The Inhibition of Bacteriochlorophyll Biosynthesis in *Rhodospseudomonas spheroides* by 8-Hydroxyquinoline

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Granick (1948, 1950, 1961) has isolated different mutants of *Chlorella* which are unable to synthesize chlorophyll and which accumulate magnesium protoporphyrin, magnesium vinyl phaeoporphyrin a_5 and a monoester of magnesium protoporphyrin, presumed to be the monomethyl ester. Granick (1961) has proposed that these compounds are intermediates in chlorophyll biosynthesis, and it has been suggested that they are also intermediates in bacteriochlorophyll biosynthesis (Mauzerall & Granick, 1961).

Although a system has been extracted from *Rhodospseudomonas spheroides* which enzymically methylates magnesium protoporphyrin (Tait & Gibson, 1961), and magnesium protoporphyrin monomethyl ester has been isolated from the medium of wild-type *R. spheroides* (Jones, 1963), there is no evidence of common intermediates in the biosynthesis of bacteriochlorophyll and chlorophyll after this stage. Studies of mutants of *R. spheroides* unable to synthesize bacteriochlorophyll have shown the accumulation of a compound resembling *Chlorobium* phaeophytin-650 (Griffiths, 1962), and of a compound differing slightly in spectroscopic properties from magnesium vinyl phaeoporphyrin a_5 (protochlorophyllide) (Stanier & Smith, 1959; Griffiths, 1962). Previous studies of a mutant of *R. spheroides* deficient in carotenoids showed the excretion into the medium of other compounds with a phaeophytin type of spectrum (Sistrom, Griffiths & Stanier, 1956), and it has been suggested that these compounds may be intermediates or possibly derivatives of intermediates in bacteriochlorophyll biosynthesis.

The effect of 8-hydroxyquinoline on the synthesis of metalloporphyrins was investigated by Lowe & Phillips (1961), who showed that certain chelating agents catalysed the insertion of metal ions into porphyrin nuclei. Those chelating agents that catalysed the reaction were also active as bactericides or fungicides (Lowe & Phillips, 1962), and it appeared possible that this biological activity was due to an interference with normal metalloporphyrin biosynthesis.

It has now been found that, when wild-type *R. spheroides* is grown in a medium containing 8-

hydroxyquinoline, bacteriochlorophyll biosynthesis is partly inhibited, and that a number of pigments related to plant and bacterial chlorophylls and protochlorophylls are secreted into the medium. The work discussed in the present paper describes the 8-hydroxyquinoline effect and the isolation and some properties of these pigments, which may be of importance in the normal biosynthesis of bacteriochlorophyll.

MATERIALS AND METHODS

Chemicals. Powdered polyethylene (melt index, 3.5), a gift from the Dow Chemical Co., was given by Dr J. M. Anderson. Other compounds used were commercial products. Ether was washed with water until free of peroxides before use. To conform with the usual convention in this field HCl concentrations are given as % (w/v).

Organism. The strain of *Rhodospseudomonas spheroides* used and growth conditions were those described by Jones (1963). Where modifications were made these are described in the text.

Harvesting of cultures. At the end of the logarithmic growth period (24–30 hr.) the organisms were harvested by centrifuging at 0°. The supernatant liquid was retained and the cells were washed twice in 0.05 M-phosphate buffer, pH 7.3. If not used immediately the cells were kept frozen at –20° until required. A sample of the washed cells was heated to constant weight at 100° for determination of dry weight.

Determination of bacteriochlorophyll. The bacteriochlorophyll in the organisms was extracted in acetone-methanol (7:2, v/v) and determined spectrophotometrically by the method of Cohen-Bazire, Sistrom & Stanier (1957).

Determination of carotenoid. Carotenoid concentrations in acetone-methanol (7:2, v/v) extracts of whole cells were determined spectrophotometrically by the method of Cohen-Bazire *et al.* (1957).

Determination of haem pigments. The washed cells from 30 ml. of culture were suspended in 6 ml. of the alkaline pyridine reagent of Paul, Theorell & Åkeson (1953) and the difference spectra (reduced-minus-oxidized values) of the alkaline pyridine haemochromogens recorded in the range 500–570 m μ . The haemochromogen difference spectrum showed strong absorption bands at 551 and 521 m μ with a very slight shoulder at 557 m μ . The absorption bands at 551 and 521 m μ were assumed to be due to a mixture of the prosthetic groups of cytochrome *c* and *Rhodospirillum* haem protein, and that at 557 m μ to be due to a small amount of cytochrome *b* (cf. Orlando & Horio, 1961).

Although the alkaline pyridine haemochromogens of *Rhodospirillum* haem protein and cytochrome *c* are identical in band position they differ in molar extinction values (Bartsch & Kamen, 1960). The value for $\Delta\epsilon_{\text{mm}}$ ($\alpha - \text{min.}$) of 22.0 for the reduced-minus-oxidized spectrum was obtained by using a sample of crystalline mammalian cytochrome *c* (cf. Porra & Jones, 1963*a*).

Molisch phase test. The phase test for an isocyclic ring was carried out on chlorophyll derivatives as described by Smith & Benitez (1955).

Preparation of protochlorophyll. Protochlorophyll was prepared from the leaves of etiolated bean plants (*Phaseolus vulgaris*) by the method described by Smith & Benitez (1955).

Preparation of chlorophyll *a* and chlorophyll *b*. Plant chlorophyll pigments were prepared from acetone extracts of spinach leaves by chromatography on polyethylene columns (cf. Anderson, Blass & Calvin, 1960; Anderson & Calvin, 1962). Chromatography columns 2 cm. in diameter were packed with 15 g. of powdered polyethylene equilibrated with aq. 70% (v/v) acetone. The extracts of spinach leaf were diluted to give a final concentration of 70% of acetone and applied to the column, which was developed with 70% acetone. Chlorophyll *b* moves ahead of chlorophyll *a* in this system, but there was some overlapping and the mixed fractions were discarded. The pigments were rechromatographed on polyethylene columns developed with the same solvent. The products were examined spectroscopically and each was found to be free of contamination by the other pigment.

Preparation of magnesium-free chlorophyll derivatives (phaeophytins). The chlorophyll pigments in ether solution were shaken with 15% HCl, which rapidly removes Mg^{2+} ions from Mg^{2+} ion complexes of porphyrins and chlorins (cf. Smith & Benitez, 1955).

Preparation of metal ion complexes of porphyrins and phaeophytins. Ferrous ions were introduced into porphyrins by the method of Morell, Barrett & Clezy (1961). Reduced-minus-oxidized pyridine haemochromogen spectra were recorded for these haem compounds by the method of Porra & Jones (1963*b*). Cupric ions were introduced into the phaeophytin-type pigments by heating them in pyridine solution with copper acetate (cf. Barnes & Dorough, 1950). After the addition of ether, unchanged phaeophytin was removed by exhaustive washing with 25% HCl.

Chromatography of 70% acetone-soluble pigments derived from the cells or medium of *Rhodospseudomonas spheroides*. Pigments obtained by ether extraction of the cell-free supernatant of *R. spheroides* medium or from acetone extraction of whole cells were made up in 70% acetone and chromatographed on a polyethylene column which was developed with 70% acetone. The order of elution from such a column was: xanthophylls, copper phaeophorbide and some free phaeophorbides, bacteriochlorophyll, protochlorophyll-like material, magnesium protoporphyrin monomethyl ester, protoporphyrin monomethyl ester and finally carotenoids.

Spectra. Spectra were usually determined with a Bausch and Lomb Spectronic 505 recording spectrophotometer, calibrated by reference to a mercury emission spectrum. Spectra at wavelengths greater than 700 m μ were determined with an Optica recording spectrophotometer fitted with a Philips type 150 CVP photomultiplier.

RESULTS

In the biosynthesis of haem pigments there is only one enzymic reaction for which a porphyrin is known to be a substrate. That is the reaction where Fe^{2+} ions are inserted into protoporphyrin (Porra & Jones, 1963*b*). It seems likely that Mg^{2+} ions are inserted into protoporphyrin by an analogous reaction in bacteriochlorophyll biosynthesis. In earlier reactions of the biosynthetic sequence, porphyrinogens and not porphyrins are the substrates (cf. Mauzerall & Granick, 1961), and these do not readily chelate metal ions (Heikel, Lockwood & Rimington, 1958). If 8-hydroxyquinoline acts as a bactericide by catalysing the insertion of metal ions, probably Cu^{2+} ions, into a porphyrin (Lowe & Phillips, 1962), it is likely that protoporphyrin is the only such porphyrin intermediate available in the organism and that copper protoporphyrin would be produced.

R. spheroides was therefore grown in media containing concentrations of 8-hydroxyquinoline varying from 0 to 50 μM , which inhibited growth up to 25% at the highest chelator concentration. The cells and media were examined for metalloporphyrins by the usual ethyl acetate-acetic acid (3:1, v/v) extraction procedure (Dresel & Falk, 1956). If a complex of protoporphyrin with a transition-metal ion were formed it would be soluble in this mixture, would not be extracted from it by concentrated hydrochloric acid and would give strong absorption bands in organic solvents in the 500–600 m μ region (Falk & Nyholm, 1958; Mauzerall & Granick, 1961). No such material was found but it was observed that a considerable amount of fluorescent substance was excreted into the medium, particularly at a chelator concentration of 25 μM . This was extracted from ethyl acetate with 15% hydrochloric acid and had an oxorhodo-type spectrum (Lemberg & Falk, 1951) in ether with maxima in the visible region at 641, 589, 566 and 527 m μ . A similar substance was extracted from the cells. Neither had properties that corresponded with those of any of the known intermediates in haem biosynthesis. Since the unusual pigment was very lipid-soluble, an attempt was made to isolate it from larger volumes of medium by simple ether extraction.

Large (10 l.) bottles of medium containing 8-hydroxyquinoline (25 μM) were inoculated and the cells removed by centrifuging in the usual way at the end of the growth period. The supernatant was extracted twice with 5 l. of ether which was then washed twice with equal volumes of water and concentrated by evaporation. The spectrum of such an ether extract is shown in Fig. 1. When compared with the spectrum of a similar extract from normal medium a considerable decrease in the

absorption due to magnesium protoporphyrin monomethyl ester (Jones, 1963) was noted and new absorption bands were found in the region 600–700 $m\mu$. The material with the oxorhodo-type porphyrin spectrum which had been detected in the ethyl acetate–acetic acid extracts was not found in this simple neutral extract.

Since 8-hydroxyquinoline caused the excretion of an unusual pigment, which gave a porphyrin type of spectrum on acid treatment, the effect of this chelator on haem and bacteriochlorophyll concentrations in the cells was investigated. After growth at various concentrations of chelator the concentrations of haem and bacteriochlorophyll were determined. Haem was determined as if only cytochrome *c* were present, since *Rhodospirillum* haem protein gives a similar pyridine haemochromogen and the concentration of cytochrome *b* was low in both treated and untreated cells.

It was found (Fig. 2) that the total concentration of haem pigments was unaffected by treatment with the chelator but that of bacteriochlorophyll was decreased.

Purification of pigments from medium. The substances secreted into the media in the presence of 8-hydroxyquinoline were extracted into ether, transferred into 70% acetone and chromatographed on a polyethylene column as described in the Materials and Methods section. A number of coloured bands developed. The first band eluted appeared from its spectrum to contain a mixture of a copper chlorin (cf. Sistrom *et al.* 1956) and some free phaeophytins or phaeophorbides. Compounds 4, 5, 6, 7 and 8 were eluted in that order with 70% acetone from the column and the spectra of 4, 5 and 7 are given in Figs. 3 (a) and 3 (b). Compounds 6 and 8 were identified from their spectra and chromatographic properties in the lutidine–water system as the monoesters of magnesium protoporphyrin and protoporphyrin respectively (cf. Jones, 1963; Granick, 1961). Compound 5 was converted into compound 7 by shaking with 5% hydrochloric acid and this suggested that compound 5 was a Mg^{2+} ion complex of either a porphyrin or chlorin. Compound 7 had properties that corresponded with those of the material with the oxorhodo-type spectrum isolated in the preliminary experiments.

Chromatography on 60% acetone. The first, mixed fraction eluted from the column was taken up into ether and washed with 15% hydrochloric acid until no further fluorescent material was extracted. The spectrum of the material remaining in the ether (compound 1) is given in Fig. 4. The acid washings were adjusted to pH 4 with sodium acetate and extracted with ether until no fluorescence remained in the acid layer, and the ether was washed with water until free of acid. The ether

layer was evaporated to dryness, made up in 60% (v/v) acetone and chromatographed on a polyethylene column which was developed with 60% acetone. Two bands developed and the spectra of these two materials (compounds 2 and 3) are given in Fig. 5.

The spectrum of compound 1 agreed very closely with that described by Sistrom *et al.* (1956) for a

Fig. 1. Absorption spectra in ether of pigments extracted from media of *Rhodopseudomonas spheroides*. Broken line: ether extract of untreated medium, 200 ml. of medium being extracted with 200 ml. of ether which was then concentrated to 20 ml. for recording of the spectrum (1 cm. light-path). Continuous line: ether extract of 8-hydroxyquinoline-treated medium, 200 ml. of medium being extracted with 200 ml. of ether which was then concentrated to 25 ml. for recording of the spectrum (1 cm. light-path).

Fig. 2. Effect of 8-hydroxyquinoline on intracellular haem and bacteriochlorophyll concentrations in *Rhodopseudomonas spheroides*. ●, Bacteriochlorophyll; ○, haem (determined as cytochrome *c*).

Cu^{2+} ion-containing chlorophyll derivative extracted from the medium of a mutant of *R. spheroides*. It was also unchanged by treatment with concentrated hydrochloric acid, as would be expected of a copper chlorin or copper phaeophorbide. The position of the band in the red region showed that the material was not a Cu^{2+} ion complex of a porphyrin or of a tetrapyrrole of the level of oxidation of bacteriochlorophyll (i.e. a tetrahydroporphyrin). The complex was digested in perchloric acid-sulphuric acid and copper determined by the method of Piper (1950). A 5 ml. sample of the ether solution, of $E_{419}^{1\text{cm.}}$ 0.96,

contained 4.3 $\mu\text{g.}$ of copper. This suggests the original Cu^{2+} ion complex had an $\epsilon_{\text{max}}^{419\text{m}\mu}$ of approximately 70.

The spectra of two of the materials isolated from the medium correspond closely with those of materials already described in the literature, namely *Chlorobium* phaeophytin-650 and plant phaeophytin *a* (Table 1). However, the solubility of all these materials in 15 % hydrochloric acid showed that no phytol or other long alkyl side chain was present and that the molecules were phaeophorbides and not phaeophytins. The spectrum of compound 3 has an unusual shape resembling that of phaeophytin-660 except that the position of band I is 5 $\text{m}\mu$ further to longer wavelength.

Fig. 3. Absorption spectra in ether of pigments extracted from the medium of 8-hydroxyquinoline-treated cells of *Rhodospseudomonas spheroides*. (a) Compound 4: λ_{max} . 667, 609, 561, 534, 506, 471 and 409 $\text{m}\mu$. (b) Compound 5 (continuous line): λ_{max} . 624, 574, 537 and 438 $\text{m}\mu$; and compound 7 (broken line): λ_{max} . 642, 590, 568, 527 and 421.5 $\text{m}\mu$.

Fig. 4. Absorption spectrum in ether of compound 1 extracted from the medium of 8-hydroxyquinoline-treated cells of *Rhodospseudomonas spheroides*. λ_{max} . 643, 598, 546, 504, 419 and 396 $\text{m}\mu$.

Fig. 5. Absorption spectra in ether of pigments extracted from the medium of 8-hydroxyquinoline-treated cells of *Rhodospseudomonas spheroides*. Compound 2 (broken line): λ_{max} . 659, 603, 554, 532, 502, 469 and 406 $\text{m}\mu$; compound 3 (continuous line): λ_{max} . 671, 611, 545, 514, 482 and 408 $\text{m}\mu$.

Table 1. *Absorption maxima and band ratios in ether of compounds isolated from the medium of Rhodopseudomonas spheroides grown in the presence of 25 μ M-8-hydroxyquinoline, compared with those of some known substances*

Experimental details are given in the text.

Compound	Compound	Spectral properties					
		$\lambda_{\max.}$ (m μ) ...					
Compound 1		$\lambda_{\max.}$ (m μ) ...	643	598	546	504	419
		Band ratios ...	69	13.3	5.5	4.4	100
Compound IIb-1c of Sistrom <i>et al.</i> (1956)		$\lambda_{\max.}$ (m μ) ...	642	597	545	500	419
		Band ratios ...	64	15.8	5.7	5.4	100
Compound 2		$\lambda_{\max.}$ (m μ) ...	659	603	554	532	502
		Band ratios ...	47.8	8.7	3.9	10.7	11.0
<i>R. spheroides</i> mutant pigment-660 (Griffiths, 1962)		$\lambda_{\max.}$ (m μ) ...	660	603	555	531	502
		Band ratios ...	37.8	7.0	2.7	8.1	8.8
Compound 3		$\lambda_{\max.}$ (m μ) ...	671	611	545	514	482
		Band ratios ...	45.7	4.17	13.3	8.4	3.7
<i>Chlorobium</i> phaeophytin-660 (Stanier & Smith, 1960)		$\lambda_{\max.}$ (m μ) ...	665	607	545	514	482
		Band ratios ...	47.9	5.7	13.3	8.9	3.3
Compound 4		$\lambda_{\max.}$ (m μ) ...	667	609	561	534	506
		Band ratios ...	46.0	8.8	3.8	9.2	10.8
Phaeophytin <i>a</i> (Smith & Benitez, 1955)		$\lambda_{\max.}$ (m μ) ...	667	609.5	560	534	505
		Band ratios ...	48.4	7.4	2.7	9.5	11.0

Table 2. *Absorption maxima and band ratios in ether of compound 5 and some of its derivatives compared with those of some known substances*

Experimental details are given in the text.

Compound	Compound	Spectral properties			
		$\lambda_{\max.}$ (m μ) ...			
Compound 5		$\lambda_{\max.}$ (m μ) ...	624	574	537
		Band ratios ...	12.2	6.8	2.8
Protochlorophyll (Koski & Smith, 1948)		$\lambda_{\max.}$ (m μ) ...	623	571	535
		Band ratios ...	12.1	4.6	2.2
<i>R. spheroides</i> mutant pigment-625 (Griffiths, 1962)		$\lambda_{\max.}$ (m μ) ...	624	573	535
		Rel. intensity ...	2	3	4
Compound 7 or acid-treated compound 5*		$\lambda_{\max.}$ (m μ) ...	642	590	568
		Band ratios ...	0.93	6.1	9.1
Protophaeophytin (Koski & Smith, 1948)		$\lambda_{\max.}$ (m μ) ...	638	585	565
		Band ratios ...	0.98	6.25	9.4
Pyridine haemochromogen derived from compound 7*		$\lambda_{\max.}$ (m μ) ...	562	530	
Pyridine haemochromogen derived from protophaeophytin		$\lambda_{\max.}$ (m μ) ...	564	530	

* Treatment of compound 5 with 15% HCl yielded a product indistinguishable from compound 7.

Although compounds 2 and 3 resemble *Chlorobium* phaeophytin-650 and *Chlorobium* phaeophytin-660 in their spectral properties, both gave a positive Molisch phase test and in this differed from the *Chlorobium* compounds (Holt & Morley, 1960). Both compounds rapidly developed a transient yellow colour at the interface followed by an intense green.

Copper complexes of all the phaeophytin-like materials isolated from the medium, and also of phaeophytins *a* and *b*, were prepared and found to differ slightly in band positions from compound 1. The Cu^{2+} ion complex of compound 3 (bands at 634, 600, 547, 505 and 413 m μ) most resembled compound 1 with respect to spectrum. The spectra of the Cu^{2+} ion complexes of compound 4 and of phaeophytin *a* were identical.

Table 2 shows some properties of compound 5 and of some of its derivatives, and these are compared with the properties of plant and bacterial protochlorophylls. The spectral properties of compound 5 are identical with those of protochlorophyll-like material found in mutant strains of *R. spheroides* (Stanier & Smith, 1959; Griffiths, 1962) and differ slightly from those of plant protochlorophyll. This difference is also evident in the Mg^{2+} ion-free pigments and in the pyridine haemochromogens prepared from them.

Effect of increasing concentrations of 8-hydroxyquinoline on the excretion of chlorophyll derivatives. The effect of increasing concentrations of chelator on the production of the plant phaeophytin *a*-like material, bacterial protochlorophyll, magnesium protoporphyrin monomethyl ester and intra-

cellular bacteriochlorophyll was investigated, by using the molar extinction coefficients of the chlorophyll compounds which they closely resemble. As shown in Fig. 6, up to an 8-hydroxyquinoline concentration of $25 \mu\text{M}$, the excretion of the unusual pigments increases as the concentration of bacteriochlorophyll declines.

Investigations of the 8-hydroxyquinoline effect. Sistrom *et al.* (1956) reported that a carotenoid-deficient mutant of *R. spheroides* excreted a number of porphyrins into the medium, some of which resemble the compounds now found in the 8-hydroxyquinoline-treated cultures. It was suggested by Sistrom *et al.* (1956) that this pigment excretion was due to an alteration in the structure of the chromatophore caused by the absence of carotenoids, which decreased the amount of bacteriochlorophyll that could fit in the malformed chromatophore. It was postulated that the surplus bacteriochlorophyll was degraded and excreted as phorbides.

Carotenoid concentrations in the chelator-treated cells, however, were not appreciably altered, but the possibility that the primary effect was on carotenoid production was further investigated. *R. spheroides* was grown in the presence of diphenylamine, which is known to inhibit carotenoid production in photosynthetic bacteria (Goodwin & Osman, 1954; Wassink & Kronenberg, 1962). Although a severe decrease of carotenoid synthesis was induced by this treatment, no excretion of porphyrins occurred, showing that

Fig. 6. Effect of 8-hydroxyquinoline on pigments produced by *Rhodospseudomonas spheroides*. ●, Intracellular bacteriochlorophyll; ○, magnesium protoporphyrin monomethyl ester in the medium; ▲, compound 5 in the medium; □, compound 4 in the medium.

excretion of chlorophyll derivatives did not necessarily follow from inhibited carotenogenesis.

The omission of metal ions from the growth medium of *R. spheroides* did not cause the excretion of phorbides. It therefore appears that the effect of the chelator was not simply to decrease the amount of metal ions available in the medium. However, experiments where metal ions were omitted, but 8-hydroxyquinoline was added, gave interesting

Fig. 7. Effect of the omission of various metal ions from the growth medium of *Rhodospseudomonas spheroides* on the secretion of ether-soluble pigments into the medium caused by the presence of 8-hydroxyquinoline. The medium (200 ml.) was extracted with an equal volume of ether which was concentrated to the volume indicated. Absorption spectra were recorded, with 1 cm. light-path, in the range 560–700 $m\mu$. (a) —, Complete medium containing no 8-hydroxyquinoline, 20 ml. of ether concentrate; ---, complete medium containing 8-hydroxyquinoline ($25 \mu\text{M}$), 15 ml. of ether concentrate; — · —, Fe^{2+} ion-deficient medium containing 8-hydroxyquinoline ($25 \mu\text{M}$), 12 ml. of ether concentrate. (b) —, Cu^{2+} ion-deficient medium containing 8-hydroxyquinoline ($25 \mu\text{M}$), 9 ml. of ether concentrate; ---, Mn^{2+} ion-deficient medium containing 8-hydroxyquinoline ($25 \mu\text{M}$), 18 ml. of ether concentrate; — · —, Zn^{2+} ion-deficient medium containing 8-hydroxyquinoline ($25 \mu\text{M}$), 9 ml. of ether concentrate.

results. The usual excretion of phorbides occurred in all cases except where Cu^{2+} ions were omitted from the medium (Figs. 7a and 7b); it appears that the chelate is effective only in the presence of Cu^{2+} ions. The detection of the copper phaeophorbide in chelator-treated cultures now appeared to be significant. It seemed possible that this compound was formed by an exchange reaction of Cu^{2+} ions for Mg^{2+} ions in an intermediate in bacteriochlorophyll biosynthesis rather than by insertion of Cu^{2+} ion into a metal-free ring system since a Mg^{2+} ion-free pigment did not seem to be a probable intermediate at this stage of biosynthesis. The work of Tait & Gibson (1961) indicates that alteration of the porphyrin side chains in chlorophyll biosynthesis occurs after the stage of insertion of Mg^{2+} ions. These workers found that magnesium protoporphyrin but not protoporphyrin was a substrate for enzymic methylation in *R. sphaeroides*. Also, the enzymic reduction from the oxidation level of protochlorophyll to chlorophyll *a* requires the Mg^{2+} ion-containing pigment. It therefore is unlikely that a Mg^{2+} ion-free chlorophyll intermediate is available for the insertion of Cu^{2+} ions.

Effect of 8-hydroxyquinoline on intracellular pyrrole pigments. Acetone extracts of normal and chelator-treated cells were chromatographed on polyethylene columns as described above. Both extracts contained large amounts of bacteriochlorophyll together with small but significant quantities of magnesium protoporphyrin monomethyl ester and the Mg^{2+} ion-free porphyrin ester. Both extracts also contained a small amount of material running behind bacteriochlorophyll with a strong absorption at $680\text{ m}\mu$ but which was not obtained free of impurities. It appears possible that this substance was 2-acetylmethyl phaeophorbide *a*, an oxidation product of bacteriophaeophytin, which has been described in other extracts of photosynthetic bacteria (Smith & Benitez, 1955), or allomerized bacteriochlorophyll (cf. Kaplan & Silberman, 1959), but insufficient material was available to permit further testing. Extracts of the inhibited cells contained in addition the compounds 1, 2, 3, 4, 5 and 7 previously (see above) found in the medium. Compound 5, resembling protochlorophyll, was present in much the greatest concentration, in some cases being present to approximately 20% of the bacteriochlorophyll concentration. It therefore appears that the porphyrin derivatives found in the medium of inhibited cells originate from inside the cells and are not further extracellular breakdown products.

DISCUSSION

The results described in the present paper show that 8-hydroxyquinoline inhibits the formation of

bacteriochlorophyll by *R. sphaeroides* and causes the formation of several unusual pigments, although haem synthesis is almost unaffected (Fig. 2). For this chelator to be effective the presence of Cu^{2+} ions was essential and none of the other metal ions normally present in the medium could substitute for it. Although such a phenomenon would be expected if 8-hydroxyquinoline acted by inserting Cu^{2+} ions into a porphyrin which then interfered with further steps in the biosynthetic sequence, no such copper porphyrin was found. The detection of a compound (compound 1) with the spectroscopic and solubility properties of a copper phaeophorbide suggested that insertion of Cu^{2+} ions may take place, not into a porphyrin, but into a dihydroporphyrin or phaeophorbide nucleus. Although no exchange of added $^{28}\text{Mg}^{2+}$ ions with chlorophyll *a* has been detected in aqueous organic solvents (Aronoff, 1962), exchanges of metal ions have been demonstrated in the tetraphenyl porphyrin series where a metal ion with a lower stability in the metal-ion-porphyrin complex was replaced by one with a higher stability (Barnes & Dorough, 1950). Also, Corwin & Melville (1955) have shown that Mg^{2+} ion-porphyrin complexes are more resistant to exchange than chlorin complexes, which suggests that in the biosynthetic pathway Cu^{2+} ions are more likely to exchange with pigments at the oxidation level of chlorins, such as chlorophyll *a* or the *Chlorobium* chlorophylls, than with pigments at the oxidation level of the porphyrins, such as protochlorophyll or magnesium protoporphyrin.

Since Cu^{2+} ions were ineffective in causing the formation of a copper phaeophorbide in the absence of 8-hydroxyquinoline, it appears possible that the chelator acts by increasing the rate of exchange of Cu^{2+} for Mg^{2+} ions in one particular susceptible pigment. This stimulation may be due simply to the role of the chelator in forming a lipid-soluble Cu^{2+} ion complex which is more accessible to the sites of bacteriochlorophyll biosynthesis or to its action in catalysing the exchange (cf. Lowe & Phillips, 1961, 1962). A combination of these effects may also occur.

The finding (Fig. 6) that in chelate-inhibited cultures the production of the protochlorophyll-like and phaeophytin *a*-like material increases as the intracellular bacteriochlorophyll concentration falls suggests that these compounds may be intermediates, or derived from intermediates, in bacteriochlorophyll biosynthesis. These may accumulate if the formation of a copper phaeophorbide or phaeophytin blocks normal biosynthesis. This hypothesis is strengthened by the finding that compounds 2 and 5 have been detected in *R. sphaeroides* mutants blocked in bacteriochlorophyll synthesis (Griffiths, 1962), which shows that

they are not decomposition products of bacteriochlorophyll. Compound 3 has not previously been detected in *R. spheroides* and, like compound 2, resembles a *Chlorobium* phaeophorbide in its spectral properties and may be closely related to these compounds although possessing an isocyclic ring. A compound resembling bacteriochlorophyll has been isolated from a strain of *Chlorobium thiosulphatophilum* in which the main pigment is chlorophyll-650 and in *Chloropseudomonas ethylicum* in which the main pigment is chlorophyll-660 (cf. Olson & Romano, 1962), showing that in one strain of photosynthetic bacteria a variety of chlorophylls can occur.

8-Hydroxyquinoline-inhibited cultures of *R. spheroides* have proved more convenient than mutants as a source of tetrapyrrole pigments of possible biosynthetic importance since there are no problems of mutant instability. It is hoped that they will prove useful in studies of pigment production but, as with mutant studies, there is always a possibility that the compounds that accumulate when one step in a biosynthetic sequence is blocked are not intermediates but more stable derivatives of the intermediates.

SUMMARY

1. The addition of 8-hydroxyquinoline to the medium of *Rhodopseudomonas spheroides* caused a decrease in the intracellular concentration of bacteriochlorophyll but not of haem pigments or carotenoids. This inhibition was accompanied by the secretion of tetrapyrrolic pigments into the medium.

2. 8-Hydroxyquinoline was ineffective in these experiments when Cu^{2+} ions were omitted from the medium, but the omission of other metal ions did not decrease the secretion of the tetrapyrrolic pigments.

3. The extracellular pigments were purified by chromatography on polyethylene columns and eight compounds were isolated; two of them were well-known compounds, magnesium protoporphyrin monomethyl ester and protoporphyrin monomethyl ester, and were found in the medium of inhibitor-free cultures. The other compounds resembled phaeophorbide *a*, *Chlorobium* phaeophorbide-660, *Chlorobium* phaeophorbide-650, magnesium vinyl phaeoporphyrin *a*₅, vinyl phaeoporphyrin *a*₆ and a copper phaeophorbide in their spectroscopic properties.

4. The cells, when grown in the presence of the chelator, were found to contain the same eight pigments also present in the medium.

5. As the concentration of 8-hydroxyquinoline in the medium was increased the intracellular bacteriochlorophyll concentrations decreased, but

the excretion of phaeophorbide *a*-like and magnesium vinyl phaeoporphyrin *a*₅-like material increased.

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A Note on the Characterization of Carbohydrate Sulphates by Acid Hydrolysis

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The recent observation (Barry & McCormick, 1957) that glucose 3-sulphate is hydrolysed significantly faster than galactose 6-sulphate has called in question the tentative conclusion drawn by Percival (1949), that differences in rates are generally too small to be useful for characterization purposes. The rates of hydrolysis of a number of carbohydrate sulphates are now reported, and it is shown that such rates can be useful for the characterization of single sulphates, and also for 'fingerprinting' compounds containing several different ester groups. The technique has helped to establish that λ -carrageenan contains galactose 2,6-disulphate units in addition to the galactose 4-sulphate units known to be present (Rees, 1963).

MATERIALS

Carbohydrate sulphates. The monosaccharide sulphates were synthesized by definitive routes (Peat, Turvey, Clancy & Williams, 1960; Turvey & Williams, 1962) and generously given by Dr J. R. Turvey. Evidence for the types of sulphate present in porphyran and the carrageenan polysaccharides is summarized elsewhere (Peat & Rees, 1961; Rees & Conway, 1962; Rees, 1961, 1963). Fucoidin and the *Enteromorpha* polysaccharide were given by Dr Elizabeth Percival. The dextran sulphate was Dr Ricketts's preparation E (Ricketts, 1956) and was known to contain a high proportion of equatorial ester. Laminarin sulphate was given by Dr D. Walker of Boots Pure Drug Co. Ltd. The mucopolysaccharides were given by Dr K. S. Dodgson (Lloyd, Dodgson, Price & Rose, 1961), the shark preparation being prepared from the head cartilage of the blue shark (*Charcharinus glaucus*) and the chondroitin sulphate A from ox cartilage by the usual methods. The presence of a small amount of 6-sulphate (probably contaminating chondroitin sulphate C) in the latter polysaccharide was confirmed by infrared spectroscopy.

METHODS

Acid hydrolysis of carbohydrate sulphates. Samples of a solution of the compound in 0.250 N-hydrochloric acid (0.250 ml., containing about 50 μ g. of esterified sulphate) were sealed in Pyrex test tubes (3 in. $\times$ $\frac{1}{8}$ in.) and heated in a boiling-water bath for varying periods. After cooling, centrifuging and opening, the contents of each tube were exactly neutralized with sodium hydroxide (2.50 N; 0.025 ml.) and a trace of Cetavlon and chloroaminodiphenyl reagent (0.250 ml.) added. The latter reagent was prepared as described by Jones & Letham (1954). After mixing, keeping for several hours, and clarification on the centrifuge, a portion of the supernatant solution was diluted and the extinction measured as described by Jones & Letham.

To avoid interference in the determination by non-hydrolysed polysaccharide sulphate, it was normally satisfactory to allow the reaction to proceed for about 30 min. to allow extensive glycosidic hydrolysis to occur, before making the first measurement. No interference through precipitation of chloroaminodiphenyl by polysaccharide was then observed. In certain cases (e.g. dextran sulphate) where the glycosidic linkages were very stable, or the sulphate very acid-labile, this was not practicable and an alternative procedure was followed (Foster, Martlew, Stacey, Taylor & Webber, 1961; K. O. Lloyd & P. F. Lloyd, unpublished work). After neutralization as above, a solution of Cetavlon (sufficiently concentrated to give an excess; 0.250 ml.) was added and the precipitate removed on the centrifuge. The supernatant solution (0.250 ml.) was then treated with chloroaminodiphenyl reagent, and the experiment completed as usual.

In certain cases, it was necessary to introduce corrections for the ultraviolet absorption of the degradation products of 3,6-anhydrogalactose (this sugar being a component of some of the polysaccharides that were examined). Larger samples were then heated in the sealed tubes and, after cooling and opening, portions (0.250 ml.) were removed and treated as usual. Further portions of each solution were diluted in the same ratio, but without the