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

5. ZINC–PROTOPORPHYRIN CHELATASE*

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Of the metal complexes of protoporphyrin which are of biological importance those of magnesium and ferrous iron are of special importance. Magnesium protoporphyrin is most probably an intermediate in the biosynthesis of chlorophyll (Granick, 1961) and of bacteriochlorophyll (Gibson, Neuberger & Tait, 1963), while ferrous protoporphyrin (protohaem) is a component of haemoglobin, myoglobin, cytochromes and other haem proteins. The enzymic formation of magnesium protoporphyrin has not been described, but that of protohaem from Fe^{2+} ions and protoporphyrin is well documented. Ferrochelatases have been found in mitochondria from rat liver (Labbe & Hubbard, 1961) and pig liver (Porra & Jones, 1963*a*, *b*) and in haemolysates from duck blood (Yoneyama, Oyama, Sugita & Yoshikaya, 1962) and chicken blood (Schwartz, Hill, Cartwright & Wintrobe, 1959). These enzymes have been partially purified and their specificities towards various porphyrins and metal ions have been tested. They act on all porphyrins with two carboxyl groups. They are, however, more specific towards the metal; the enzyme in duck blood (Oyama, Sugita, Yoneyama & Yoshikaya, 1961) uses Zn^{2+} ions at 10% and Co^{2+} ions at 2% the rate of Fe^{2+} ions, the one from rat-liver mitochondria (Labbe & Hubbard, 1961) uses Co^{2+} ions more efficiently than the one in duck blood but is completely inactive with Zn^{2+} ions; it also uses Mn^{2+} ions to a small extent.

During the course of unsuccessful experiments designed to detect a magnesium-incorporating enzyme in chromatophores from *Rhodopseudomonas spheroides* the production of zinc protoporphyrin was noted, and the rate of its formation was greatly stimulated by bringing about the reaction in a water–ether emulsion. Under these conditions a marked synthesis of zinc protoporphyrin was also detected with mitochondria from a number of mammalian tissues. The properties of these zinc–protoporphyrin chelatases, particularly the one in *R. spheroides*, are described in detail and their possible metabolic functions discussed.

* Part 4: Gibson, Neuberger & Tait (1963).

MATERIALS AND METHODS

Chemicals. [^{65}Zn]Zinc chloride was obtained from The Radiochemical Centre, Amersham, Bucks. Tween 20 and Tween 80 were gifts from Honeywell and Stein Ltd., London. De-ionized water, which was used throughout, was prepared from distilled water by passing it through a column of Bio-Deminrolit (The Permutit Co. Ltd., London).

Ether was dried over anhydrous Na_2SO_4 and redistilled before use.

All other chemicals were obtained or prepared as described by Gibson *et al.* (1963).

Organisms. The strain of *Rhodopseudomonas spheroides* and the conditions of growth have been described by Gibson *et al.* (1963). Except where stated otherwise the organisms used to make enzyme extracts were grown semi-anaerobically in the light as described by Gibson, Neuberger & Tait (1962). For certain experiments organisms were grown aerobically in the dark. 'High' or 'low' content of O_2 obtained by varying the extent of aeration gave unpigmented or pigmented organisms respectively (cf. Lascelles, 1959). In the experiments described here the content of O_2 was not measured, but this factor was controlled empirically by observing the extent of pigmentation of the organisms.

Enzyme sources

Chromatophores. These were prepared according to Gibson *et al.* (1963) from *R. spheroides*. In all experiments except where stated otherwise the chromatophores were prepared from organisms grown semi-anaerobically in the light in the malate medium of Lascelles (1956).

Protoplasts. These were prepared as described by Karunairatnum, Spizizen & Gest (1958). They were lysed by dilution of the suspension in 20% sucrose with a large volume of water. The lysed particles were collected by centrifuging, washed with 0·05M-potassium phosphate, pH 7·4, and resuspended in the same buffer to a concentration of 10–30 mg. of protein/ml. and kept frozen at −20°.

Mitochondria. These were prepared from guinea-pig liver and from rabbit liver, heart and kidney as described by Hogeboom (1955). After being washed twice in 0·25M-sucrose the mitochondrial pellet was suspended in 0·05M-potassium phosphate, pH 7·4, to a concentration of 10–20 mg. of protein/ml. and kept frozen at −20°.

Estimations

Protein. The protein content of chromatophores, protoplasts and mitochondria was determined by the method of Lowry, Rosebrough, Farr & Randall (1951), with crystalline bovine plasma albumin (Armour and Co. Ltd.) as standard.

Porphyrins. The concentration of porphyrins and of metalloporphyrins was determined by measuring the extinction of solutions in ether at the absorption maximum in the Soret band, and by using the appropriate molar extinction coefficient.

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. One μc of ^{65}Zn gave 24400 counts/min. under these conditions. Alternatively, solutions (0·5 ml.) were mixed with 5 ml. of a dioxan-based scintillator (Bray, 1960). Radioactivity was measured in a I.D.L. scintillation counter with the upper-gate-discriminator bias set at 50 v. Under these conditions 1 μc of ^{65}Zn gave 253800 counts/min.

Radioactive spots on paper chromatograms were located by radioautography.

Infrared-absorption spectra. Porphyrin (50–100 μg.) in a small volume of ether was mixed with 6 mg. of powdered KCl and the suspension was evaporated to dryness. The resulting powder was pressed to form a micro-disk. The infrared spectrum was measured on a Unicam SP.200 spectrophotometer.

Assay of enzyme activities

Zinc–protoporphyrin chelatase. To incubation mixtures (vol. 0·5 ml.; cf. legends to Figs. 1–3 and Table 1) was added 4 ml. of acetone–0·1 N-NH_3 (9:1, v/v). After centrifuging, the supernatant was extracted twice with 3 ml. of light petroleum (b.p. 60–80°). This removes the bacteriochlorophyll and carotenoids of the chromatophores. To the aqueous phase, containing the porphyrins, were added 0·3 ml. of saturated NaCl, 0·5 ml. of 0·5 M-KH_2PO_4 and 4 ml. of ether. After thorough mixing the ether layer was removed and analysed for porphyrin and zinc porphyrin. This extraction method is modelled on that of Granick (1961). In assays with $^{65}ZnCl_2$ this ether solution was washed with 3 ml. of water before portions of it were taken for measurement of radioactivity. Most of the assays were done with protophorphyrin as substrate and therefore the extinction of the ether solution was measured at 404 mμ and 415 mμ, which are the absorption maxima of protoporphyrin and zinc protoporphyrin respectively. When other porphyrins were used as substrates the wavelengths at which readings were measured were chosen accordingly. From the known molar extinction coefficients in ether of protophorphyrin (404 mμ, $1{\cdot}35 \times 10^5$; 415 mμ, $0{\cdot}94 \times 10^5$) and zinc protoporphyrin (404 mμ, $0{\cdot}75 \times 10^5$; 415 mμ, $2{\cdot}40 \times 10^5$) the concentration of each component was calculated in a manner similar to that described by Dresel & Falk (1956). It was assumed that protoporphyrin and zinc protoporphyrin behave in identical fashion during the extraction and that the relative amounts are the same in the final ether solution as they are at the end of the experiment in the incubation mixture. To correct for slight differences in the recovery of porphyrins (usually about 60% of the total) in the final ether solution the activity is expressed in terms of the theoretical amount of zinc protoporphyrin formed in the assay.

Ferrochelatase. Tris buffer, pH 8·4 (150 μmoles), protoporphyrin (50 μm-moles), $FeSO_4$ (50 μm-moles), ascorbic acid or GSH (10–20 μmoles) and enzyme extract (0·5–2·0 mg. of protein) were incubated in a total volume of 0·5 ml. under N_2 for 2 hr. at 37°. The treatment of the mixture followed exactly that described for the assay of zinc–protoporphyrin chelatase. Since haemin is insoluble in ether the extent of the reaction was taken as the difference between the amounts of protoporphyrin recovered in the final ether solution when experiments were done in the presence and absence of $FeSO_4$ respectively.

RESULTS

Preliminary observations

The finding that the enzymic conversion of protoporphyrin, but not of magnesium protoporphyrin, into the corresponding monomethyl ester was strongly inhibited by EDTA suggested that protoporphyrin had first to be converted into a metalloporphyrin before it could be methylated (Gibson *et al.* 1963). That the insertion of the metal was catalysed by an enzyme was indicated by the following experimental results. Protoporphyrin and chromatophores in tris buffer, pH 8·4, were incubated at 37° for periods up to 2 hr., after which EDTA was added, followed by *S*-adenosyl[*Me*-^{14}C]-methionine. The mixture was incubated for a further 2 hr. The amount of radioactivity incorporated into porphyrin was proportional to the duration of the first incubation in the absence of EDTA. The formation of metalloporphyrin was also pH-dependent; the greatest incorporation of radioactivity into porphyrin took place when the chromatophores and protoporphyrin were incubated at pH 8·4 before addition of EDTA. A large-scale experiment was performed in which protoporphyrin, *S*-adenosyl[*Me*-^{14}C]methionine and chromatophores from *R. spheroides*, and tris buffer, pH 8·4, were incubated for 4 hr. After incubation, the porphyrin mixture was extracted and purified by the method of Granick (1961), which does not hydrolyse acid-labile metalloporphyrins. This porphyrin mixture was chromatographed on paper in the lutidine–ammonia system of Granick (1961). All the radioactivity was located in an area corresponding to a porphyrin with one free carboxyl group. This portion of the paper was cut out and the eluted porphyrin was identified as a metalloporphyrin on the basis of its spectrum in ethereal solution which, in the visible region, had peaks of almost equal intensity at about 545 mμ and 590 mμ. Zinc, magnesium and calcium protoporphyrins all have similar spectrum bands but there are slight differences in the intensities of the two peaks. Probably owing to the presence of some impurity the nature

of the experimental metalloporphyrin could not be deduced.

It was found that formation of metalloporphyrin could be estimated directly, without coupling it to the methylation, by measuring the extinction of the extracted porphyrins at two different wavelengths in the Soret region of the spectrum (see the Materials and Methods Section). For metalloporphyrin formation only tris buffer, protoporphyrin and chromatophores were required and it was considered that Mg^{2+} ions, or some magnesium complex present in the chromatophores, might be the metal substrate. It was subsequently found that the metal ion was present in the tris buffer. With fresh tris buffer made up in de-ionized water no metalloporphyrin was formed unless Zn^{2+} ions were added; moreover, no other metal ions could replace Zn^{2+} ions. Therefore in all further experiments on the enzymic formation of zinc protoporphyrin suitable controls were included to check that contamination of the reagents with Zn^{2+} ions was small. It was not found necessary to take any precautions other than using de-ionized water for making up all solutions.

Zinc–protoporphyrin chelatase in chromatophores

The formation of zinc protoporphyrin in the presence of increasing amounts of chromatophores is shown in Fig. 1. The small but significant formation of zinc protoporphyrin in the absence of chromatophores was no greater than was obtained with chromatophores previously boiled for 3 min. or with the supernatant obtained on centrifuging crushed organisms at 105000*g* for 90 min. The progress of the reaction was approximately linear for 60 min., when using up to 1·1 mg. of protein/assay. The reaction was carried out at temperatures from 5° to 38°, and the heat of activation was calculated to be 14800 kcal./mole. About 70% of the activity was lost by heating the chromatophores in tris buffer, pH 8·4, for 5 min. at 55°. This loss in activity could be abolished by heating the chromatophores at 55° in the presence of protoporphyrin (50 μm-moles/0·5 ml.), but not in the presence of zinc chloride (15 μm-moles/0·5 ml.).

The formation of zinc protoporphyrin in the presence of increasing amounts of Zn^{2+} ions is shown in Fig. 2. There is a significant formation, about 20% of the maximum value, in the absence of added Zn^{2+} ions, and with low concentrations of added Zn^{2+} ions more than the theoretical amount of zinc protoporphyrin is formed. The K_m for protoporphyrin was 200 μM, and that for zinc chloride, which cannot be calculated accurately because of the contamination, is probably about 40–50 μM. Under the conditions of assay none of the following ions (tested at 100 μm-moles/assay) gave rise to significant formation of metalloprotoporphyrins either in the presence or absence of chromatophores: Cu^{2+}, Co^{2+}, Al^{3+}, Mn^{2+}, Fe^{2+}, Fe^{3+}, Ca^{2+}, Ba^{2+}, Sr^{2+}, Mg^{2+}. Even though the spectra of some of the expected metalloprotoporphyrins are not known, any formation would be expected to change the ratio of extinctions at 404 mμ and 415 mμ from

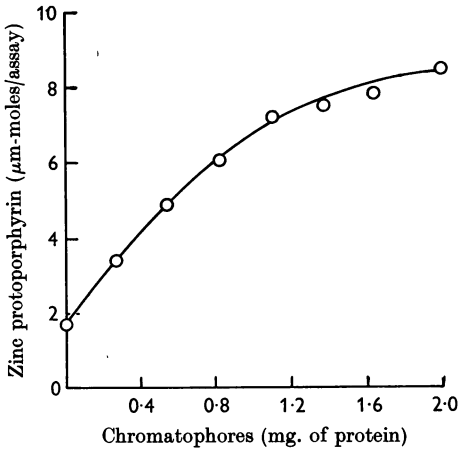

Fig. 1. Formation of zinc protoporphyrin in the presence of different amounts of chromatophores. Tris buffer, pH 8·4 (150 μmoles), protoporphyrin (34 μm-moles), $ZnCl_2$ (20 μm-moles) and chromatophores (as stated) in a total volume of 0·5 ml. were incubated at 37° for 1 hr. The porphyrins were isolated and the amount of zinc protoporphyrin formed was estimated as described in the Materials and Methods section.

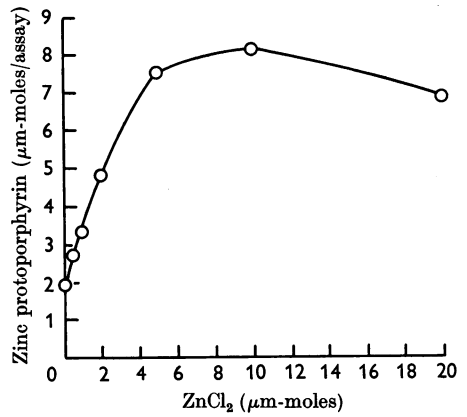

Fig. 2. Formation of zinc protoporphyrin in the presence of different amounts of $ZnCl_2$. Tris buffer, pH 8·4 (150 μmoles), protoporphyrin (39 μm-moles), $ZnCl_2$ (as stated) and chromatophores (0·97 mg. of protein) in a total volume of 0·5 ml. were incubated at 37° for 1 hr. The porphyrins were isolated and the amount of zinc protoporphyrin formed was estimated as described in the Materials and Methods section.

that obtained with protoporphyrin itself. The same ions were also tested, at 100 μm-moles/assay, in the presence of 10 μm-moles of zinc chloride. The Co^{2+} salt inhibited zinc protoporphyrin formation by 70 % and the Mn^{2+} salt by 50 %; other cations were apparently without effect (but see below for experiments with [^{65}Zn]zinc chloride). A number of agents known to form metal complexes tested at 0·3 mM (diphenylthiocarbazone, 1,10-phenanthroline and 8-hydroxyquinoline) were inactive, but EDTA at 30 μm-moles/assay inhibited by 60 % the formation of zinc protoporphyrin promoted by 10 μm-moles of zinc chloride.

The pH–activity curve in tris buffer (Fig. 3) shows an optimum in the region of pH 8. The activity was the same in triethanolamine of the same pH, but was considerably lower in phosphate, probably owing to formation of zinc phosphate. The effects of ascorbic acid and GSH were examined, since these reducing agents are necessary for demonstrating ferrochelatase activity (Labbe & Hubbard, 1961). GSH markedly inhibited the reaction, but ascorbic acid at concentrations up to 20 μmoles/assay had no effect. Protoporphyrinogen could be used as substrate instead of protoporphyrin: however, the former was completely inactive in the presence of ascorbic acid. This is probably because ascorbic acid inhibits the oxidation of protoporphyrinogen to the true substrate protoporphyrin.

When zinc protoporphyrin is formed enzymically there is a change in the spectrum in the visible region between 490 mμ and 630 mμ (Fig. 4). The spectrum of the final ether solution (see the Materials and Methods section) obtained from incubations containing protoporphyrin, Zn^{2+} ions and chromatophores is intermediate between that of protoporphyrin and zinc protoporphyrin. The formation of zinc protoporphyrin was further confirmed in experiments with $^{65}Zn^{2+}$ ions, where the increase in the ether-soluble radioactivity paralleled the formation of zinc protoporphyrin as measured by extinction change (Table 1). The non-enzymic formation of zinc protoporphyrin noted above

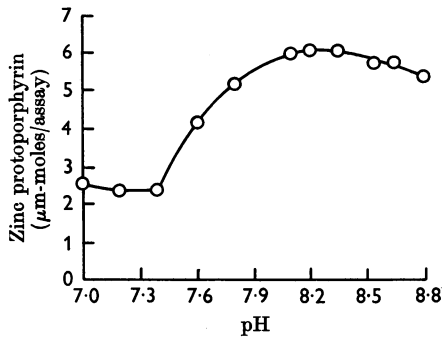

Fig. 3. pH–activity curve for zinc–protoporphyrin chelatase. Tris of pH stated (150 μmoles), protoporphyrin (34 μm-moles), $ZnCl_2$ (10 μm-moles) and chromatophores (0·75 mg. of protein) in a total volume of 0·5 ml. were incubated at 37° for 1 hr. The porphyrins were isolated and the amount of zinc protoporphyrin formed was estimated as described in the Materials and Methods section.

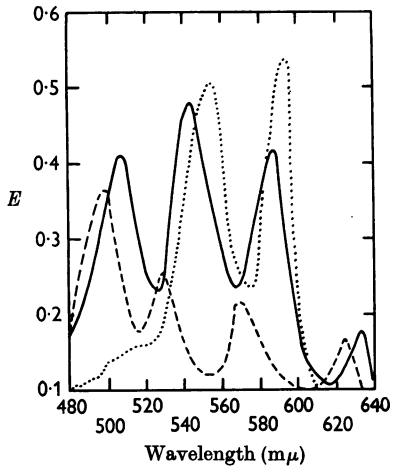

Fig. 4. Spectra in ether solution of protoporphyrin (- - - -), zinc protoporphyrin (· · · · ·) and porphyrins extracted after incubating protoporphyrin, $ZnCl_2$ and chromatophores (———).

Table 1. *Assay of zinc–protoporphyrin chelatase in the presence of* [^{65}Zn]*zinc chloride*

Tris buffer, pH 8·4 (100 μmoles), protoporphyrin (30 μm-moles), $^{65}ZnCl_2$ (15·6 μm-moles; 0·04 μc/μm-mole; 980 counts/min./μm-mole at infinite thinness on gas-flow counter) and chromatophores (3·4 mg. of protein) in a total volume of 0·5 ml. were incubated for the times stated at 37°. The porphyrins were isolated and the amount of zinc protoporphyrin and the radioactivity in the final ether solution were estimated as described in the Materials and Methods section.

Time of incubation (min.)	Zinc protoporphyrin (μm-moles/ml. of ether)	Counts/min./ml. of ether	Counts/min./μm-mole of zinc protoporphyrin
0	0·07	4	55
25	0·68	256	392
50	1·01	416	414
75	1·43	616	432
100	1·50	608	405
100 (no chromatophores)	0·53	272	518
100 (no protoporphyrin)	0·00	15	—

(Fig. 1) was also confirmed. That the ether-soluble zinc is all in the form of zinc protoporphyrin was shown by chromatography followed by radioautography (see below). It may also be noted that the molar radioactivity of the zinc protoporphyrin is lower than expected.

Assay of zinc–protoporphyrin chelatase in the presence of ether

Under the assay conditions described above the maximum conversion of protoporphyrin into zinc protoporphyrin was about 30%. No magnesium protoporphyrin formation was detected and it was thought that magnesium in the ionic form might first have to be transferred to a lipid phase before being incorporated into protoporphyrin. Therefore the reaction was carried out in the presence of ether. To the 0·5 ml. of assay system was added 0·2 ml. of ether. The tube was stoppered, shaken vigorously to obtain an emulsion and incubated. From time to time the tube was shaken to keep the emulsion intact. No formation of magnesium protoporphyrin was detected but when these conditions were tried with Zn^{2+} ions instead of Mg^{2+} ions a large stimulation of zinc protoporphyrin formation was found (Fig. 5). Ether had no effect on the non-enzymic formation of zinc protoporphyrin but consistently stimulated the enzymic reaction by a factor of five to six. With methanol (0·2 ml.) or Tween 80, at a final concentration of 0·5%, instead of ether the stimulation was two- to three-fold. Butanol, acetone, ethylene glycol, propylene glycol (all 0·2 ml.) were inactive, as was deoxycholate (0·5%, w/v). In the presence of ether, as in its absence, the incorporation of $^{65}Zn^{2+}$ ions followed closely the formation of zinc protoporphyrin as measured spectrophotometrically (Fig. 5). That the ether-soluble ^{65}Zn is actually in the form of zinc protoporphyrin was shown as follows. An incubation was done with protoporphyrin, $^{65}Zn^{2+}$ ions and chromatophores in the presence of ether, and, as judged from the visible spectrum, 80% of the porphyrin was in the form of the zinc complex. The porphyrin was purified and chromatographed on paper in lutidine–0·05N-ammonia (10 : 7, v/v) (Granick, 1961). It ran as a single spot, having an R_F identical with that of zinc protoporphyrin and protoporphyrin (these two compounds not being separated by this solvent) and on radioautography all the radioactivity was associated with this spot.

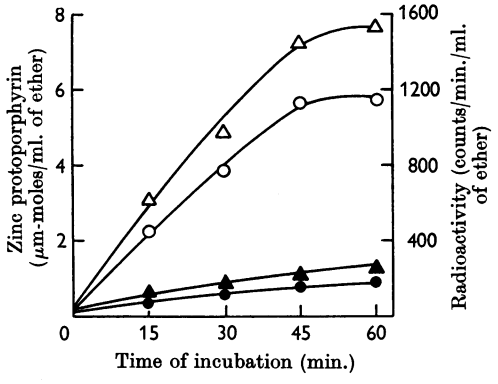

Fig. 5. Effect of ether on zinc–protoporphyrin-chelatase activity. Tris buffer, pH 8·4 (100 μmoles), protoporphyrin (39 μm-moles), $ZnCl_2$ (30 μm-moles; 0·02 μc/μm-mole; 429 counts/min./μm-mole at infinite thinness) and chromatophores (1·4 mg. of protein) were mixed in a total volume of 0·5 ml. To one set of tubes 0·2 ml. of ether was added, the tubes were stoppered and the contents mixed thoroughly. All tubes were incubated at 37° for the times stated. The porphyrins were extracted as described in the Materials and Methods section and assayed for zinc protoporphyrin (○, in the presence of ether; ●, in the absence of ether) and radioactivity (△, in the presence of ether; ▲, in the absence of ether).

In Table 1 and Fig. 5 it can be seen that the molar radioactivity of the isolated zinc protoporphyrin is considerably lower than that of the [^{65}Zn]zinc chloride added. There are a number of possible reasons for this, but the most obvious one is the presence of non-radioactive Zn^{2+} ions as a contaminant of the reagents. This explanation agrees with the findings discussed above that a small quantity of zinc protoporphyrin is formed in the absence of added Zn^{2+} ions and that when small amounts of Zn^{2+} ions are added the formation of zinc protoporphyrin is often higher than the theoretical amount (cf. Fig. 2). An experiment in the presence of ether was performed under the conditions described in Fig. 5 but with quantities of [^{65}Zn]zinc chloride varying from 8 to 100 μm-moles. By comparing the molar radioactivities of the zinc chloride and the zinc protoporphyrin, and by the use of values obtained on the gas-flow counter and the scintillation counter, it was calculated that there were about 20 μmg. ions of Zn^{2+} contaminating each 0·5 ml. of assay mixture. However, from the low blanks obtained in the absence of added Zn^{2+} ions (cf. Fig. 2) all the contaminating zinc does not appear to be 'free' to form zinc protoporphyrin unless additional Zn^{2+} ions are added. For instance, in an experiment with ether only 3·6 μm-moles of zinc protoporphyrin were formed in the absence of added Zn^{2+} ions, whereas 20 μm-moles were formed on the addition of 10 μmg. ions. On the other hand the low molar radioactivity of the zinc protoporphyrin may be due to the fact that all the metalloporphyrin formed is not zinc protoporphyrin or that all the radioactivity in the solution of zinc chloride supplied is not due to $^{65}Zn^{2+}$ ions.

In an attempt to determine how ether increases the rate of the reaction the various properties of

the enzyme were retested in the presence of ether. The heat of activation was 14800 kcal./mole, as it was in the absence of ether, and the K_m values for the substrates were virtually unchanged. The pH optimum in tris buffer was the same but the shape of the curve was different, the activity at pH 7 being higher compared with that at pH 8 than it was without ether (cf. Fig. 3). Omission of tris buffer had no effect on the enzyme activity. This last point is of importance with regard to the specificity for different porphyrins which will be discussed below. As in the absence of ether, no other metal ions could replace Zn^{2+} ions as substrate. A number of metal salts were tested for their ability to inhibit formation of zinc protoporphyrin from [^{65}Zn]zinc chloride. At 100 μm-moles/0·5 ml. in the presence of 30 μm-moles of zinc chloride the Cu^{2+} salt inhibited by 45 %, the Co^{2+} salt by 80 % and the Ca^{2+} salt by 20 %.

After incubations in the presence of ether, in which over 80 % of the protoporphyrin was converted into zinc protoporphyrin (as indicated by spectrophotometric measurements), the porphyrins were purified (see the Materials and Methods section) and the infrared spectrum was measured. This spectrum was identical in every respect with that of authentic zinc protoporphyrin prepared by alkaline hydrolysis of zinc protoporphyrin dimethyl ester, synthesized according to Fischer & Pützer (1926).

When synthetic zinc protoporphyrin, magnesium protoporphyrin or haemin was incubated with $^{65}Zn^{2+}$ ions and chromatophores, the isolated metalloporphyrins were unlabelled. Also, if the reaction with protoporphyrin, [^{65}Zn]zinc chloride and chromatophores, in the presence of ether, was allowed to proceed until about 80 % of the porphyrin was present as zinc protoporphyrin and then unlabelled Zn^{2+} ions were added and the incubation was continued, the molar radioactivity of the isolated zinc protoporphyrin was the same as that obtained before adding unlabelled Zn^{2+} ions. Similarly, if the first incubation was with unlabelled Zn^{2+} ions and subsequently $^{65}Zn^{2+}$ ions were added, only a small amount of radioactivity was found in the isolated porphyrins, equivalent in amount to the extra zinc protoporphyrin formed during that period of the incubation. These experiments show conclusively that there was no exchange between zinc protoporphyrin and Zn^{2+} ions.

As mentioned above, only a small amount of zinc protoporphyrin was formed in the absence of chromatophores and in the presence of tris buffer, and this reaction was not affected by ether. Also, the enzymic formation of zinc protoporphyrin proceeded very well in the absence of tris buffer within the range pH 7·4–9.0. When other porphyrins were tested for their ability to form zinc porphyrins, the situation was much more complex (Table 2). With coproporphyrin and haematoporphyrin all that was required to form the zinc complex was to incubate the porphyrin with Zn^{2+} ions at neutral pH. After incubations for 2 hr. a large percentage of the porphyrin was converted into zinc porphyrin, as judged from the peak in the Soret region of the spectrum, the visible spectrum and the radio-

Table 2. *Enzymic and non-enzymic formation of zinc porphyrins*

Porphyrins (~50 μm-moles), $^{65}ZnCl_2$ (30 μm-moles; 0·02 μc/mole; 429 counts/min./μm-mole at infinite thinness) and where stated tris buffer, pH 8·4 (100 μmoles), and chromatophores (1·5 mg. of protein), in a total volume of 0·5 ml. (with the addition of 0·2 ml. of ether where stated), were incubated at 37° for 2 hr. The porphyrins were isolated and the final ether solution containing them was assayed spectrophotometrically in the Soret and visible regions of the spectrum and the radioactivity was determined. —, Not determined.

Substrate	Ether			No ether		
Additions ...	None	Enzyme	Tris	Tris	Enzyme	Not incubated
Protoporphyrin						
Soret peak	404	412	404	404	404	404
Counts/min./ml. of ether	101	970	101	100	150	22
Deuteroporphyrin						
Soret peak	397	405	404	404	406	396
Counts/min./ml. of ether	373	1760	1126	—	1736	—
Mesoporphyrin						
Soret peak	395	407	406	396	398	396
Counts/min./ml. of ether	85	1010	879	—	290	—
Haematoporphyrin						
Soret peak	410	—	410	410	—	396
Counts/min./ml. of ether	804	—	830	—	—	—
Coproporphyrin						
Soret peak	407	407	408	408	—	396
Counts/min./ml. of ether	2010	—	1950	—	—	—

activity in the purified porphyrin fraction when the reaction was carried out with $^{65}Zn^{2+}$ ions. As with zinc protoporphyrin the maxima of absorption in the Soret region of other zinc porphyrins are about 10 mμ higher than those of the corresponding porphyrins. Tris buffer, chromatophores and ether had no effect on these reactions. An incubation was carried out with coproporphyrin and Zn^{2+} ions in the presence of ether, and as judged from the visible spectrum most of the porphyrin was in the form of the zinc complex. The infrared spectrum showed a peak at 5·9 μ characteristic of un-ionized carboxyl groups but there were no peaks at 6·4 and 7·1 μ, which are characteristic of ionized carboxyl groups (Schwartz, Berg, Bossenmaier & Dinsmore, 1960), indicating that the material is a zinc–coproporphyrin complex and not a zinc coproporphyrin salt (Schwartz *et al.* 1960). With deuteroporphyrin and mesoporphyrin only a small amount of zinc complex was formed in the absence of tris and chromatophores, and the reaction could be speeded by adding either of these components. With deuteroporphyrin ether had no effect on the enzymic or non-enzymic reaction, but with mesoporphyrin virtually no zinc mesoporphyrin was formed in the absence of ether.

Zinc–protoporphyrin-chelatase activity in Rhodopseudomonas spheroides *grown under different conditions*

Activity of zinc–protoporphyrin chelatase was examined (Table 3) in lysed protoplasts from organisms grown at 'high' oxygen partial pressure in the dark (unpigmented), at a 'low' oxygen partial pressure in the dark (pigmented) and anaerobically in the light (pigmented). The enzyme activity in chromatophores from both sets of pigmented organisms was also tested. The enzyme activity in protoplasts and chromatophores from both types of pigmented organisms was the same, but that in protoplasts from unpigmented organisms was markedly lower.

The chromatophore preparations used in the experiments described in this paper are not pure and consist of at least two types of particles termed 'heavy' and 'light' by Cohen-Bazire & Kunisawa (1960). 'Pure' chromatophores have been prepared by Dr A. Gorchein in this Laboratory by sucrose-gradient centrifugation of crude 'chromatophores'. The 'light' pigmented particles accumulate in 0·66 M-sucrose and consist of spheres 400–800 Å in diameter (A. Gorchein, unpublished work; cf. Schachman, Pardee & Stanier, 1952). The activity of zinc–protoporphyrin chelatase in these particles was as high, on a protein basis, as that of 'crude' chromatophores.

Ferrochelatase in chromatophores

Ferrochelatase activity was detected in chromatophores and was 6·4 μm-moles/mg. of protein/hr. There was no activity in the absence of ferrous sulphate or of ascorbic acid or GSH. Chromatophores previously heated to 55° for 5 min. lost about 80 % of their activity. The assay was repeated in the presence of 0·2 ml. of ether, which increased the rate of the reaction from 6·4 to 19·7 μm-moles/mg. of protein/hr. With mesoporphyrin, deuteroporphyrin and haematoporphyrin there was a small disappearance of porphyrin in the absence of chromatophores but a considerably greater disappearance in their presence. With these porphyrins the en-

Table 3. *Activity of zinc–protoporphyrin chelatase in fractions of* Rhodopseudomonas spheroides *grown under different conditions*

Organisms were grown, and lysed protoplasts and 'crude' chromatophores were prepared as described in the Materials and Methods section. Pure chromatophores were prepared by Dr A. Gorchein by sucrose-gradient centrifugation (cf. Bull & Lascelles, 1963). Enzymic assay: tris, pH 8·4 (150 μmoles), protoporphyrin (50 μm-moles), $ZnCl_2$ (30 μm-moles) and enzyme source (0·5–3·0 mg. of protein), in a volume of 0·5 ml. plus 0·2 ml. of ether, were mixed thoroughly and incubated at 37° for 60 min. The porphyrins were isolated and purified and the amount of zinc protoporphyrin formed was estimated as described in the Materials and Methods section.

Expt.	Preparation	Growth conditions	Zinc protoporphyrin (μm-moles/mg. of protein/hr.)
1 (A)	Lysed protoplasts	High O_2, dark	2·8
	Lysed protoplasts	Anaerobic, light	7·3
1 (B)	Lysed protoplasts	Low O_2, dark	11·6
	Lysed protoplasts	Anaerobic, light	13·9
	Crude chromatophores	Low O_2, dark	22·0
	Crude chromatophores	Anaerobic, light	21·6
2	Crude chromatophores	Anaerobic, light	22·2
	Pure chromatophores	Anaerobic, light	18·0

Table 4. *Comparison of chelatase activities in mitochondria and chromatophores*

The assay conditions for zinc-protoporphyrin chelatase are those described in Table 3 and those for ferrochelatase are described in the Materials and Methods section. To the 0·5 ml. assay system 0·2 ml. of ether was added where stated. The rates of the reactions were calculated as described in the Materials and Methods section. —, Not determined.

	Production (μm-moles/mg. of protein/hr.)			
	Zinc–protoporphyrin chelatase		Ferrochelatase	
Enzyme source	Without ether	With ether	Without ether	With ether
Chromatophores	4·8	22·5	6·4	19·7
Guinea-pig liver mitochondria	1·9	18·3	4·4	10·7
Rabbit-liver mitochondria	—	2·5	1·8	3·2
Rabbit-kidney mitochondria	—	2·6	—	4·0
Rabbit-heart mitochondria	—	6·3	5·0	9·0

zymic activity was slightly less than with protoporphyrin. Coproporphyrin formed coprohaem non-enzymically and addition of chromatophores did not speed this reaction.

Inhibition of zinc–protoporphyrin chelatase in chromatophores by Fe^{2+} *ions and of ferrochelatase by* Zn^{2+} *ions*

Although Fe^{2+} ions do not inhibit zinc–protoporphyrin chelatase under the assay conditions described in Fig. 1, a marked inhibition is observed in the presence of 20 μmoles of ascorbic acid. In the presence of 12 μm-moles of zinc chloride, 15 μm-moles of ferrous sulphate inhibited by 50 %. Under these conditions only a small amount of haem was formed. When the reaction was carried out in the presence of different amounts of Zn^{2+} and Fe^{2+} ions the inhibition was competitive, and K_i for ferrous sulphate was 50 μM. The inhibition was much less marked in the presence of ether: for instance, 100 μm-moles of ferrous sulphate in the presence of 10 μm-moles of zinc chloride inhibited zinc protoporphyrin formation by 85 % in the absence of ether and by only 15 % in its presence. When ferrochelatase activity was assayed in the presence of ether Zn^{2+} ions were inhibitory. Zinc chloride (20 μm-moles) inhibited the reaction by about 30 % and 100 μm-moles by about 63 % in the presence of amounts of ferrous sulphate varying between 20 and 100 μm-moles. Thus inhibition by Zn^{2+} ions is non-competitive.

Zinc–protoporphyrin-chelatase and ferrochelatase activities in mitochondria

Tests for zinc–protoporphyrin-chelatase activity were carried out with tissues which are known to contain ferrochelatase, although previous work with rat-liver mitochondria (Labbe & Hubbard, 1961) and duck blood (Oyama *et al.* 1961) had indicated little or no zinc–protoporphyrin-chelatase activity. Both zinc–protoporphyrin-chelatase and ferrochelatase activities could be detected in mitochondria from guinea-pig liver and from rabbit liver, heart and kidney (Table 4). The activities were low in the absence of ether, but as with chromatophores they were markedly increased in the presence of ether.

When rabbit-liver mitochondria were preheated at 55° for 5 min. there was almost complete inactivation of ferrochelatase activity. The zinc–protoporphyrin-chelatase activity was not affected under these conditions but was reduced to 20 % after heating at 70° for 5 min.

Both enzyme activities could be completely extracted from guinea-pig liver and rabbit-liver mitochondria by freezing in the presence of Tween 20 (10 mg./ml.) followed by thawing and centrifuging at 36000*g* for 1 hr. (Labbe & Hubbard, 1961). After the preparation from rabbit-liver mitochondria had been stored for 2 weeks at −20° all the zinc–protoporphyrin-chelatase activity had disappeared, whereas the ferrochelatase was still almost as active as before. This finding, together with the difference in heat inactivation, is evidence that in rabbit-liver mitochondria two different enzymes catalyse the two reactions, i.e. incorporation of Zn^{2+} and Fe^{2+} ions into protoporphyrin.

DISCUSSION

It is proposed that the incorporation of zinc into protoporphyrin, which is demonstrated in the present experiments, like that of the incorporation of ferrous iron, is an enzyme-catalysed reaction. The evidence is that the activity is heat-labile, that it has a heat of activation of 14800 cal./mole, which is similar to that for some other enzyme reactions, that it has a pH optimum, and that it is specific for the metal.

Although the formation of zinc protoporphyrin and haem is catalysed by the same extracts, our evidence indicates that two different enzymes are responsible.

(1) In both chromatophores and mitochondria zinc protoporphyrin is formed in the presence and

the absence of ascorbic acid, whereas haem is formed only in its presence.

(2) GSH inhibits the biosynthesis of zinc protoporphyrin but not that of haem.

(3) Extracts prepared from mitochondria by treatment with Tween 20 lose their zinc–protoporphyrin-chelatase activity but not their ferrochelatase activity after storage at −20° for about 14 days.

(4) With chromatophores Fe^{2+} ions competitively inhibit zinc protoporphyrin formation, whereas Zn^{2+} ions are a non-competitive inhibitor of ferrochelatase.

Final proof for the existence of two enzymes will, however, have to await further purification.

The marked activity of zinc–protoporphyrin chelatase demonstrated, in the present study, in a number of animal tissues is in contrast with the rather low incorporation of zinc into protoporphyrin reported by previous workers. These differences may be due to the fact that they used Tween in the preparation of the enzyme and GSH or cysteine in its assay. These agents have been found, in the present work, to destroy or inhibit zinc–protoporphyrin-chelatase activity respectively. Moreover none of the previous workers incorporated ether into their incubation mixtures.

The enzyme in chromatophores is specific for Zn^{2+} ions; no other metalloporphyrins were formed when the assay was performed in the absence of ascorbic acid. Under these conditions only Co^{2+}, Mn^{2+} and Cu^{2+} ions, and to a lesser extent Ca^{2+} ions, were inhibitory.

Many porphyrins rapidly form complexes with zinc in the absence of an enzyme. With mesoporphyrin and Zn^{2+} ions both tris buffer and ether are required, with deuteroporphyrin only tris buffer is necessary, and with coproporphyrin and haematoporphyrin neither tris nor ether needs to be present for complex-formation. Zinc mesoporphyrin and zinc deuteroporphyrin can also be formed in the absence of tris buffer but in the presence of chromatophores. It is possible that the different behaviour of protoporphyrin as compared with that of the other porphyrins is connected with a more extensive resonance that can occur in the former porphyrin, which contains two vinyl groups.

The reason for the stimulation of zinc–protoporphyrin-chelatase activity by ether may be that the enzyme is a lipoprotein or the enzyme may be buried in the chromatophores and mitochondria, both of which have a high content of lipids. The involvement of lipid is also shown by the finding that the enzyme, along with the ferrochelatase, can be extracted from mitochondria by treatment with Tween 20. Ether may also be acting to transfer protoporphyrin and Zn^{2+} ions into a lipophilic phase. Protoporphyrin is ether-soluble and Ward & Fantl (1963) have shown that a number of hydrophilic cations can be transferred from an aqueous to a lipophilic phase by shaking a solution of the cation salt with phosphatidic acid and ether.

The finding of an enzyme catalysing the formation of zinc protoporphyrin raises the problems of its function and of the role of the product of the reaction. Although much work has been done on the metabolism of zinc in animals, plants and micro-organisms and the importance of zinc as a component of a number of enzymes is known (cf. Vallee, 1959), there is very little evidence for any connexion between the metabolism of zinc and that of porphyrin. Zinc uroporphyrin and zinc coproporphyrin have been identified in the urine of patients with acute porphyria (Watson & Schwartz, 1941) and in culture filtrates of *Corynebacterium diphtheriae* (Coulter & Stone, 1938). Such complexes can, however, be formed non-enzymically (Heikel, Lockwood & Rimington, 1958; and the present paper). Zinc deficiency in plants is known to interfere with chlorophyll synthesis (Vallee, 1959), but the exact role of zinc has not been elucidated. Zinc has also been implicated in photosynthesis in *Chlorella* (Schwartz, 1956). Feeding excess of zinc to rats resulted in retarded growth and also gave rise to a microcytic hypochromic anaemia (Smith & Larson, 1946). A mixture of iron, copper and cobalt raised the haemoglobin to normal amounts. The anaemia could be caused by zinc inhibiting the biosynthesis of haem as has been shown in this work with chromatophores. Dancewicz & Malinowska (1958) reported that zinc protoporphyrin dimethyl ester, but not the methyl esters of protoporphyrin, copper protoporphyrin or cobalt protoporphyrin, at concentrations of between 3 and 100 μm-moles/ml., caused a 19% increase in oxygen consumption by rat-liver homogenates. These results do not give much insight into the possible functions of zinc protoporphyrin. Extensive experiments carried out in this Laboratory with cell-free extracts have failed to provide any evidence that, in *R. spheroides*, zinc protoporphyrin is an intermediate in the biosynthesis of bacteriochlorophyll.

SUMMARY

1. An enzyme, zinc–protoporphyrin chelatase, has been detected in chromatophores from *Rhodopseudomonas spheroides*. It catalyses the formation of zinc protoporphyrin from zinc chloride and protoporphyrin.

2. Zinc–protoporphyrin chelatases are also present in mitochondria from guinea-pig liver and from rabbit liver, heart and kidney.

3. The rate of the reaction is about five times as high in a water–ether emulsion as in an aqueous medium. Methanol and Tween 80 also stimulate,

but to a smaller extent. The possible significance of these findings is discussed.

4. The enzyme from *R. spheroides* is specific for Zn^{2+} ions. Of the salts tested as inhibitors only those with Cu^{2+}, Co^{2+}, Mn^{2+} and Ca^{2+} ions are active.

5. When ascorbic acid is present in the assay mixture ferrochelatase activity can be demonstrated in all the above extracts. Under these conditions Fe^{2+} ions competitively inhibit zinc protoporphyrin formation and Zn^{2+} ions are a non-competitive inhibitor of haem formation.

6. The specificity of the zinc–protoporphyrin chelatase towards other porphyrins has been tested. Unlike protoporphyrin some porphyrins readily form a zinc complex in the absence of enzyme.

REFERENCES

Bray, G. A. (1960). *Analyt. Biochem.* **1**, 279.

Bull, M. J. & Lascelles, J. (1963). *Biochem. J.* **87**, 15.

Cohen-Bazire, G. & Kunisawa, R. (1960). *Proc. nat. Acad. Sci., Wash.*, **46**, 1543.

Coulter, C. B. & Stone, F. M. (1938). *Proc. Soc. exp. Biol., N.Y.*, **38**, 423.

Dancewicz, A. M. & Malinowska, T. (1958). *Postępy Hig. Med. dośw.* **12**, 447; cited from *Chem. Abstr.* (1962), **56**, 4046h.

Dresel, E. I. B. & Falk, J. E. (1956). *Biochem. J.* **63**, 388.

Fischer, H. & Pützer, B. (1926). *Hoppe-Seyl. Z.* **154**, 62.

Gibson, K. D., Neuberger, A. & Tait, G. H. (1962). *Biochem. J.* **83**, 539.

Gibson, K. D., Neuberger, A. & Tait, G. H. (1963). *Biochem. J.* **88**, 325.

Granick, S. (1961). *J. biol. Chem.* **236**, 1168.

Heikel, T., Lockwood, W. H. & Rimington, C. (1958). *Nature, Lond.*, **182**, 313.

Hogeboom, G. H. (1955). In *Methods in Enzymology*, vol. 1, p. 16. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.

Karunairatnum, M. C., Spizizen, J. & Gest, H. (1958). *Biochim. biophys. Acta*, **29**, 649.

Labbe, R. F. & Hubbard, N. (1961). *Biochim. biophys. Acta*, **52**, 130.

Lascelles, J. (1956). *Biochem. J.* **62**, 78.

Lascelles, J. (1959). *Biochem. J.* **72**, 508.

Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951). *J. biol. Chem.* **193**, 265.

Oyama, H., Sugita, Y., Yoneyama, Y. & Yoshikaay, H. (1961). *Biochim. biophys. Acta*, **47**, 413.

Porra, R. J. & Jones, O. T. G. (1963*a*). *Biochem. J.* **87**, 181.

Porra, R. J. & Jones, O. T. G. (1963*b*). *Biochem. J.* **87**, 186.

Schachman, H. K., Pardee, A. B. & Stanier, R. Y. (1952). *Arch. Biochem. Biophys.* **38**, 245.

Schwartz, H. C., Hill, R. L., Cartwright, G. E. & Wintrobe, M. M. (1959). *Fed. Proc.* **18**, 545.

Schwartz, M. (1956). *Biochim. biophys. Acta*, **22**, 463.

Schwartz, S., Berg, M. H., Bossenmaier, I. & Dinsmore, H. (1960). *Meth. biochem. Anal.* **8**, 221.

Smith, S. E. & Larson, E. J. (1946). *J. biol. Chem.* **163**, 29.

Vallee, B. L. (1959). *Physiol. Rev.* **39**, 443.

Ward, H. A. & Fantl, P. (1963). *Arch. Biochem. Biophys.* **100**, 338.

Watson, C. J. & Schwartz, S. (1941). *J. clin. Invest.* **20**, 440.

Yoneyama, Y., Oyama, H., Sugita, Y. & Yoshikaya, H. (1962). *Biochim. biophys. Acta*, **62**, 261.

Biochem. J. (1964) **90**, 616

The Mechanism of Carbohydrase Action

10. ENZYMIC SYNTHESIS AND PROPERTIES OF 6-α-MALTOSYLGLUCOSE*

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The main polymeric linkages of amylopectin and glycogen are the chain-forming α-(1→4)-bond and the branch-forming α-(1→6)-bond. Partial acid hydrolysis of the polysaccharides yields glucose, maltose and isomaltose. Of the expected four trisaccharides [maltotriose, 4-α-isomaltosylglucose (panose), 6-α-maltosylglucose and 4,6-di-α-glucosylglucose], only maltotriose and panose have been isolated despite a thorough search for the isomers of panose (Edwards, 1955; see also Whelan, 1958). Isomaltotriose would also be formed if two (1→6)-bonds were in juxtaposition and was indeed isolated from glycogen by Wolfrom & Thompson (1957). The question therefore arises whether the trisaccharides not detected are not formed, or are particularly labile to acid and so do not accumulate. A partial solution to the problem would be obtained by examination of the trisaccharides in question. Summer & French (1956) indicated that these compounds, 6-α-maltosylglucose and 4,6-di-α-glucosylglucose, could be synthesized by the coupling reaction between cyclomaltohexaose (Schardinger α-dextrin) and isomaltose, catalysed

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