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INTERMEDIATES IN CHLOROPHYLL BIOSYNTHESIS IN *RHODOPSEUDOMONAS SPHEROIDES*: EFFECTS OF SUBSTRATES AND INHIBITORS

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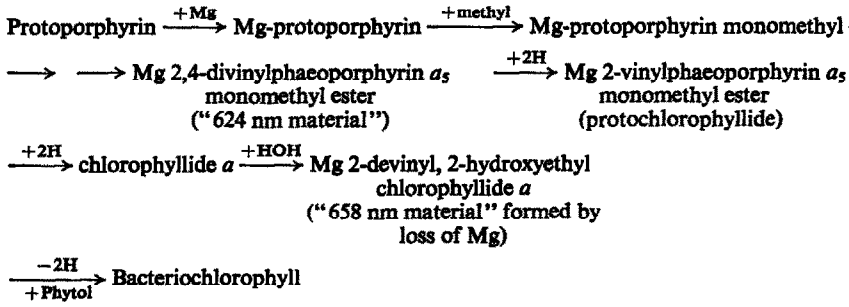
Abstract—Washed cells of *Rhodopseudomonas spheroides* when suspended in a simple medium excreted magnesium protoporphyrin monomethyl ester. Addition of 8-hydroxyquinoline and copper to the suspending medium led to the excretion of pigments more closely related to chlorophyll, the nature of which depended upon the substrate provided. When α-oxoglutarate was supplied the main pigment was at the oxidation level of chlorophyll; when succinate was supplied, the main pigment was at the oxidation level of protochlorophyll. Malonate increased the tendency to accumulate the less reduced pigment at the protochlorophyll oxidation level. The possibility that the biosynthesis of chlorophyll in *R. spheroides* is dependent upon the availability of NADH is discussed. Extracts of *R. spheroides* catalysed the incorporation of Cu^{2+} into porphyrins: the rate of copper porphyrin formation was increased by the addition of 8-hydroxyquinoline. The possibility that this reaction is implicated in the inhibitory action of copper-8-hydroxyquinoline complexes is also discussed.

INTRODUCTION

THE biosynthesis of chlorophylls in both green plants and the purple non-sulphur bacteria, the Athiorhodaceae, is believed to follow a common pathway, probably as far as chlorophyllide *a* (Scheme 1). The evidence for such a scheme is based largely upon studies of pigments accumulated in cultures of *Rhodopseudomonas spheroides* treated with 8-hydroxyquinoline,[1,2,3,4] and in mutants of *Chlorella*[5,6,7] and mutants of *R. spheroides*.[8,9,10] Only



SCHEME 1.

[1] O. T. G. JONES, *Biochem. J.* **86**, 429 (1963).
[2] O. T. G. JONES, *Biochem. J.* **88**, 335 (1963).
[3] O. T. G. JONES, *Biochem. J.* **89**, 182 (1963).
[4] O. T. G. JONES, *Biochem. J.* **91**, 572 (1964).
[5] S. GRANICK, *J. Biol. Chem.* **175**, 333 (1948).
[6] S. GRANICK, *J. Biol. Chem.* **236**, 1168 (1961).
[7] S. GRANICK, *J. Biol. Chem.* **183**, 713 (1950).
[8] R. Y. STANIER and J. H. C. SMITH, *Yearbook Carneg. Instn* **58**, 336 (1959).
[9] M. GRIFFITHS, *J. Gen. Microbiol.* **27**, 427 (1962).
[10] J. LASCELLES, *Biochem. J.* **100**, 175 (1966).

one step, the methylation of magnesium protoporphyrin, has been studied enzymically,[11] in extracts of *R. spheroides*, but there is now evidence that magnesium 2,4-divinylphaeoporphyrin a_5 monomethyl ester, formerly called bacterial protochlorophyll[8] can be formed by higher plants[12] and some marine algae,[13] supporting the view[3] that this compound is an intermediate on a pathway common to all chlorophyll-forming organisms.

It will be shown in this communication that the nature of the pigments, postulated as intermediates in chlorophyll synthesis, formed when washed cells of *R. spheroides* are treated with 8-hydroxyquinoline can be altered by changing the substrates available and that these effects may be related to processes normally controlling chlorophyll formation. In this organism chlorophyll synthesis appears to be controlled by oxygen tension in the growth medium[14] and these substrate effects may be linked to the availability of reducing power for the reduction of porphyrins to chlorins.

The effect of 8-hydroxyquinoline upon the enzymic formation of metalloporphyrins and the metal specificity of the metalloporphyrin-forming enzyme was investigated in attempts to elucidate the possible mechanism whereby 8-hydroxyquinoline inhibited bacteriochlorophyll synthesis. It was found that copper porphyrins were formed enzymically and that their rate of formation was increased by the addition of 8-hydroxyquinoline. The significance of these observations is discussed.

RESULTS

When washed cells of *Rhodopseudomonas spheroides* were incubated in Lascelles' Mixture I with α-oxoglutarate as carbon source,[15] the ether-soluble pigment magnesium protoporphyrin monomethyl ester was excreted into the medium. This has characteristic absorption maxima at 550 and 591 nm (Fig. 1). The addition of 8-hydroxyquinoline and Cu (20:1) to these washed cells greatly reduced the formation of this compound and caused the production of a pigment with an absorption maximum at 658 nm, previously identified as 2-hydroxyethylphaeophorbid *a*.[4] This pattern of pigment excretion was much simpler than that found in cells grown in the presence of 8-hydroxyquinoline, where a complex mixture of pigments was formed.[2] If succinate was substituted for α-oxoglutarate then the pattern of pigment excretion caused by 8-hydroxyquinoline changed, a new pigment absorbing at 624 nm was formed, and less 658 nm pigment was formed (Fig. 2). This 624 nm pigment was found after purification on polyethylene columns[2] to be magnesium 2,4-divinylphaeoporphyrin a_5 monomethyl ester, a pigment at the oxidation level of protochlorophyll, i.e. less reduced than that formed when α-oxoglutarate was supplied. This observation suggested that α-oxoglutarate was supplying the succinyl CoA necessary for synthesis of δ-amino laevulinic acid and also supplying $NADH_2$ to be used in the later, reductive steps in chlorophyll synthesis. It is known that succinate can be directly converted to succinyl CoA by *R. spheroides*;[16] any additional reducing power would have to be supplied by the metabolism of succinate through other pathways, possibly by photosynthetically driven oxidation or via the tricarboxylic acid cycle. In order to block this source of reducing power, malonate was added to the 8-hydroxyquinoline treated suspensions of *R. spheroides*. It was found (Fig. 3) this did indeed lower the formation of the more reduced 658 nm material with a

[11] K. D. GIBSON, A. NEUBERGER and G. H. TAIT, *Biochem. J.* **88**, 325 (1963).
[12] O. T. G. JONES, *Biochem. J.* **101**, 153 (1966).
[13] T. R. RICKETTS, *Phytochem.* **5**, 223 (1966).
[14] G. COHEN-BAZIRE, W. R. SISTROM and R. Y. STANIER, *J. Cell. Comp. Physiol.* **49**, 25 (1957).
[15] J. LASCELLES, *Biochem. J.* **62**, 78 (1956).
[16] B. BURNHAM, *Acta Chem. Scand.* **17**, S.123 (1963).

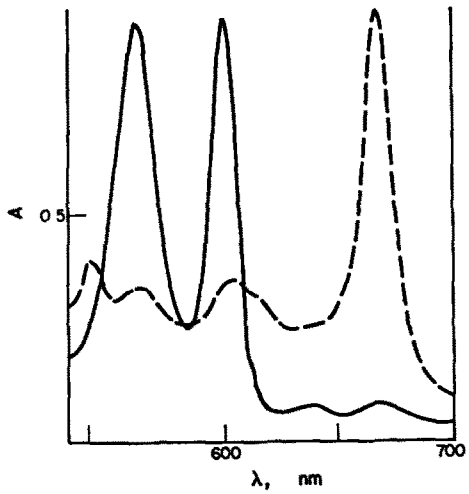


FIG. 1. SPECTRA, IN ETHER, OF ETHER-SOLUBLE PIGMENTS EXCRETED INTO THE MEDIUM BY WASHED CELLS OF *R. spheroides* SUSPENDED IN LASCELLES' α-OXOGLUTARATE MEDIUM.[15]

After 16 hr anaerobic incubation, in light, the suspension was centrifuged, 50 ml supernatant extracted with ether and concentrated to 2 ml for recording spectra (1 cm light path). —— Extract from cells suspended in normal medium; ---- extract from cells suspended in medium containing 8-hydroxyquinoline (100 μM) and Cu^{2+} (5 μM).

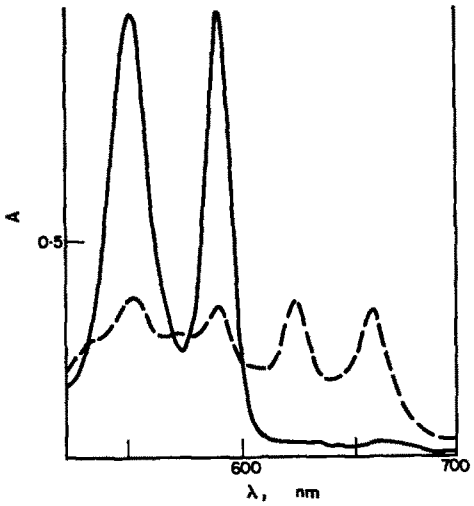


FIG. 2. AS FOR FIG. 1, EXCEPT THAT CELLS WERE SUSPENDED IN SUCCINATE MEDIUM.

—— Extract from cells suspended in normal medium; ---- extract from cells suspended in 8-hydroxyquinoline (40 μM) and Cu^{2+} (5 μM).

corresponding increase in the 624 nm material when either succinate or α-oxoglutarate was supplied. Using malate as the substrate the pattern of pigment formation more closely resembled that in the presence of succinate than that of α-oxoglutarate.

Phillips and Lowe[17] have suggested that certain toxic chelating agents act by interfering

[17] M. B. LOWE and J. N. PHILLIPS, *Nature* **190**, 262 (1961).

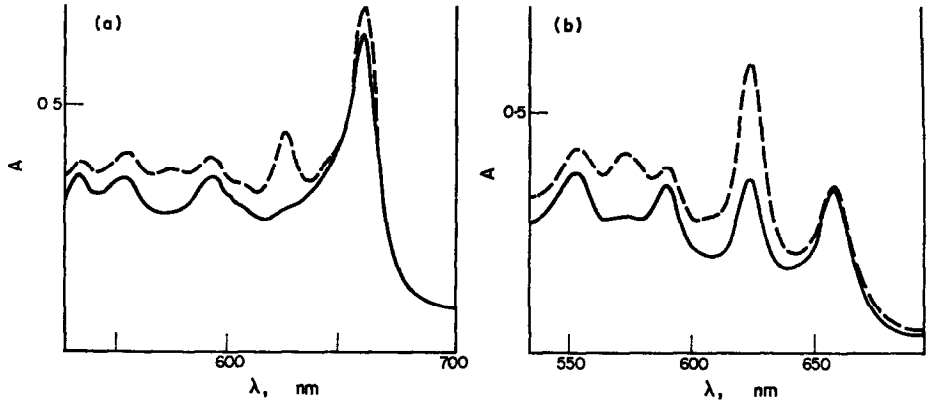


FIG. 3. EFFECT OF MALONATE UPON ETHER-SOLUBLE PIGMENTS EXCRETED BY WASHED CELLS OF *R. spheroides* SUSPENDED IN MEDIA CONTAINING 8-HYDROXYQUINOLINE AND Cu^{2+}.

(a) Conditions as for Fig. 1, —— extract from cells suspended in Lascelles' α-oxoglutarate medium containing 8-hydroxyquinoline (100 μM) and Cu^{2+} (5 μM). ---- extract from cells suspended in Lascelles' α-oxoglutarate medium containing 8-hydroxyquinoline (100 μM), Cu^{2+} (5 μM) and malonate (20 mM).

(b) Conditions as for Fig. 2, —— extract from cells suspended in Lascelles' succinate medium containing 8-hydroxyquinoline (40 μM) and Cu^{2+} (5 μM); ---- extract from cells suspended in Lascelles' succinate medium containing 8-hydroxyquinoline (40 μM), Cu^{2+} (5 μM) and malonate (20 mM).

with normal haem metabolism by catalysing the insertion of Cu^{2+} into porphyrins in place of the usual Fe^{2+} required in haem synthesis. It was therefore interesting to examine the influence of added iron upon the effect of copper and 8-hydroxyquinoline upon washed cells. The results of such an experiment (Table 1) show that ferric citrate inhibited the excretion

TABLE 1. EFFECT OF FERRIC CITRATE UPON THE FORMATION OF PIGMENTS BY 8-HYDROXYQUINOLINE-TREATED SUSPENSIONS OF *Rhodopseudomonas spheroides*

Additions to incubation mixture	mμmoles/mg dry wt. cells			
	658 nm material in medium	Copro-porphyrin in medium	Magnesium proto-porphyrin ester in medium	Bacterio-chlorophyll in cells
None	0·02	17	1·4	20
Cu^{2+} (0·5 μM) + 8-hydroxyquinoline (100 μM)	0·55	2·5	0·12	16
Ferric citrate (10 μM)	0·02	0·1	0·25	37
Cu^{2+} (0·5 μM) + 8-hydroxyquinoline (100 μM) + ferric citrate (10 μM)	0·05	0·4	0·025	21
Cu^{2+} (5 μM) + 8-hydroxyquinoline (100 μM) + ferric citrate (10 μM)	0·15	0·2	0·025	16

Washed cells of *R. spheroides* were suspended in the α-oxoglutarate medium of Lascelles[15] to which various additions were made, as shown. The suspensions were incubated for 16 hr at 30° in stoppered tubes in an illuminated water-bath. The porphyrins in the medium and chlorophyll in cells were determined as described in the Experimental section.

of tetrapyrrole pigments into the medium, even in the presence of 8-OH quinoline, but it did not completely overcome the effect of the chelator upon bacteriochlorophyll synthesis. Ferric citrate was active against the copper chelate at concentrations lower than those that might be explained from consideration of stability constants: 8-hydroxyquinoline was present in much more than a three-fold excess of the ferric citrate concentration. No other metal ion had this action (Mn^{2+}, Mg^{2+}, Ni^{2+}, Co^{2+} and Zn^{2+} were examined).

The observation that added iron reduced the excretion of magnesium protoporphyrin monomethyl ester by washed cells (Table 1) was confirmed in a further experiment (Fig. 4) and confirms the earlier observation with growing cells.[1]

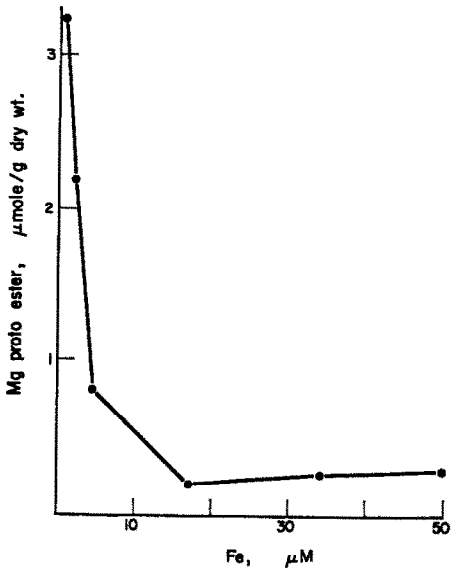


FIG. 4. EFFECT OF ADDED FERRIC CITRATE UPON THE EXCRETION OF MG PROTOPORPHYRIN MONOMETHYL ESTER BY WASHED CELLS OF *R. spheroides* SUSPENDED IN LASCELLES' α-OXOGLUTARATE MEDIUM.

Formation of Metalloporphyrins by Extracts of R. spheroides *and the Effect of 8-Hydroxyquinoline upon this Process*

Since it was possible that a copper porphyrin might be formed within the organism in the presence of the copper chelate, extracts of cells were prepared and the formation of metalloporphyrins examined.

By marking the disappearance of the characteristic absorption at Band IV of a porphyrin it is possible to determine its rate of conversion into a metalloporphyrin which normally has only two major bands in the visible region and this has been used as the basis of an assay of enzymic metalloporphyrin formation.[18] In experiments on copper–porphyrin formation the identity of the product was confirmed by its characteristic absorption spectrum after extraction into ethylacetate/acetic acid and washing with 10% HCl. Using this assay it was shown that extracts of *R. spheroides* catalysed the formation of copper porphyrins, and that the rate of copper incorporation was dependent upon the enzyme concentration. The rates of Cu^{2+} incorporation were of the same order as Fe^{2+} incorporation (Fig. 5), but differed

[18] A. JOHNSON and O. T. G. JONES, *Biochim. Biophys. Acta* **93**, 171 (1964).

in that O_2 completely inhibited the incorporation of Fe^{2+} but did not affect that of Cu^{2+} (Fig. 5).

When 8-hydroxyquinoline was added to extracts of *R. spheroides* there was initially a rapid rise in the rate of Cu-porphyrin formation (Fig. 5). Increasing additions of the chelate had the reverse effect: incorporation of Cu^{2+} into porphyrin was completely inhibited, presumably as a result of the formation of the catalytically inactive 2:1 copper chelate. Thus it seems possible that if Cu^{2+} is carried into the region of the cell where porphyrin synthesis is taking place then a certain amount of copper porphyrin may be formed. Burnham and Lascelles[19] have shown that the enzyme δ-amino laevulinic synthetase, a key enzyme in the control of tetrapyrrole pigment formation, is inhibited by low concentrations of free metalloporphyrins, including Cu-protoporphyrin, and it seems possible that this may explain some of the effect of copper chelates in reducing porphyrin excretion by *R. spheroides* in conditions of iron deficiency. A number of copper porphyrins and phaeophorbids were added to intact cells under conditions where added protohaem was inhibitory but they were

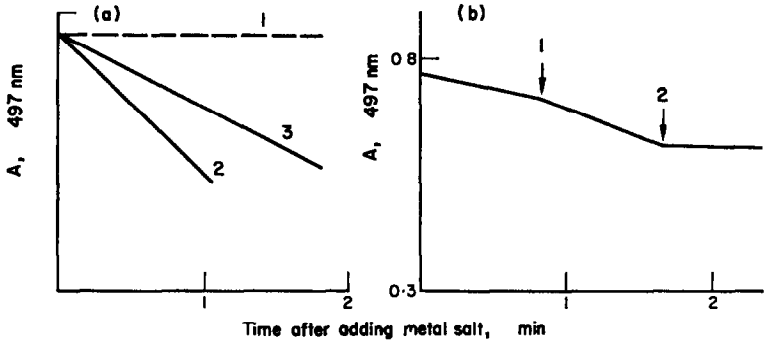


FIG. 5. (a) INCORPORATION OF Cu^{2+} AND Fe^{2+} INTO DEUTEROPORPHYRIN BY EXTRACTS OF *R. spheroides*. 1. AEROBIC Fe^{2+}. 2. ANAEROBIC Fe^{2+}. 3. Cu^{2+}. Cu^{2+} GAVE THE SAME RATE, EITHER AEROBICALLY OR ANAEROBICALLY.

(b) EFFECT OF ADDED 8-HYDROXYQUINOLINE UPON THE INCORPORATION OF Cu^{2+} INTO DEUTEROPORPHYRIN. AT 1, 8-HYDROXYQUINOLINE WAS ADDED AT A CONCENTRATION EQUIMOLAR WITH THE Cu^{2+} PRESENT. AT 2, A SECOND ADDITION OF EQUIMOLAR 8-HYDROXYQUINOLINE WAS MADE.

found to have no effects on porphyrin excretion: presumably they failed to penetrate to the sensitive site within the organism.

Specificity of the Chelating Agent Effect upon R. spheroides

Since it appears possible that the effect of 8-hydroxyquinoline upon chlorophyll synthesis in *R. spheroides* is associated within its catalysis of the incorporation of Cu^{2+} into porphyrins a study was made of a range of chelating agents to determine those that were effective in producing this excretion of new pigments. In order to test the chelating agents under the most favourable conditions, they were added to media lacking the usual high concentrations of the chelating agents EDTA, nitrilotriacetic acid and glutamic acid. Under these conditions four chelating agents were effective (in increasing order of efficiency), kojic acid, salicylaldehyde, sodium diethyldithiocarbamate and 8-OH quinoline. All these four were found by Phillips and Lowe[17] to catalyse the non-enzymic insertion of metals into porphyrins.

[19] B. F. BURNHAM and J. LASCELLES, *Biochem. J.* **87**, 462 (1963).

DISCUSSION

The experiments described here are an extension of those of Lascelles[15] who showed that washed cells of *Rhodopseudomonas spheroides* excreted porphyrins, mostly coproporphyrin when suspended in a medium containing a carbon source, such as α-oxoglutarate, and glycine. It has now been found that the addition of 8-hydroxyquinoline to the suspending medium caused the formation of porphyrins and magnesium porphyrins more closely related to chlorophyll. The material absorbing at 658 nm, identified as 2-devinyl,2-hydroxyethyl-phaeophorbid *a*, is presumably formed from the corresponding 2-devinyl,2-hydroxyethyl-chlorophyllide *a* by the loss of magnesium, a reaction that occurs readily in light.[10] The 624 nm material and the 658 nm material are presumed to fit on the scheme for bacterio-chlorophyll biosynthesis (Scheme 1), where it can be seen that the conversion of the magnesium phaeoporphyrin to the chlorophyllide involves two reductive steps requiring 4H.

The observation (Fig. 2) that relatively much more 624 nm material is formed from succinate than from α-oxoglutarate suggests that the availability of NADH may be a limiting factor in the synthesis of bacteriochlorophyll. Succinate can be converted directly to succinyl CoA without yielding NADH whereas α-oxoglutarate yields one molecule of NADH during this conversion. This suggestion is supported by the results of the experiments where malonate was added to the medium. This would inhibit the photoxidation of succinate to NADH mediated by the bacterial photosynthetic system and would thus reduce the available reducing power of both succinate and α-oxoglutarate. As can be seen in Fig. 3, malonate caused a decrease in the 658 nm material relative to the 624 nm material. Although enzymic evidence is not available it seems possible that the availability of NADH may be important in controlling chlorophyll formation in these photoheterotrophs, which form no photosynthetic pigments under conditions of vigorous aeration, where NADH levels would be expected to be low. Such a control mechanism requires that the magnesium 2,4-divinylphaeoporphyrin acts as a repressor of an earlier stage in chlorophyll biosynthesis. This hypothesis is reasonable since such a mechanism must exist in higher plants where only relatively small quantities of protochlorophyll accumulate in the dark where no chlorophyll formation takes place; no other intermediates accumulate in significant quantities.

As a result of experiments where photopigment formation by *R. spheroides* was measured in cultures containing inhibitors of NADH oxidation such as amytal and atebrin or uncouplers such as 2,4-dinitrophenol and dicoumarol Sistrom[20] also has suggested that the ratio of NADH to NAD may control pigment synthesis.

The effect of small additions of iron in overcoming some of the effects of 8-hydroxy-quinoline (Table 1) suggest that this material may be interfering with normal iron metabolism, possibly by interfering with the incorporation of iron or magnesium into porphyrins through the catalytic formation of a copper porphyrin, as suggested by Phillips and Lowe.[17] Copper porphyrins themselves did not reproduce the effects of 8-hydroxyquinoline when added to the medium, but it was found that in broken cell preparations, Cu^{2+} incorporation into porphyrins proceeded almost as rapidly as Fe^{2+} incorporation and that it was stimulated by 8-hydroxyquinoline (Fig. 5). It may be that the effect of this chelator in whole cells of *R. spheroides* is to cause the formation of some copper porphyrin that can interfere with the normal organization of chlorophyll (or haem) molecules in photosynthetic units leading to an excretion of pigment molecules.

[20] W. R. SISTROM, *Bacterial Photosynthesis*, p. 53. Antioch Press, Yellow Springs (1963).

EXPERIMENTAL

Organism. Rhodopseudomonas spheroides was grown as described previously.[14] The medium for inducing porphyrin synthesis by washed cells was Mixture I of Lascelles,[15] modified as indicated in the text.

Determination of porphyrins in growth medium. After removal of cells by centrifuging, ether-soluble porphyrins were extracted from the supernatant; the medium was twice extracted with equal volumes of ether which was then washed twice with water. Magnesium protoporphyrin monomethyl ester normally present in the ether extract[1] was usually determined from its absorption maximum at 591 nm (see Fig. 1) using the value of 18·2 for ϵmM obtained by Granick.[5] When a mixture of pigments was present in the ether extract, magnesium protoporphyrin monomethyl ester was extracted into 8% HCl, which caused the removal of magnesium, and the resulting protoporphyrin ester was determined using a value of 275 for the ϵmM at 408 nm in 2·5% HCl.[21] The determination of 2-hydroxyethylphaeophorbide *a* was based upon its absorption at 658 nm and an extinction coefficient at this maximum in the red region assumed equal to that of the absorption maximum of phaeophorbide *a* in this region. Over 95 per cent of the porphyrin remaining in the medium after ether extraction was coproporphyrin and was determined as coproporphyrin in 0·1 N HCl using an ϵmM of 489 at 400 nm.[21]

Bacteriochlorophyll content of cells was determined after extraction into acetone/methanol.[14]

Preparation of Extracts for Determination of Enzymic Metalloporphyrin Formation

Washed cells of *R. spheroides* suspended in 0·1 M phosphate buffer, pH 7·4, at a concentration of 0·1 g wet wt./ml were treated for 20 min in the Raytheon sonic oscillator (250 W, 10 kc). The extract was centrifuged at 70,000 *g* for 120 min and the supernatant used for assay.

Assay of Formation of Copper- and other Metallo-porphyrins

The assay method of Johnson and Jones[18] was used. Incubations were carried out in glass cuvettes of 1 cm light path. The incubation mixture contained 200 mμmoles of porphyrin, 200 μmoles tris buffer, pH 7·9, enzyme extract (1·0 ml) and water to a volume of 4·0 ml. The spectrophotometer was fixed on the absorption maximum of Band IV of the porphyrin (for mesoporphyrin this was at 498 nm) and the rate of decrease in absorption measured following the addition of 100 mμmoles $CuSO_4$. In order to determine the rate for insertion of Fe^{2+} anaerobic conditions were essential[22] and incubations were carried out under oxygen-free nitrogen in cuvettes fitted as continuations of the main well of Thunberg tubes. The reaction was started by tipping the metal salt from the side-arm. Inconsistent results were obtained if the porphyrin substrate was not in true solution and it was necessary to add sufficient detergent (0·5% Emasol 4130 or Tween 80) to maintain the porphyrin in solution at pH 7·9.

[21] C. RIMINGTON, *Biochem. J.* **75**, 620 (1960).
[22] R. J. PORRA and O. T. G. JONES, *Biochem. J.* **87**, 181 (1963).