Ferrochelatase of *Rhodopseudomonas spheroides*

By M. S. JONES AND O. T. G. JONES

Department of Biochemistry, University of Bristol, Bristol BS8 1TD, U.K.

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Extracts of *Rhodopseudomonas spheroides* contain two ferrochelatases: one is soluble and forms metalloporphyrins from deuteroporphyrin and haematoporphyrin; the other is particulate and forms metalloporphyrins from protoporphyrin, mesoporphyrin, deuteroporphyrin and haematoporphyrin. Neither enzyme incorporates Mg^{2+} into porphyrins or Fe^{2+} into porphyrin cytochrome *c*. By using the particulate enzyme, plots of $1/v$ versus $1/s$ when one substrate was varied and the other kept constant showed that neither substrate affected the K_m of the other. The suggested sequential mechanism for the reaction is supported by derivative plots of slopes and intercepts. The K_m for deuteroporphyrin was $21.3 \mu M$ and that for Co^{2+} was $6.13 \mu M$. The enzyme incorporated Co^{2+} , Fe^{2+} , Zn^{2+} , Ni^{2+} and Mn^{2+} ; Cd^{2+} was not incorporated and was an inhibitor, competitive with respect to Co^{2+} , non-competitive with respect to deuteroporphyrin. The K_i for Cd^{2+} was $0.73 \mu M$. Ferrochelatase was inhibited by protohaem, non-competitively with respect to Co^{2+} or with respect to deuteroporphyrin. Inhibition by magnesium protoporphyrin was non-competitive with respect to deuteroporphyrin, uncompetitive with respect to Co^{2+} . The inhibitory concentrations of the metalloporphyrins are lower than those required for the inhibition of δ -aminolaevulinate synthetase by protohaem. Fe^{3+} is not incorporated aerobically into porphyrins unless an electron donor, succinate or NADH, is supplied; the low aerobic rate of metalloporphyrin synthesis obtained is insensitive to rotenone and antimycin. The rate of Fe^{3+} incorporation increases as anaerobic conditions are achieved.

Ferrochelatase (protohaem ferro-lyase, EC 4.99.1.1) catalyses the insertion of Fe^{2+} ions into porphyrins to form haems. It has been found not only in erythropoietic tissues such as bone marrow (Kasagar, Konno & Sakai, 1954), reticulocytes (Clark & Walsh, 1959) and avian erythrocytes (Krueger, Melnick & Klein, 1956), but also in yeast and bacteria (Porra & Jones, 1963b), where it presumably functions in the biosynthesis of cytochrome prosthetic groups. More recently this enzyme has been found in isolated chloroplasts of higher plants (Jones, 1968) and in proplastids and plant mitochondria (Porra & Lascelles, 1968). In all these cases the enzyme appears to be associated with a particulate fraction of the cell, but Johnson & Jones (1964) reported that in the photosynthetic bacterium *Rhodopseudomonas spheroides* a soluble ferrochelatase was present that differed in porphyrin specificity from the ferrochelatase found in the particulate chromatophore fraction present in this organism. We have examined the ferrochelatase of *R. spheroides* in some detail, not only because the existence of two ferrochelatases may be important in the formation of magnesium porphyrins and hence chlorophylls or in the forma-

tion of the cytochromes *c* or *a*, which do not have protohaem prosthetic groups, but also because the enzyme from this source is particularly active in our spectrophotometric assay system (Jones & Jones, 1969) and so is well suited to the kinetic studies we describe in this paper. The ferrochelatase of chloroplasts, although of equal importance in its position at the branch point of haem and chlorophyll synthesis, is difficult to assay with precision because of interference by carotenoid and chlorophyll pigments. In *R. spheroides* these problems can be overcome by using a carotenoid-less mutant.

EXPERIMENTAL

Organism. The blue-green mutant of *Rhodopseudomonas spheroides* (strain R.22, a gift from Dr W. R. Sistrom) was grown anaerobically in the light, harvested and washed as described previously (Jones, 1969).

Preparation of cell-free extracts. Cell suspensions in 100 mM-tris-HCl buffer, pH 7.5, were disrupted by ultrasonic treatment at maximum power for 2 min at $0^\circ C$ by using a Dawes Soniprobe (100 W). The suspension was centrifuged at 25000g for 10 min to remove unbroken cells and debris and the supernatant was recentrifuged

at 100 000g for 1 h. The high-speed supernatant was retained and the particles were washed once by resuspension in 100 mM-tris-HCl buffer, pH 7.5, followed by centrifugation at 100 000g for 1 h; the washed particles were resuspended in the same buffer. The ferrochelatase in the washed particles was solubilized by stirring particles in 0.3% sodium deoxycholate for 30 min and then taking the supernatant after centrifuging at 100 000g for 1 h.

Ferrochelatase assay. Details of the dual-wavelength spectrophotometric assay have been given elsewhere (Jones & Jones, 1969). Although the natural substrate for ferrochelatase is presumed to be Fe^{2+} , the ready autoxidizability of this material at the alkaline pH optimum of the enzyme (Jones, 1968) makes its use in kinetic experiments inconvenient and we have used Co^{2+} as the metal substrate as a routine. The formation of cobalt deuteroporphyrin, and that of cobalt haematoporphyrin, was measured by using the wavelength pair 498 nm minus 510.5 nm (ϵ_{mM} 10.00).

Rates of non-enzymic metalloporphyrin formation were negligible in comparison with enzymic rates.

Enzyme kinetics. Lines were fitted to experimental points and kinetic constants calculated by using a computer program modified by Dr P. J. England of this Department from Cleland (1963c).

δ -Aminolaevulinate synthetase. This was assayed by the method of Lascelles (1960). Glutathione was omitted from the mixture and the incubation time was 30 min. Cells were disrupted by ultrasonic treatment as described previously, the suspension was centrifuged at 20 000g for 10 min, and the supernatant was allowed to stand at 0°C for 2 h to allow spontaneous activation of the enzyme to occur (Marriott, Neuberger & Tait, 1969).

Protohaem and magnesium protoporphyrin micelles. Phosphatidylcholine was dissolved in an ether solution of the metalloporphyrin, the ether evaporated to dryness and the residue suspended in tris-HCl buffer as described by Jones & Jones (1969) for porphyrin micelle preparation.

Chemicals. Crystalline protohaem was prepared from whole blood by the method of Labbe & Nishida (1957); magnesium protoporphyrin dimethyl ester (a gift from Dr R. J. Porra) was hydrolysed to the free acid in methanolic 1% (w/v) KOH (Falk, 1964); no free protoporphyrin could be detected in it by absorption spectroscopy. Mesoporphyrin IX, deuteroporphyrin IX and haematoporphyrin IX were obtained from Koch-Light Laboratories Ltd., Colnbrook, Bucks., U.K. Deuterohaem, mesohaem, protoporphyrin and cobalt deuteroporphyrin were prepared as described by Falk (1964). Approx. 1 mM solutions of porphyrin were prepared in 2% (v/v) Tween 80 in 40 mM-tris-HCl buffer, pH 8.2. The porphyrin concentrations of the solutions were calculated from the intensity of the Soret maximum in acid solution (Falk, 1964); protohaem concentration was calculated after preparing an alkaline pyridine haemochrome and measuring the reduced-minus-oxidized spectrum (cf. Porra & Jones, 1963a); magnesium protoporphyrin concentration was determined from measurement of the extinction at 408 nm in 2.7 M-HCl (Falk, 1964) after its conversion into protoporphyrin. Porphyrin cytochrome *c* was prepared by removal of iron from mammalian cytochrome *c* by a method suggested to us by Mr R. V. Belcher. Cytochrome *c* (10 mg) was dissolved in 0.5 ml of pyridine. Acetic acid (9.5 ml) was added and the solution

Fig. 1. Visible-absorption spectrum of porphyrin cytochrome *c*. Porphyrin cytochrome *c* was prepared as described in the Experimental section. After being freeze-dried, the protein was dissolved in water and its spectrum recorded. Absorption maxima were at 504, 538, 567 and 620 nm.

gassed with N_2 for 5 min. A saturated solution of FeSO_4 in conc. HCl (0.4 ml) was added and gassing with N_2 continued for 10 min. Ethanol (10 ml) and water (10 ml) were added and the mixture was neutralized with Na_2CO_3 . The mixture was added to a column of Sephadex G-25 and eluted in water. The resulting purple fluorescent protein was concentrated by freeze-drying. The visible-absorption spectrum is given in Fig. 1. The addition of sodium dithionite caused no change in this spectrum, indicating that no unchanged cytochrome *c* remained.

Protein. Concentration of protein in extracts was measured by the method of Lowry, Rosebrough, Farr & Randall (1951).

RESULTS

Existence of more than one ferrochelatase in *R. spheroides*. After the 250 000g supernatant from the sonicated *R. spheroides* suspension was centrifuged at 100 000g for 60 min, three fractions were obtained in the centrifuge tube: S1, a clear straw-coloured upper layer; S2, a blue-green middle layer containing small membrane particles; and the particulate fraction that formed the pellet. The S1, S2 and pellet fractions were assayed for ferrochelatase activity, by using several different porphyrin substrates. The porphyrin specificities of these fractions were different (Table 1). Activity with protoporphyrin as substrate was present only in the particulate fraction, whereas the S1 fraction was active only with haematoporphyrin and deuteroporphyrin. Of the four porphyrins used in making metalloporphyrins, protoporphyrin is the only natural substrate that directly yields the prosthetic group of a natural haemoprotein. It therefore appeared possible that the activity in the

Table 1. *Porphyrin specificities of soluble and particulate ferrochelatase activities in R. spheroides*

Fractions were prepared as described in the Experimental section. Samples of each fraction (1–10 mg of protein) and porphyrin (100 nmol) in 2.5 ml of 100 mM-tris-HCl buffer, pH 8.2, containing 0.1% Tween 80, were incubated in the dual-wavelength spectrophotometer at 37°C. The $\Delta(E_{\max} - E_1)$ was recorded after addition of Co^{2+} to a final concentration of 40 μM and the rates of metal ion incorporation were calculated by using extinction coefficients determined as described by Jones & Jones (1969). Specific activities of ferrochelatase are expressed as nmol of metalloporphyrin formed/min per mg of protein.

Fraction	Ferrochelatase specific activities with different porphyrin substrates				
	Haemato- porphyrin	Deutero- porphyrin	Mesoporphyrin	Proto- porphyrin	Porphyrin cytochrome c
Sonicated cells	1.04	3.58	0.375	0.231	0.0
Supernatant (S1)	0.41	0.102	0.0	0.0	0.0
Middle layer (S2)	0.635	0.716	0.105	0.0	0.0
Pellet	0.885	7.0	0.983	0.983	0.0

soluble fractions was normally connected with the formation of other metalloporphyrins such as magnesium protoporphyrin or cytochrome c. In repeated experiments in varied conditions none of the fractions was active in inserting magnesium into porphyrins or in inserting iron into porphyrin cytochrome c. Magnesium incorporation was attempted by using the free Mg^{2+} ion as substrate or, in a lipid-soluble form, complexed with cardiolipin or as a water-soluble complex with ATP. Porphyrins were provided in phospholipid micelles, in detergent or in colloidal suspension at pH 8.0. These negative results led us to examine the possibility that the activity in the soluble fractions was an artifact produced by partial denaturation of the particulate ferrochelatase on sonication. The particulate fraction was further sonicated and the chelatase activities of the supernatant and pellet fractions, obtained after centrifuging, were again assayed. No further release of haemato-porphyrin-specific activity into the soluble fraction was found, and the pattern of porphyrin specificity of the particulate ferrochelatase was also unchanged when this enzyme was solubilized by treatment with deoxycholate.

Because of the uncertainty about the natural function, if any, of the enzyme in the supernatant fractions we concentrated our further attention on the properties of the particulate enzyme.

Kinetic properties of the particulate ferrochelatase. In an attempt to distinguish between the possible reaction mechanisms for ferrochelatase, the effects of varying the concentration of each substrate while the other was kept constant were examined (Figs. 2 and 3). The lack of effect of one substrate on the K_m for the other suggests that the reaction proceeds by a sequential mechanism, with both substrates bound before product release (Cleland, 1963a). This conclusion is supported by the plots

Fig. 2. Double-reciprocal plots of initial reaction velocity against Co^{2+} concentration for various concentrations of deuteroporphyrin. Deuteroporphyrin and solubilized ferrochelatase preparation (0.5–2.0 mg of protein) in 2.5 ml of 100 mM-tris-HCl buffer, pH 8.2, containing 0.1% Tween 80, were incubated in the dual-wavelength spectrophotometer at 37°C. The $\Delta(E_{498} - E_{510.5})$ was recorded after the addition of Co^{2+} , and the rates of metal ion incorporation were calculated by using ϵ_{nm} 10.0 (Jones & Jones, 1969). Deuteroporphyrin concentrations were 4 μM ($\circ$), 6 μM ($\square$), 10 μM ($\triangle$) and 20 μM ($\bullet$).

(Dalziel, 1957; Florini & Vestling, 1957) shown in Figs. 4 and 5, where intercepts, e.g.:

$$\frac{1}{V} + \frac{K_m(\text{Co}^{2+})}{[\text{Co}^{2+}] \cdot V}$$

and slopes, e.g.:

$$\frac{K_m(\text{Co}^{2+})}{V} + \frac{K_i(\text{Co}^{2+}) \cdot K_m(\text{porphyrin})}{[\text{Porphyrin}] \cdot V}$$

derived from Figs. 2 and 3 were plotted against the reciprocal of substrate concentration. The secondary plots of slopes gave identical slopes, and the secondary plots of intercepts had the same vertical

Fig. 3. Double-reciprocal plots of initial reaction velocity against deuteroporphyrin concentration for various concentrations of Co^{2+} . Conditions for determination of rates of metal ion incorporation were as indicated in Fig. 2. Co^{2+} concentrations were $2.5 \mu\text{M}$ (○), $3.5 \mu\text{M}$ (□), $5 \mu\text{M}$ (△), $7.5 \mu\text{M}$ (●), $12.5 \mu\text{M}$ (■) and $25 \mu\text{M}$ (▲).

Fig. 4. Plots of the values for the vertical intercepts derived from Figs. 2 and 3 against the reciprocal of the fixed substrate concentration. Intercepts from Fig. 2 are plotted against the reciprocal of the deuteroporphyrin concentration (○); intercepts from Fig. 3 are plotted against the reciprocal of the Co^{2+} concentration (□).

intercept. These results also made it possible to determine very accurately the K_m for Co^{2+} ($6.13 \mu\text{M}$) and for deuteroporphyrin ($21.3 \mu\text{M}$). The lower rates

Fig. 5. Plots of the values for the slopes derived from Figs. 2 and 3 against the reciprocal of the fixed substrate concentration. Slopes from Fig. 2 are plotted against the reciprocal of the deuteroporphyrin concentration (○); slopes from Fig. 3 are plotted against the reciprocal of the Co^{2+} concentration (□).

Table 2. *Metal ion specificity of ferrochelatase from the particulate fraction of R. spheroides*

Deuteroporphyrin (100 nmol) and particulate fraction (3–15 mg of protein) in 2.5 ml of 100 mM-tris-HCl buffer, pH 8.2, containing 0.1% Tween 80, were incubated in the dual-wavelength spectrophotometer. The $\Delta(E_{498} - E_{510.5})$ was recorded after addition of various metal ions to the concentrations shown. Rates of metal ion incorporation were calculated by using extinction coefficients determined as described by Jones & Jones (1969).

Metal ion	Specific activity (nmol/min per mg of protein)
Fe^{2+} ($40 \mu\text{M}$)	4.900
Co^{2+} ($40 \mu\text{M}$)	4.900
Zn^{2+} ($5 \mu\text{M}$)	3.020
Cu^{2+} ($40 \mu\text{M}$)	0.246
Mn^{2+} ($200 \mu\text{M}$)	0.109
Ni^{2+} ($120 \mu\text{M}$)	0.084

obtained with protoporphyrin did not permit such extensive kinetic studies.

Metal ion specificity of the particulate ferrochelatase. The particulate ferrochelatase of *R. spheroides* is not specific for Co^{2+} or Fe^{2+} ; activity with Zn^{2+} (Johnson & Jones, 1964; Neuberger & Tait, 1964) and also with Cu^{2+} (Jones, 1967) had previously been described. We have confirmed these reports and have also found low rates of activity with Ni^{2+} and Mn^{2+} (Table 2). These metals were all mutually competitive for ferrochelatase. Care had to be taken to test metal substrates over a wide concentration range; high

Fig. 6. Effect of high concentrations of Co^{2+} and Zn^{2+} on particulate ferrochelatase from *R. spheroides*. Deuteroporphyrin (100 nmol) and particulate fraction (2–4 mg of protein) in 2.5 ml of 100 mM-tris-HCl buffer, pH 8.2, containing 0.1% Tween 80, were incubated in the dual-wavelength spectrophotometer at 37°C. The $\Delta(E_{498} - E_{510.5})$ was recorded after addition of Co^{2+} (○) or Zn^{2+} (□) to the concentrations shown. Rates of metal ion incorporation were calculated by using ϵ_{max} 10.0.

Fig. 8. Double-reciprocal plots of initial reaction velocity against deuteroporphyrin concentration to show the inhibition by Cd^{2+} of ferrochelatase from the particulate fraction of *R. spheroides*. Conditions for determination of rates of cobalt deuteroporphyrin formation were as described in Fig. 2. The concentration of Co^{2+} was 12.5 μM . The concentrations of Cd^{2+} were 7.0 μM (○), 3.5 μM (□), 1.75 μM (Δ) and 0.70 μM (●); ■, control with no added Cd^{2+} .

Fig. 7. Double-reciprocal plots of initial reaction velocity against Co^{2+} concentration to show the inhibition by Cd^{2+} of ferrochelatase from the particulate fraction of *R. spheroides*. Conditions for the determination of rates of cobalt deuteroporphyrin formation were as described in Fig. 2. The concentration of deuteroporphyrin was 20 μM . The concentrations of Cd^{2+} were 7.0 μM (○), 3.5 μM (□), 1.4 μM (Δ), 0.7 μM (●) and 0.35 μM (■); ▲, control with no added Cd^{2+} .

concentrations of Zn^{2+} but not of Co^{2+} were inhibitory to the enzyme from *R. spheroides* (Fig. 6) and the enzyme differed in this from the mito-

chondrial enzyme, where high concentrations of Co^{2+} but not of Zn^{2+} were inhibitory (M. S. Jones & O. T. G. Jones, unpublished work). Fe^{3+} , Mg^{2+} and Sr^{2+} were not incorporated and were not inhibitors of the chelatase action, but Cd^{2+} , which also was not incorporated, inhibited the incorporation of other metals. Double-reciprocal plots (Figs. 7 and 8) show that this inhibition is competitive with the metal substrate but not with the porphyrin (Cleland, 1963b). The K_i for Cd^{2+} determined from the intercepts on the abscissa in Fig. 7 was 0.73 μM .

Effect of metalloporphyrins on ferrochelatase. Preliminary studies had shown that the rate of incorporation of Fe^{2+} into porphyrins catalysed by *R. spheroides* extracts was not linear with time: the rate tended to diminish, possibly owing to product inhibition of the ferrochelatase. This effect was not found with the other metals used as substrates. Further investigation with protohaem micelles or in alkaline solution showed that ferrochelatase was severely inhibited by relatively low concentrations of protohaem when deuteroporphyrin was substrate (Fig. 9a) and similar results were obtained with protoporphyrin as substrate. The concentration of protohaem that was 50% inhibitory (10 μM) was a little lower than that found to inhibit aminolaevulinate synthetase by 50% in our extracts (24 μM). Magnesium protoporphyrin

Fig. 9. (a) Inhibition of ferrochelatase activity by protohaem. Deuteroporphyrin (100nmol) and *R. spheroides* particulate fraction (2–4mg of protein) in 2.5 ml of 100mM-tris-HCl buffer, pH 8.2, containing 0.1% Tween 80, were incubated in the dual-wavelength spectrophotometer at 37°C. The $\Delta(E_{498} - E_{510.5})$ was recorded after the addition of Co^{2+} to 40 μM and the rates of metal ion incorporation were calculated by using ϵ_{mM} 10.0. Protohaem was included in the assay mixture at the concentrations shown. (b) Inhibition of ferrochelatase activity by magnesium protoporphyrin. Conditions for the determination of rates of cobalt deuteroporphyrin formation were as described in (a). Magnesium protoporphyrin was included in the assay mixture at the concentrations shown.

Table 3. *Effect of various metalloporphyrins on ferrochelatase activity*

Conditions for the determination of rates of cobalt deuteroporphyrin formation were as described in Fig. 9. The metalloporphyrins shown were included in the assay mixture at a concentration of 20 μM .

Addition to assay mixture	Inhibition of enzyme activity at 20 μM (%)
Protohaem	67
Magnesium protoporphyrin	75
Deuterohaem	15
Mesohaem	20
Cobalt deuteroporphyrin	22
Cytochrome c	0
Haemoglobin	0
Magnesium protoporphyrin dimethyl ester	5
Protoporphyrin dimethyl ester	0

was found to be a potent inhibitor of metal incorporation into deuteroporphyrin (Fig. 9b) or protoporphyrin, with 50% inhibition occurring at about 4 μM . At higher concentrations of magnesium protoporphyrin (80 μM) there was almost complete inhibition of activity. Other metalloporphyrins examined (Table 3) were found to be less inhibitory when compared at concentrations of 20 μM . The dimethyl ester of magnesium protoporphyrin was ineffective.

We have studied the inhibitions by magnesium

Fig. 10. Double-reciprocal plots of initial reaction velocity against deuteroporphyrin concentration to show the inhibition by protohaem of ferrochelatase from the particulate fraction of *R. spheroides*. Conditions for determination of rates of cobalt deuteroporphyrin formation were as described in Fig. 2. The concentration of Co^{2+} was 12.5 μM . The concentrations of protohaem were 29.0 μM ($\circ$), 20.3 μM ($\square$), 11.6 μM ($\triangle$) and 5.8 μM ($\bullet$); $\blacksquare$, control with no added protohaem.

protoporphyrin and iron protoporphyrin in some detail, and have shown by standard reciprocal plots (Figs. 10 and 11) that the inhibition by protohaem is non-competitive with respect to porphyrin and non-competitive with respect to Co^{2+} . The interpretation of the inhibition of cobalt

Fig. 11. Double-reciprocal plots of initial reaction velocity against Co^{2+} concentration to show the inhibition by protohaem of ferrochelatase from the particulate fraction of *R. spheroides*. Conditions for determination of rates of cobalt deuteroporphyrin formation were as described in Fig. 2. The concentration of deuteroporphyrin was $19 \mu\text{M}$. The concentrations of protohaem were $46.4 \mu\text{M}$ ($\circ$), $23.2 \mu\text{M}$ ($\square$), $11.6 \mu\text{M}$ ($\triangle$) and $5.8 \mu\text{M}$ ($\bullet$); $\blacksquare$, control with no added protohaem.

Fig. 12. Double-reciprocal plots of initial reaction velocity against deuteroporphyrin concentration to show the inhibition by magnesium protoporphyrin of ferrochelatase from the particulate fraction of *R. spheroides*. Conditions for determination of rates of cobalt deuteroporphyrin formation were as described in Fig. 2. The concentration of Co^{2+} was $12.5 \mu\text{M}$. The concentrations of magnesium protoporphyrin were $8.15 \mu\text{M}$ ($\circ$), $4.89 \mu\text{M}$ ($\square$), $3.26 \mu\text{M}$ ($\triangle$) and $1.63 \mu\text{M}$ ($\bullet$); $\blacksquare$, control with no added magnesium protoporphyrin.

deuteroporphyrin formation by protohaem is less certain than would be the case if it were true product inhibition. It may, however, be due to ferrochelatase having an ordered mechanism (Cleland, 1963b). The inhibition by magnesium

Fig. 13. Double-reciprocal plots of initial reaction velocity against Co^{2+} concentration to show the inhibition by magnesium protoporphyrin of ferrochelatase from the particulate fraction of *R. spheroides*. Conditions for the determination of rates of cobalt deuteroporphyrin formation were as described in Fig. 2. The concentration of deuteroporphyrin was $19 \mu\text{M}$. The concentrations of magnesium protoporphyrin were $9.78 \mu\text{M}$ ($\circ$), $6.52 \mu\text{M}$ ($\square$) and $4.08 \mu\text{M}$ ($\triangle$); $\bullet$, control with no added magnesium protoporphyrin.

protoporphyrin is more complex (Figs. 12 and 13); it is non-competitive with respect to porphyrin and apparently uncompetitive with respect to Co^{2+} . The minimal interpretation is that the inhibition by magnesium protoporphyrin is of a completely different character from that by protohaem. The K_i values derived from the intercepts of plots of $1/v$ versus $1/s$ are given in Table 4.

*Availability of Fe^{3+} for haem synthesis in particulate preparations of *R. spheroides*.* Since Fe^{2+} is readily oxidized in aqueous solution it appears unlikely that much of this form of iron is available free within the cell, particularly when *R. spheroides* is growing aerobically. We have found, however, that particulate preparations are able to utilize Fe^{3+} if an oxidizable substrate such as succinate or NADH is provided. Presumably Fe^{3+} then acts as an electron acceptor. If Fe^{3+} is added to a particulate preparation containing porphyrin, no haem synthesis occurs until succinate or NADH is added (Fig. 14, trace b). A low rate of haem synthesis is then observed, even under aerobic conditions. The point at which the cuvette becomes anaerobic is indicated by a sudden dip in the trace, due, we believe, to the 'tail' of the spectroscopic change caused by a cytochrome reduction. This dip was found at the same interval after substrate addition in the absence of porphyrin and metal and coincided

Table 4. Constants for the inhibition of ferrochelatase by protohaem and magnesium protoporphyrin

Values were derived from data for the plots shown in Figs. 10–13 by using a computer program based on that of Cleland (1963c). Conditions for the determination of rates of cobalt deuteroporphyrin formation were as described in Fig. 2.

Inhibitor	Variable substrate	Fixed substrate	Type of inhibition	K_i (μM)
Protohaem	Deuteroporphyrin	Co^{2+} ($12.5\mu\text{M}$)	Non-competitive	15.1
Protohaem	Co^{2+}	Deuteroporphyrin ($19\mu\text{M}$)	Non-competitive	26.9
Magnesium protoporphyrin	Deuteroporphyrin	Co^{2+} ($12.5\mu\text{M}$)	Mixed	3.9 (slope), 9.1 (intercept)
Magnesium protoporphyrin	Co^{2+}	Deuteroporphyrin ($19\mu\text{M}$)	Uncompetitive	6.5

Fig. 14. Availability of reduced iron for haem synthesis. *R. spheroides* particulate fraction (6mg of protein) in 2.5ml of 100mM-tris-HCl buffer, pH 8.2, containing 0.1% Tween 80, was incubated in the dual-wavelength spectrophotometer at 37°C. In (a) and (b) deuteroporphyrin (100nmol) was included in the assay mixture. Haem formation (a and b) or cytochrome reduction (c) was recorded at the wavelengths shown and additions were made as indicated. The final concentrations of added compounds were: Fe^{3+} , $80\mu\text{M}$; succinate, 8mM ; sodium azide, 3mM ; rotenone, $25\mu\text{M}$; antimycin A, $25\mu\text{M}$.

with the much larger changes observed at 552nm minus 544nm due to cytochrome *c* reduction (Fig. 14, trace c). At this point, where anaerobic conditions are achieved, the rate of haem synthesis is greatly increased, presumably because oxygen no longer competes with ferrochelatase for Fe^{2+} , and all the Fe^{3+} reduced by the electron-transport chain becomes available for haem synthesis. The sequence of carriers in the respiration of *R. spheroides* particles is uncertain, although the cytochrome pattern and response to inhibitors of the aerobically grown form shows many similarities to the mito-

chondrial system. It is significant that neither rotenone nor antimycin A inhibits the flow of electrons to Fe^{3+} from NADH or succinate as judged by the rate of haem synthesis (Fig. 14, trace a), and indicates that iron may be reduced by the flavoprotein dehydrogenases or by an adjacent non-haem-iron protein.

DISCUSSION

Although interpretation of negative results must be cautious, our failure to detect the enzymic incorporation of Fe^{2+} into porphyrin cytochrome *c* and the earlier failure of *R. spheroides* extracts to form haem *c* or haem *c* 2,4-diethyl ester from the corresponding porphyrins (Johnson & Jones, 1964) would support a scheme in which cytochrome *c* is synthesized by the addition of protohaem to a preformed apoprotein. Evidence for such a scheme has been obtained by Kadenbach (1970), who followed the time-course of incorporation of [^{14}C]lysine into mammalian microsomal fractions, and by Lascelles, Rittenberg & Clark-Walker (1969), who showed that in the bacterium *Spirillum itersonii* [^{59}Fe]protohaem was directly incorporated into cytochrome *c* with high efficiency. Since Sinclair, White & Barrett (1967) have found that ^{14}C -labelled protohaem was incorporated into haem *a*, without loss of specific radioactivity, by haematin-requiring mutants of *Staphylococcus aureus*, it appears possible that protohaem is an obligatory intermediate in the biosynthesis of all cytochromes and that modification of side chains takes place after insertion of Fe^{2+} .

The kinetic properties of the particulate enzyme (Figs. 2, 3, 4 and 5) show that the ferrochelatase reaction has a sequential, and not a non-sequential (Ping Pong), mechanism (Cleland, 1963a). This means that, in metalloporphyrin formation, proton dissociation from the central nitrogen atoms does not precede reaction of the metal cation with the

resulting porphyrin anion. Instead some form of displacement mechanism must operate in which porphyrin and metal ion form a complex from which protons subsequently dissociate.

There have been various studies of the rates of incorporation of metals into porphyrins both in model non-enzymic systems and in enzymic systems. There is general agreement that Zn^{2+} is enzymically incorporated by mammalian mitochondria (Neuberger & Tait, 1964), erythrocytes (Johnson & Jones, 1964), barley mitochondria (Goldin & Little, 1969), yeast mitochondria (Labbe, Volland & Chaix, 1968) and bacteria (Neuberger & Tait, 1964; Johnson & Jones, 1964). It seems very likely that this formation of zinc porphyrins is a result of the lack of specificity of the haem-synthesizing enzyme. The enzymic formation of cobalt porphyrins has been measured in avian erythrocytes (Oyama, Sugita, Yoneyama & Yoshikaya, 1961), yeast and bacteria (Porra & Ross, 1965), rat liver mitochondria (Labbe & Hubbard, 1961) and photosynthetic bacteria and erythrocytes (Johnson & Jones, 1964). As a result of their studies of competition between different metal substrates Jones & Jones (1969) concluded that the same enzyme was responsible for the incorporation of Fe^{2+} , Zn^{2+} or Co^{2+} in rat liver mitochondria. Porra & Ross (1965), however, found that in *Clostridium tetanomorphum*, a bacterium that synthesizes vitamin B_{12} but no haems, Co^{2+} incorporation rates far exceeded rates of Fe^{2+} incorporation, suggesting the presence of a specific Co^{2+} chelatase. There have been other reports on the enzymic formation of manganese porphyrins (Labbe & Hubbard, 1961). The results we have obtained show Fe^{2+} , Co^{2+} and Zn^{2+} as much the best substrates, with Cu^{2+} , Ni^{2+} and Mn^{2+} less good (Table 2). There is no obvious correlation between the physical properties of these ions and their rates of incorporation. With the exception of Fe^{2+} and Co^{2+} the rates are roughly in the order that has been described for the aqueous non-enzymic formation of metalloporphyrin, when the metal ions that give measurable rates are $\text{Cu}^{2+} > \text{Zn}^{2+} > \text{Mn}^{2+} > \text{Co}^{2+} > \text{Fe}^{2+} > \text{Ni}^{2+} > \text{Cd}^{2+}$ (Fleischer, Choi, Hambricht & Stone, 1964; Phillips, 1967). Although Mg^{2+} , Co^{2+} and Fe^{2+} may be non-enzymically incorporated in a non-aqueous environment, they are only slowly incorporated in aqueous physiological conditions, which suggests that the active site of the enzyme may provide a hydrophobic environment for the chelation step; there is evidence that phospholipids are important for the activity of ferrochelatase (Sawada, Takeshita, Sugita & Yoneyama, 1969).

Cd^{2+} can be incorporated non-enzymically into porphyrins at low rates in an aqueous environment and appears similar enough in physical properties to act as a competitive inhibitor of the normal

substrates in the enzyme-catalysed reaction; it may prove useful in further studies on the mode of action of ferrochelatase. Metal ions such as Ni^{2+} and Mn^{2+} that are incorporated at low rates are also competitive inhibitors of Co^{2+} or Fe^{2+} incorporation. It appears to us that one problem that nearly all organisms have to solve is to keep unwanted metal ions away from the site of ferrochelatase action. Zinc and copper porphyrins may be found in bacterial and yeast culture media, although these may have been formed non-enzymically.

Inhibition of ferrochelatase by metalloporphyrins. The original observation by Burnham & Lascelles (1963) that aminolaevulate synthetase in *R. spheroides* is subject to negative-feedback inhibition by protohaem has been frequently confirmed; this inhibition may be of key importance in the regulation of haem and chlorophyll synthesis in this organism (Lascelles, 1968). Our observation that ferrochelatase is even more sensitive than aminolaevulate synthetase to protohaem inhibition and is also strongly inhibited by magnesium protoporphyrin opens up many possible refinements in the regulation of haem and chlorophyll synthesis in *R. spheroides*, but the overall control will probably not be understood until the mechanism of Mg^{2+} incorporation and its regulation have been characterized. There is indirect evidence that the Mg^{2+} -incorporation step is sensitive to oxygen. Lascelles & Altshuler (1969) have shown that an aminolaevulate-requiring mutant of *R. spheroides* grown in a medium containing aminolaevulate stops the synthesis of magnesium tetrapyrroles when the medium is oxygenated, although normal haem synthesis still occurs. There is another form of regulatory mechanism found in these bacteria; oxygenation of cultures leads to the formation of a low-molecular-weight inhibitor of the activation of aminolaevulate synthetase (Marriott *et al.* 1969). When the cells become anaerobic, and chlorophyll synthesis is required, this last-mentioned control over aminolaevulate synthetase and also the inhibition of Mg^{2+} incorporation will disappear. The effect of the inhibition of ferrochelatase by magnesium protoporphyrin and protohaem will then be to divert porphyrin intermediates to the chlorophyll pathway, which is where the main requirement would be at that time.

The specificity of the inhibition by metalloporphyrins (Table 3) suggests that, for inhibition to occur, the porphyrin ring must bear two free propionic acid side chains and both co-ordination positions of the iron in the porphyrin ring must be available (cytochrome *c* and haemoglobin were ineffective). The presence of the two vinyl groups at positions 2 and 4 of the tetrapyrrole ring increases inhibitory activity.

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