Haemoproteins and Haem Synthesis in Facultative Photosynthetic and Denitrifying Bacteria

By R. J. PORRA* AND JUNE LASCELLES

Microbiology Unit, Department of Biochemistry, University of Oxford

(Received 27 April 1964)

1. A simple spectrophotometric method is described for the measurement of various haemoproteins in extracts of photosynthetic and non-photosynthetic bacteria. The method is based on measurements of difference spectra at the Soret maxima. 2. In photosynthetic bacteria of the Athiorhodaceae group the concentration of carbon monoxide-binding haemoprotein and of cytochromes of the *b* and *c* types is two to three times as high in anaerobically grown cells as in those grown aerobically. 3. During the adaptation of *Rhodospseudomonas spheroides* 8253 to form photosynthetic pigments the concentration of each of these haemoproteins increases in parallel with that of the bacteriochlorophyll. 4. The carbon monoxide-binding haemoprotein in aerobically grown *Rps. spheroides* 8253, in contrast with anaerobically grown cells, is predominantly in the particulate fraction of extracts prepared by ultrasonic vibration. The *b*- and *c*-type cytochromes are approximately equally distributed between each fraction in extracts from both types of cell. 5. Extracts of *Micrococcus denitrificans* grown anaerobically on nitrate contain more cytochromes of the *b* and *c* types, as well as of the carbon monoxide-binding pigment, than do those from aerobically grown cells. 6. The activity of ferrochelatase in both *Rps. spheroides* 8253 and *M. denitrificans* was similar in extracts from cells grown aerobically and anaerobically, though the haemoprotein content was higher under the latter conditions. Coproporphyrinogen oxidative decarboxylase could not be demonstrated in cell-free extracts of either organism.

Bacteria of the Athiorhodaceae group, when grown under photosynthetic conditions, contain catalase and *b*- and *c*-type cytochromes as well as a carbon monoxide-binding haemoprotein originally referred to as *Rhodospirillum* haem protein but more recently designated cytochromoid *c* (Kamen, 1963; Bartsch, 1963). Organisms of this group grow anaerobically in the light, when the cells are rich in photosynthetic pigments (pigmented cells), or aerobically in the dark, when the cells contain little photosynthetic pigment (non-pigmented cells).

Quantitative determination of the various haemoproteins in *Rhodospirillum rubrum* and *Rhodospseudomonas spheroides* grown under the different conditions have given conflicting results (Clayton, 1959; Geller, 1962). These discrepancies can probably be attributed to difficulties in the methods used to determine the haemoproteins; in both investigations these were based on extraction and fractionation techniques. In the present work, spectrophotometric techniques have been developed

for estimating the individual haemoproteins in cell-free extracts of the Athiorhodaceae. Although several assumptions are made in this assay the results were reproducible and satisfactory for comparative purposes. These methods have been used to determine the concentration of haemoproteins in organisms grown under various conditions, and in suspensions of *Rps. spheroides* as they become adapted from the non-pigmented to the pigmented state to see if there was any correlation between haemoprotein formation and the development of photosynthetic pigments. Since an increase in haemoprotein was observed during this adaptation, the activities of several enzymes concerned in haem biosynthesis were also investigated. In addition the haemoproteins and biosynthetic enzymes of the denitrifying organism *Micrococcus denitrificans* were examined.

EXPERIMENTAL

Chemicals. Triton X-100, a neutral detergent of the alkyl-phenoxypolyethoxyethanol group produced by Rohm and Haas Co., Philadelphia, Pa., U.S.A., was obtained from

* Present address: Division of Plant Industry, C.S.I.R.O., Canberra, A.C.T., Australia.

Charles Lennig and Co., London, W.C. 1. Mesoporphyrin IX, used in the ferrochelatase assay, was obtained from Fluka, A.-G., Basle, Switzerland. Coproporphyrinogen III, the substrate of coproporphyrinogen oxidative decarboxylase, was prepared as described by Porra & Falk (1964) from coproporphyrin III isolated from culture fluids of *Rps. spheroides* (Lascelles, 1956). Carbon monoxide was supplied by Imperial Chemical Industries, Pilkington-Sullivan Works, Widnes, Lancs.

Organisms. The following organisms were used: *Rhodospseudomonas spheroides* strain N.C.I.B. 8253 (from the National Collection of Industrial Bacteria), strain CC1 (high-catalase mutant, from Dr R. Clayton; see Clayton & Smith, 1960) and strain Ga (from Dr W. R. Sistrom); *Rhodospirillum rubrum* and *Rhodospseudomonas capsulata* were strains no. 1.1.1 and no. 2.3.11 respectively from the collection of Professor C. B. van Niel. *Micrococcus denitrificans* was supplied by Dr W. Verhoeven. Stock cultures of the photosynthetic organisms were maintained as described by Lascelles (1959); *M. denitrificans* was maintained as described by Chang & Morris (1962).

Growth of organisms. *Rps. spheroides* and *Rsp. rubrum* were grown on the malate-glutamate medium described by Lascelles (1959), *Rps. capsulata* on the succinate-glutamate medium of Carr & Lascelles (1961) and *M. denitrificans* on the succinate medium with KNO_3 (10 mM) as described by Chang & Morris (1962). Conditions for growth of the facultative photosynthetic organisms in the light and in the dark were as described by Lascelles (1959). Aerobic and anaerobic cultures of *M. denitrificans* were as described by Chang & Morris (1962).

Preparation of cell-free extracts. (a) Crude extract. The organisms were suspended in 0.04 M-tris buffer, pH 7.5, to a final cell concentration of 15–25 mg. dry wt./ml. and disrupted by ultrasonic vibration for 5 min. at 600 w and 25 kcyc./sec. with a Mullard ultrasonic generator type E7590B: the transducer assembly was cooled in an ice bath. Whole organisms and debris were removed by centrifugation at 6000 g for 15 min. at 4°. The supernatant (crude extract) contained about 10 mg. of protein/ml. Before use in enzyme assays the extracts were dialysed overnight at 0° against 0.04 M-tris buffer, pH 7.5.

(b) Soluble and particulate fractions. Crude extracts, prepared as above, were divided into soluble and particulate fractions by centrifugation at 105 000 g for 2 hr. at 0° in the Spinco model L ultracentrifuge. The supernatant (soluble fraction) was decanted and the pellet resuspended in an equal volume of 0.04 M-tris buffer, pH 7.5 (particulate fraction).

Clarification of cell-free extracts. For determining the haemoprotein content, cell-free extracts were clarified by the addition of 2% (v/v) Triton X-100. Goldberger, Smith, Tisdale & Bomstein (1961) showed that treatment of cytochrome *b* from ox-liver mitochondria with this detergent does not cause denaturation leading to the formation of a carbon monoxide-binding pigment, and we have now shown that pure catalase preparations are similarly unaffected by treatment with Triton X-100.

Assay of haemoproteins. The method below describes simple spectrophotometric techniques to determine the concentrations of both carbon monoxide-binding haemoproteins and cytochromes. The latter were determined by a method adapted from the spectrophotometric technique used by Chance (1952) in which the α -peaks of the cyto-

chromes in the difference spectra of the reduced and oxidized cells were measured. Although this method can be used to study the cytochromes of *Rps. spheroides* (Table 2), it was found to be unsatisfactory for routine use: the α -peaks were small and there was interference to the spectra between 550 and 600 m μ caused by a reaction between bacteriochlorophyll and the potassium ferricyanide used to oxidize the cytochromes. To overcome these difficulties measurements were made in the Soret region. All spectra were obtained with an Optica model CF4DR recording spectrophotometer.

Expression of results. All extinction coefficients represent the extinctions of solutions of the various haemoproteins in which the concentration of the haem prosthetic group is millimolar; thus to obtain the concentration of cytochromoid *c*, which contains two haem prosthetic groups, the relevant data, expressed in terms of cytochromoid *c* haem, should be halved.

Carbon monoxide-binding haemoproteins. The cell-free extracts, in suitable dilutions, were assayed spectrophotometrically at pH 4.75 for carbon monoxide-binding haemoproteins by using the carbon monoxide-minus-reduced difference spectrum (Bartsch & Kamen, 1958). To 1.0 ml. of a non-pigmented extract or to 1.0 ml. of an aqueous 1:3 (v/v) dilution of pigmented extract was added 0.5 ml. of the 0.1 M-citrate-tris buffer, pH 4.75, described by Bartsch & Kamen (1958). This buffered solution was then mixed with sodium dithionite (approx. 1 mg.) and 0.4 ml. was transferred to a micro-cuvette (10 mm. light-path and 0.5 ml. capacity). Carbon monoxide was gently bubbled through the remainder of the solution in a test tube, and 0.4 ml. of this solution transferred to a second micro-cuvette and the difference spectrum recorded. The difference in extinction between the Soret peak of the carbon monoxide-haemoprotein complex and the trough caused by the Soret absorption of the reduced haemoprotein ($\Delta E_{1\text{cm}}^{\text{CO-red.}}$) was measured. Cytochromoid *c*, isolated from *Rsp. rubrum*, has $\Delta \epsilon_{\text{cm}}^{\text{CO-red.}}$ 233 (calculated from the data of Bartsch & Kamen, 1958); the peak and trough occurred at 413 and 432 m μ respectively. The same $\Delta \epsilon_{\text{cm}}^{\text{CO-red.}}$ was used for the carbon monoxide-binding haemoproteins of *Rps. spheroides*, although they exhibited peaks at 418 m μ in soluble preparations and at 423 m μ in particulate preparations. The concentration of carbon monoxide-binding haemoprotein in the extracts was calculated as $\Delta E_{1\text{cm}}^{\text{CO-red.}}/0.233$ m μ moles/ml.

Total cytochrome content. The total cytochrome content (*b* plus *c*) of the cell-free extracts was estimated spectrophotometrically from the reduced-minus-oxidized difference spectra by measuring the difference in extinction between the Soret absorption of the reduced haemoprotein and the trough caused by the Soret absorption of the oxidized haemoproteins ($\Delta E_{1\text{cm}}^{\text{red.-ox.}}$). Equal volumes (0.4 ml.) of undiluted non-pigmented extract or of a 1:3 (v/v) aqueous dilution of pigmented extract were added to two micro-cuvettes (10 mm. light-path and 0.5 ml. capacity). To one cuvette was added sodium dithionite (approx. 0.3 mg.) and to the other 0.02 ml. of 1 mM-potassium ferricyanide. After the difference spectrum was recorded, the cuvette containing the oxidized haemoproteins was examined by hand spectroscopy to check that re-reduction if the haemoproteins had not occurred with the reappearance of the α - and β -bands between 500 and 570 m μ . Cytochromoid *c*, isolated from *Rsp. rubrum*, has a reduced-minus-oxidized difference spectrum with a Soret peak at 420 m μ , a trough at 390 m μ and $\Delta \epsilon_{\text{cm}}^{\text{red.-ox.}}$ 140.5 (calculated from the data of

Bartsch & Kamen, 1958). There are no reduced-minus-oxidized difference spectra available for the cytochromes of *Rps. spheroides*; only absolute spectra are available for cytochrome *c* (Orlando, 1962) and cytochrome *b* (Orlando & Horio, 1961). It was assumed that the cytochrome *c* present in *Rps. spheroides* was similar to *Chromatium* cytochrome *c*₂, with a reduced-minus-oxidized difference spectrum revealing a peak at 422 mμ, a trough at 406 mμ and $\Delta\epsilon_{1\text{cm}}^{\text{red.}-\text{ox.}}$ 175.7 (Bartsch & Kamen, 1960). It was also assumed that the cytochrome *b* present had $\Delta\epsilon_{1\text{cm}}^{\text{red.}-\text{ox.}}$ 175.7 like cytochrome *c*₂ but a peak in the reduced-minus-oxidized spectrum at about 428 mμ and a trough at about 420 mμ like cytochrome *b* from *Rsp. rubrum* (Chance & Smith, 1955).

The total cytochrome content (*b* plus *c*) of the extracts was calculated as:

$$\frac{X(\Delta E_{1\text{cm}}^{\text{red.}-\text{ox.}}) - \frac{140.5}{233}(\Delta E_{1\text{cm}}^{\text{CO}-\text{red.}})}{0.1757} \text{ m}\mu\text{moles/ml.}$$

where $\frac{140.5}{233}(\Delta E_{1\text{cm}}^{\text{CO}-\text{red.}})$ represents the contribution made

by the carbon monoxide-binding haemoprotein to the reduced-minus-oxidized difference spectrum. The derivation of the expression $X(\Delta E_{1\text{cm}}^{\text{red.}-\text{ox.}})$ is described below.

Correction factor. The reduced-minus-oxidized difference spectrum of a mixed solution of cytochrome *c* and cytochromoid *c* would exhibit a peak at about 422 mμ and a trough at about 400 mμ. When mixed with cytochrome *b* the peak would become broader and displaced towards longer wavelengths. The difference in extinction $X(\Delta E_{1\text{cm}}^{\text{red.}-\text{ox.}})$ represents the extinction difference in the Soret region that would be expected if all the haemoproteins absorbed at the same wavelength. The correction factor, *X*, was derived from the Soret region of the reduced-minus-oxidized difference spectra of the pyridine haemochromogens of deuterohaem (Soret peak 406 mμ) and of protohaem (419 mμ). Deuterohaemochromogen was used to represent both the solubilized carbon monoxide-binding haemoprotein and cytochrome *c*, and protohaemochromogen was used to represent cytochrome *b*, since the Soret bands of these haemochromogens, like those of the haemoproteins, are separated by about 10 mμ.

Solutions of deuterio- and proto-haemochromogens were prepared in the alkaline pyridine reagent described by Paul, Theorell & Åkesson (1953) and the reduced-minus-oxidized difference spectra were recorded from 375 to 580 mμ by the method of Porra & Jones (1963*a*). The concentrations of the solutions were adjusted so that the $\Delta E_{1\text{cm}}^{\text{red.}-\text{ox.}}$ values were identical and equal to 0.465. These two solutions were then mixed in various proportions to represent the two classes of haemoproteins mixed in the same proportion. If the peaks and troughs of the two haemochromogens occurred at identical wavelengths then $\Delta E_{1\text{cm}}^{\text{red.}-\text{ox.}}$ for all these mixtures would be 0.465; since the peaks are separated by about 10 mμ the observed $\Delta E_{1\text{cm}}^{\text{red.}-\text{ox.}}$ of the mixtures was always less than 0.465. The correction factor (Fig. 1) was calculated from:

$$X = \frac{0.465}{\text{observed } \Delta E_{1\text{cm}}^{\text{red.}-\text{ox.}}}$$

Individual concentrations of cytochromes *b* and *c*. Approximate concentrations of cytochromes *b* and *c* were estimated by comparison of the profile of the reduced-minus-oxidized

Fig. 1. The α -peak profiles with the corresponding correction factor, *X*. A series of α -peak profiles of deuterio- and protohaemochromogen mixtures was obtained (see the text), and the correction factor, *X*, calculated as described in text, is given for each mixture, which is defined by its deuteriohaemochromogen:protohaemochromogen ratio.

difference spectra in the α -peak region with a series of α -peak profiles obtained from the haemochromogen mixtures (Fig. 1). The α -peaks of deuterohaemochromogen (545 mμ) and protohaemochromogen (557 mμ), like those of cytochrome *c*₂ from *Chromatium* (553 mμ) (Bartsch & Kamen, 1960) and cytochrome *b* from *Rsp. rubrum* (563 mμ) (Chance & Smith, 1955), are separated by about 10 mμ.

Assay of enzymes. Ferrochelatase was determined by the method of Porra & Jones (1963*a*), except that phosphate buffer was replaced by tris buffer and GSH was omitted.

Coproporphyrinogen oxidative decarboxylase (coprogenase) was measured by the methods of Sano & Granick (1961) and also by the method of Porra & Falk (1964).

Determination of protein. Protein concentrations were determined by the method of Lowry, Rosebrough, Farr & Randall (1951), with crystalline bovine serum albumin as standard.

RESULTS

Total haem content of Rhodospseudomonas spheroides. This was determined by the pyridine haemochromogen method in cell-free extracts prepared from *Rps. spheroides* 8253 and *Rps. spheroides* CC1 grown anaerobically in the light (pigmented cells) and aerobically in the dark (non-pigmented cells). The extracts from the pigmented cells contained about twice as much haem as the extracts from the non-pigmented cells (Table 1).

The haemochromogen difference spectra of *Rps. spheroides* 8253 revealed typical α - and β -bands at

552 and 522 $m\mu$ with a shoulder at 557 $m\mu$. The shoulder at 557 $m\mu$ was presumably due to the presence of a mixture of cytochrome *b* and catalase, both of which contain protohaem prosthetic groups; the main peak at 552 $m\mu$ was assumed to be due to a mixture of cytochromoid *c* and cytochrome *c*.

The total haem content of extracts of *Rps. spheroides* CC1 (high-catalase mutant) was much higher than that in extracts of *Rps. spheroides* 8253. The haemochromogen spectra revealed typical α - and β -bands; the α -band, however, showed an α -peak at 557 $m\mu$ and a shoulder at 552 $m\mu$; the peak at 557 $m\mu$ was presumed to be due to a mixture of cytochrome *b* and catalase in which the catalase is predominant. This is supported by the results in Table 2, which show very little difference in the cytochrome or carbon monoxide-binding haemoprotein content of the two strains; the method used to estimate these haemoproteins would not detect catalase, which does not react with potassium ferri-cyanide, sodium dithionite or carbon monoxide under the conditions of the assays.

In strain 8253 the total haem content as determined by the pyridine haemochromogen method was slightly higher than the sum of the cytochrome and carbon monoxide-binding haemoproteins in the same extract (Expt. 1 in Table 2). This could be due to the small amount of catalase known to be present in the wild-type organism (Clayton, 1959).

Haemoprotein content of various photosynthetic bacteria. The concentration of haemoproteins was determined in cell-free extracts of pigmented and

Table 1. *Total haem content of crude extracts of Rhodospseudomonas spheroides*

Total haem content was determined from the difference spectra of the reduced and oxidized pyridine haemochromogens (Porra & Jones, 1963*a*) by using $\Delta\epsilon_{\text{max}}$ 22.0, the value obtained by Jones (1963) for the pyridine haemochromogen of cytochrome *c*.

	Haem content ($m\mu$ moles/ mg. of protein)
<i>Rps. spheroides</i> 8253	
Pigmented extract	0.82
Non-pigmented extract	0.32
<i>Rps. spheroides</i> CC1 (high-catalase mutant)	
Pigmented extract	2.2
Non-pigmented extract	1.07

Table 2. *Haemoprotein content of crude extracts of various bacteria of the Athiorhodaceae*

The haemoproteins were determined as described in the Experimental section except in Expt. 1*a* in which the method of Chance (1952) was used.

Expt. no.	Organism	Type of extract	Haemoprotein content ($m\mu$ mole of haem/mg. of protein)				
			CO-binding haemoprotein	Total cytochrome	Cytochrome <i>b</i>	Cytochrome <i>c</i>	Total haemoprotein
1	<i>Rps. spheroides</i> 8253	Non-pigmented	0.08	0.16	0.10	0.06	0.24
		Pigmented	0.22	0.38	0.19	0.19	0.60
1 <i>a</i>	<i>Rps. spheroides</i> 8253	Non-pigmented	—	0.09	0.06	0.03	—
		Pigmented	—	0.50	0.20	0.30	—
2	<i>Rps. spheroides</i> 8253	Non-pigmented	0.05	0.09	0.07	0.02	0.14
		Pigmented	0.22	0.22	0.11	0.11	0.44
3	<i>Rps. spheroides</i> 8253	Non-pigmented	0.04	0.22	0.10	0.12	0.26
		Pigmented	0.25	0.35	0.18	0.17	0.60
4	<i>Rps. spheroides</i> CC1 strain	Non-pigmented	0.08	0.12	0.06	0.06	0.20
		Pigmented	0.22	0.36	0.18	0.18	0.58
5	<i>Rps. spheroides</i> Ga strain	Non-pigmented	0.10	0.31	0.12	0.19	0.40
		Pigmented	0.23	0.70	0.28	0.42	0.93
6	<i>Rps. capsulata</i>	Non-pigmented	0.12	0.23	0.05	0.18	0.35
		Pigmented	0.26	0.27	0.06	0.21	0.53
7	<i>Rsp. rubrum</i>	Non-pigmented	0.03	0.17	0.03	0.14	0.21
		Pigmented	0.09	0.23	0.05	0.18	0.32

non-pigmented forms of several organisms of the Athiorhodaceae group (Table 2). The haemoprotein content of the different batches of *Rps. spheroides* 8253, grown under the same conditions, were of the same order, and the variations that occurred might be attributed to small variations in experimental conditions. The extracts used in Expt. 1 were also assayed for cytochromes *b* and *c* by the method of Chance (1952), and the values so obtained (see Expt. 1a) were in reasonable agreement with those of Expt. 1.

In all the organisms examined the total haemoprotein content was higher in extracts from pigmented cells than non-pigmented cells. The higher concentration of total haemoprotein in the pigmented cells was reflected in each of the individual haemoproteins.

Haemoprotein synthesis during adaptation to form photosynthetic pigments. When suspensions of aerobically grown cells of *Rps. spheroides* 8253 are incubated under low aeration the photosynthetic pigments are rapidly synthesized (Lascelles, 1959). Incubation of cells under these conditions resulted in an increase in total haemoproteins of about 20%,

Fig. 2. Increase in haemoprotein and bacteriochlorophyll content of *Rps. spheroides* 8253 during adaptation from the non-pigmented to pigmented condition. Aerobically grown (non-pigmented) cells were exposed to conditions of low aeration for 1½ hr. The cells were then harvested and resuspended in fresh medium and incubated anaerobically in the light. Samples were removed at intervals, the cells harvested and washed, and crude extracts prepared for determination of haemoprotein content (see the Experimental section). The haemoprotein content (●) is expressed as μmole of haem/mg. of protein. The bacteriochlorophyll content (○) was determined by the method of Cohen-Bazire, Sistrom & Stanier (1957) and is expressed as μmoles of bacteriochlorophyll/ml. of bacterial suspension.

whereas the bacteriochlorophyll concentration increased about tenfold (Fig. 2). On transfer of such suspensions to anaerobic conditions in the light the concentration of haemoproteins increased in parallel with bacteriochlorophyll (Fig. 2). The cytochromes and carbon monoxide-binding haemoproteins all increased to the same extent in the adapting cells.

Location of haemoproteins in extracts of Rhodospseudomonas spheroides 8253. Study of the distribution of haemoproteins between the soluble and particulate fractions of *Rps. spheroides* 8253 showed that the carbon monoxide-binding pigment in extracts from anaerobically grown cells is more tightly bound to particulate matter than that from photosynthetically grown organisms (Table 3). The carbon monoxide-binding haemoproteins in both types of extracts, when bound to particles, had a Soret absorption at 422 mμ, but solubilization by ultrasonic vibration or treatment with Triton X-100 displaced the Soret maxima to 418 mμ. Cytochromes *b* and *c* are more evenly distributed after ultrasonic treatment between particulate and soluble fractions in extracts of cells grown under both conditions (Table 3).

The distribution of haemoproteins was also examined in cell-free extracts of *Rps. spheroides* 8253 prepared by treatment with lysozyme (Orlando, Levine & Kamen, 1961). In preparations from both aerobically and anaerobically grown cells 70% of the haemoproteins were found to be associated with the particulate fraction (compare with Table 3).

Enzymes of haem biosynthesis in Rhodospseudomonas spheroides 8253. δ-Aminolaevulate synthase and δ-aminolaevulate dehydratase increase in activity when suspensions of *Rps. spheroides* 8253 become adapted from the non-pigmented to the pigmented state (Lascelles, 1959, 1960). However, the ferrochelatase activity (Porra & Jones, 1963a,b) was identical in extracts of non-pigmented and pigmented cells of *Rps. spheroides* 8253; both extracts formed 4.0 μmoles of mesohaem/mg. of protein/hr. In mammalian tissue ferrochelatase is mainly associated with mitochondria (Lochhead & Goldberg, 1961); in both the pigmented and non-pigmented extracts of *Rps. spheroides* 8253, 75% of the ferrochelatase activity was in the particulate fraction.

Coproporphyrinogen oxidative decarboxylase (coprogenase) (Porra & Falk, 1961, 1964) could not be demonstrated in cell-free extracts of either pigmented or non-pigmented cells of *Rps. spheroides* 8253 prepared by ultrasonic vibration, or by passage through the pressure cell described by Milner, Lawrence & French (1950) or by treatment with lysozyme (Orlando *et al.* 1961).

Haem formation in Micrococcus denitrificans. This organism was selected as an example of a non-

Table 3. *Distribution of haemoprotein in crude extracts of Rhodopseudomonas spheroides 8253*

Extracts prepared from pigmented and non-pigmented cells of *Rps. spheroides* 8253 containing 278 and 248 mg. of protein respectively were divided into soluble and particulate fractions by centrifugation (see the Experimental section). The haemoprotein content was determined in each fraction after clarification with Triton X-100 (see the Experimental section). The recovery of protein and of each haemoprotein was over 90% of that originally present in the crude extract.

Type of extract	Haemoprotein	Amount of haemoprotein (μ moles)		Percentage of haemoprotein in particulate fraction
		Soluble fraction	Particulate fraction	
Non-pigmented	CO-binding haemoprotein	< 2	11	> 85
	Cytochromes <i>b</i> and <i>c</i>	25	29	54
	Total	~ 27	40	~ 75
Pigmented	CO-binding haemoprotein	34	31	48
	Cytochromes <i>b</i> and <i>c</i>	38	54	59
	Total	72	85	54

Table 4. *Haemoproteins of Micrococcus denitrificans*

The haemoproteins of crude extracts were determined as described in the Experimental section. Total haem determinations were carried out by the pyridine haemochromogen method described in Table 1.

Extract	Haem content (μ mole of haem./mg. of protein)					
	CO-binding pigment	Total cytochrome	Cytochrome <i>b</i>	Cytochrome <i>c</i>	Total haemoprotein	Total haem
From aerobically grown cells	0.1	0.26	0.13	0.13	0.36	0.49
From anaerobically grown cells	0.14	0.64	0.32	0.32	0.78	0.87

photosynthetic bacterium that can grow both aerobically and anaerobically. It grows anaerobically with nitrate as terminal electron acceptor or aerobically with oxygen. The total haem content, as measured by the pyridine haemochromogen method, in extracts from anaerobically grown cells was almost twice that in aerobic extracts (Table 4). As in the *Athiorhodaceae*, this increase was reflected in each of the individual haemoproteins (Table 4). The carbon monoxide-binding pigment, like that of *Rps. spheroides* 8253, had a Soret peak in the carbon monoxide-minus-reduced difference spectrum at 423 $m\mu$ when associated with particles or at 418 $m\mu$ when solubilized with Triton X-100. Cytochromes of the *b* and *c* types have been described in *M. denitrificans* (Verhoeven & Takeda, 1956; Vernon, 1956; Chang, 1963), and a carbon monoxide-binding haemoprotein with a similar carbon monoxide-minus-reduced difference spectrum has been reported in a related halo-tolerant species (Hori, 1963).

Although extracts from anaerobically grown cells contained more haemoprotein, there was little difference between the ferrochelatase activities in extracts from the two types of cells. In extracts from aerobically and anaerobically grown cells the specific activities were respectively 6.9 and 7.6 μ moles of

mesohaem formed/mg. of protein/hr. As with *Rps. spheroides* 8253 this enzyme was associated mainly with the particulate fraction.

Coprogenase activity could not be detected in cell-free preparations of *M. denitrificans*.

DISCUSSION

In the photosynthetic bacteria examined the haemoprotein content of cells grown anaerobically in the light was two to three times as high as that of aerobically grown organisms (Tables 1 and 2). The ubiquinones, like the haemoproteins, are involved in electron transport, and the content of these compounds in the *Athiorhodaceae* is two to four times as high as that in the non-pigmented cells (Sugimura & Rudney, 1962; Carr, 1964).

There is no evidence that the increased haemoprotein associated with photosynthetic conditions can be attributed to one particular type; the carbon monoxide-binding haemoprotein increased in parallel with cytochromes *b* and *c*. These results appear to be in conflict with those of Clayton (1959), who found a preferential increase in cytochromoid *c* in pigmented cells of *Rps. spheroides* but not in *Rsp. rubrum*; Geller (1962), on the contrary, found a

tenfold increase in cytochromoid *c* in the latter organism. Kamen (1963), however, has stated that the non-pigmented cells of facultative photosynthetic bacteria contain no cytochromoid *c*, but such cells have been shown to contain a similar carbon monoxide-binding haemoprotein both by Nishimura & Chance (1963) and by us (Table 2). Our methods do not discriminate between various carbon monoxide-binding haemoproteins with a carbon monoxide-minus-reduced difference spectrum similar to that of cytochromoid *c*. If the carbon monoxide-binding pigment in the non-pigmented extracts is not cytochromoid *c* then the increase in cytochromoid *c* in pigmented extracts may be much greater than is suggested by Table 2.

The carbon monoxide-binding pigment in the non-pigmented extracts may be a cytochrome oxidase like the cytochrome *o* described by Castor & Chance (1959) in various non-photosynthetic bacteria; these pigments have a carbon monoxide-minus-reduced difference spectrum similar to that of cytochromoid *c*. The carbon monoxide-binding haemoprotein in the non-pigmented extract of *Rps. sphaeroides*, unlike that in the pigmented extracts, differs from cytochromoid *c* in at least one property: it is not readily solubilized by ultrasonic vibration (Table 3), whereas cytochromoid *c* of *Rsp. rubrum* is readily solubilized by this treatment (Orlando *et al.* 1961).

It has been suggested that the carbon monoxide-binding pigment present in a halo-tolerant *Micrococcus* sp. may be a cytochrome *o*-type oxidase (Hori, 1963). It is possible that the carbon monoxide-binding pigment in *M. denitrificans* may be a similar bacterial oxidase.

The authors thank Professor D. D. Woods, F.R.S., for encouragement and advice. The authors are also grateful to Miss Patricia Conibear for skilled technical assistance. R. J. P. thanks the Commonwealth Scientific and Industrial Research Organization of Australia for a Postgraduate Overseas Studentship. The research was aided by grants to the Department from the Rockefeller Foundation and from the U.S. Department of Health, Education and Welfare.

REFERENCES

- Bartsch, R. G. (1963). In *Bacterial Photosynthesis*, p. 475. Ed. by Gest, H., San Pietro, A. & Vernon, L. P. Yellow Springs, Ohio: The Antioch Press.
- Bartsch, R. G. & Kamen, M. D. (1958). *J. biol. Chem.* **230**, 41.
- Bartsch, R. G. & Kamen, M. D. (1960). *J. biol. Chem.* **235**, 825.
- Carr, N. G. (1964). *Biochem. J.* **91**, 28 p.
- Carr, N. G. & Lascelles, J. (1961). *Biochem. J.* **80**, 70.
- Castor, L. N. & Chance, B. (1959). *J. biol. Chem.* **234**, 1587.
- Chance, B. (1952). *Nature, Lond.*, **169**, 215.
- Chance, B. & Smith, L. (1955). *Nature, Lond.*, **175**, 803.
- Chang, J. P. (1963). D.Phil. Thesis: University of Oxford.
- Chang, J. P. & Morris, J. G. (1962). *J. gen. Microbiol.* **29**, 301.
- Clayton, R. K. (1959). *Arch. Mikrobiol.* **33**, 265.
- Clayton, R. K. & Smith, C. (1960). *Biochim. biophys. Res. Commun.* **3**, 143.
- Cohen-Bazire, G., Sistrom, W. R. & Stanier, R. Y. (1957). *J. cell. comp. Physiol.* **49**, 25.
- Geller, D. M. (1962). *J. biol. Chem.* **237**, 2947.
- Goldberger, R., Smith, A. L., Tisdale, H. & Bomstein, R. (1961). *J. biol. Chem.* **236**, 2788.
- Hori, K. (1963). *J. Biochem., Tokyo*, **53**, 354.
- Jones, O. T. G. (1963). *Biochem. J.* **88**, 335.
- Kamen, M. D. (1963). In *Bacterial Photosynthesis*, p. 61. Ed. by Gest, H., San Pietro, A. & Vernon, L. P. Yellow Springs, Ohio: The Antioch Press.
- Lascelles, J. (1956). *Biochem. J.* **62**, 78.
- Lascelles, J. (1959). *Biochem. J.* **72**, 508.
- Lascelles, J. (1960). *J. gen. Microbiol.* **23**, 487.
- Lochhead, A. C. & Goldberg, A. (1961). *Biochem. J.* **78**, 146.
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951). *J. biol. Chem.* **193**, 265.
- Milner, H. W., Lawrence, N. S. & French, C. S. (1950). *Science*, **111**, 633.
- Nishimura, M. & Chance, B. (1963). *Plant & Cell Physiol.* **4**, 239.
- Orlando, J. A. (1962). *Biochim. biophys. Acta*, **57**, 373.
- Orlando, J. A. & Horio, T. (1961). *Biochim. biophys. Acta*, **50**, 367.
- Orlando, J. A., Levine, L. & Kamen, M. D. (1961). *Biochim. biophys. Acta*, **46**, 126.
- Paul, K.-G., Theorell, H. & Åkesson, Å. (1953). *Acta chem. scand.* **7**, 1284.
- Porra, R. J. & Falk, J. E. (1961). *Biochem. biophys. Res. Commun.* **5**, 179.
- Porra, R. J. & Falk, J. E. (1964). *Biochem. J.* **90**, 69.
- Porra, R. J. & Jones, O. T. G. (1963a). *Biochem. J.* **87**, 181.
- Porra, R. J. & Jones, O. T. G. (1963b). *Biochem. J.* **87**, 186.
- Sano, S. & Granick, S. (1961). *J. biol. Chem.* **236**, 1173.
- Sugimura, T. & Rudney, H. (1962). *Biochim. biophys. Acta*, **62**, 167.
- Verhoeven, W. & Takeda, Y. (1956). In *Inorganic Nitrogen Metabolism*, p. 159. Ed. by McElroy, W. D. & Glass, B. Baltimore: The Johns Hopkins Press.
- Vernon, L. P. (1956). *J. biol. Chem.* **222**, 1035.