester groups of pectin are formed from methionine *in vivo* (Sato, Byerrum, Albersheim & Bonnar, 1958) and it may be suggested that in this system also the actual donor is *S*-adenosylmethionine.

The ethyl derivative of a compound can be formed instead of the usual methyl derivative if *S*-adenosylethionine is used in place of *S*-adenosylmethionine in some transmethylating systems (Parks, 1958*b*; Tuppy & Dus, 1958) or if ethionine replaces methionine in whole organisms (Dulanay *et al.* 1962). In the present study no magnesium protoporphyrin monoethyl ester could be detected when *S*-adenosylethionine was used as a substrate. However, *S*-adenosylethionine is a strong competitive inhibitor of the methylation of magnesium protoporphyrin by *S*-adenosylmethionine. This finding explains the observation made by Gibson *et al.* (1962*b*) that bacteriochlorophyll synthesis is inhibited when even small amounts of ethionine are added to suspensions of *Rps. spheroides* incubated anaerobically in the light. A similar explanation

can account for the concomitant inhibition of carotenoid synthesis by ethionine, since the two major carotenoids of *Rps. spheroides* are known to contain a methoxyl group (Stanier, 1960), which is probably donated by *S*-adenosylmethionine.

SUMMARY

1. An enzyme, *S*-adenosylmethionine–magnesium protoporphyrin methyltransferase, has been detected in *Rhodopseudomonas spheroides*. It catalyses the transfer of the methyl group from *S*-adenosylmethionine to magnesium protoporphyrin to form a compound that has been identified as magnesium protoporphyrin monomethyl ester.

2. The enzyme is confined to the chromatophores, to which it is firmly bound. It is also present in chromatophores from *Rhodospirillum rubrum*. No activity could be detected in extracts from *Rps. spheroides* grown under high aeration in the dark.

3. Zinc protoporphyrin, calcium protoporphyrin, magnesium mesoporphyrin and magnesium deuteroporphyrin are also substrates for the enzyme. Ferrous, ferric, manganous and manganic protoporphyrins are not substrates, but are strong inhibitors of the methylation of magnesium protoporphyrin. Metal-free porphyrins are believed not to be substrates, but some of them are inhibitors.

4. Both *S*-adenosylhomocysteine and *S*-adenosylethionine inhibit the reaction competitively. The inhibition by the latter compound explains the observation made by Gibson *et al.* (1962*b*) that the addition of ethionine to whole organisms inhibited bacteriochlorophyll synthesis.

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