BBA 46088

SOME EFFECTS OF IRON DEFICIENCY ON *RHODOPSEUDOMONAS SPHEROIDES* STRAIN Y

F. REISS-HUSSON, H. DE KLERK*, G. JOLCHINE, E. JAUNEAU AND M. D. KAMEN**
Laboratoire de Photosynthèse, CNRS, 91-Gif-sur-Yvette (France)
(Received November 2nd, 1970)

SUMMARY

Rhodopseudomonas spheroides strain Y was grown photosynthetically in a synthetic medium containing defined amounts of iron. Two chromatophore fractions ("light" and "heavy") were prepared by sucrose density gradient centrifugation of CsCl-treated chromatophores. They were found to differ in homogeneity, stability, specific bacteriochlorophyll content and infrared absorption spectra. Iron deficiency was found to cause excretion of porphyrins by whole cells and accumulation of poly-β-hydroxybutyrate. Inhibition of bacteriochlorophyll synthesis and decrease in the total iron and heme iron contents were also observed in whole cells and light particles; the total heme of low-iron cells and the heme bound to light particles were decreased, respectively, by factors of 20 and 16 whereas the total iron was much less affected. Variations in the infrared absorption of whole cells, light and heavy particles were caused by iron deficiency.

INTRODUCTION

A number of iron-containing proteins are known to be part of the photosynthetic bacterial electron transport chain. The variety and abundance of these compounds are striking[1,2]. Most of the researches devoted to their study use as starting material bacteria grown in media supplemented with excess amounts of substrates and iron. However, little is known about their minimal level and distribution in depleted cells. LASCELLES[3,4] has shown that in iron-deficient *Rhodopseudomonas spheroides* syntheses of both heme and bacteriochlorophyll are inhibited, and porphyrins are excreted into the growth medium, but no investigation of the heme and non-heme iron, nor of the soluble and membrane bound hemes has been reported. Hence we have grown *R. spheroides* strain Y in media containing defined amounts of iron and report some observations on the effects of such growth conditions on properties of whole cells and sub-cellular particles.

* Present address: Shell Research Laboratories, Amsterdam, Holland.
** Present address: Department of Chemistry, University of California, La Jolla, Calif., U.S.A.

METHODS

Cultures

We have used *R. spheroides* strain Y, a recent isolate by one of us (H. DE KLERK), in view of its ready growth on a number of substrates, particularly in the low-iron media described here.

The synthetic medium used in these studies and called "L" contained per l 0.02 M potassium phosphate (C. Erba) buffer (pH 6.8): 3.5 g ammonium succinate (C. Erba); 1 g sodium glutamate (Merck); 20 ml of HUTNER's base[5] in which $FeSO_4$ was omitted (all chemicals from Merck); 25 μg nicotinic acid; 12.5 μg thiamine; 0.25 μg biotin. Residual iron in this medium was 1 μM. We did not try to eliminate it because of the difficulty of avoiding recontamination with traces of iron in large scale cultures. Preparations derived from this medium are termed "low iron". Iron was added to this medium as $FeSO_4$ before autoclaving as required; in these cases, the final molarity of iron is indicated.

Bacteria were grown at 33° with continuous bubbling of CO_2–N_2 (5:95, by vol.) and magnetic stirring, in either 2-l or 10-l bottles thermostated in water baths. Illumination was provided by incandescent bulbs, and measured at the surface of the bottles. Low-iron cultures were inoculated with cells adapted to the L medium by three successive transfers. Sterile aliquots were withdrawn to follow growth, which was routinely measured by the absorbance at 1200 nm; at this wavelength optical absorption was absent. The absorbance thus was a measure of turbidity and was found to be directly proportional to cell mass.

Particle preparation

Bacteria were harvested by centrifugation, washed and suspended in 0.1 M potassium phosphate buffer (pH 7.5) (4 vol./wet wt. cells). They were disrupted in a Ribi cell at 10–15° and 20000 lb/inch^2. After 15 min incubation with deoxyribonuclease, unbroken cells and debris were eliminated by a 20 min centrifugation at 30000 $\times$ *g*. Usually two passages in the Ribi cell were necessary to disrupt about 80 % of the cells (that is, to recover 80 % of the bacteriochlorophyll in the supernatant of the 30000 $\times$ *g* centrifugation).

Particle preparation was performed following the method used by CUSANOVICH AND KAMEN[6] for *Chromatium* with minor variations. Soluble components were eliminated by 3 h centrifugation of the cell-free extract at 350000 $\times$ *g*; lower centrifugal fields resulted in incomplete centrifugation of the pigmented particles. The pellet was then layered on tubes containing 30 % CsCl, and centrifuged 1 h at 350000 $\times$ *g*. The pellet of ribosomes was discarded. Membranes floating in the top layer were recovered and washed with 0.1 M potassium phosphate buffer (pH 7.5), then applied to a linear sucrose gradient (0.5–1.5 M) and centrifuged in a Sw-25-2 rotor, at 22000 rev./min for 90 min. The pigmented material was separated in two bands, at 0.67 M and 1.02 M sucrose, respectively. These were recovered, dialysed against 0.1 M potassium phosphate buffer (pH 7.5), concentrated by centrifugation, and stored in the dark at 5°.

Chemical analyses

Bacteriochlorophyll was determined spectroscopically after extraction with 20 vol. of ice-cold absolute methanol (ε_{mM} at 720 nm = 60, see ref. 7).

Protein was measured by the method of LOWRY *et al.*[8] after 12 h digestion of the samples in 1 M NaOH at 35°, using bovine serum albumin as a standard.

Heme was determined on acetone–methanol (7:2, v/v) residues by the pyridine hemochrome method ($\varepsilon_{mM} = 23$ for ΔA (550—575 nm) in a reduced *minus* oxidized difference spectrum). As the total heme content was very difficult to measure in whole low-iron cells, it was determined on the cell-free extract obtained after breakage and elimination of whole cells and debris (see above). Its value was based on dry weight of the original cell suspension.

Iron was determined by the method of COLLINS AND DIEHL[9] as a 2,4,6-tripyridyl-*s*-triazine complex, after wet-ashing of the samples in H_2SO_4–$HClO_4$.

Dry weight of cells was determined after centrifugation, distilled water washes, and drying at 100°.

Poly-β-hydroxybutyrate was isolated from whole cells by the method of STANIER *et al.*[10], and measured by gravimetry of its chloroform solution. We confirmed that, on heating with concentrated H_2SO_4 it was converted to crotonic acid, as described by LAW AND SLEPECKY[11].

Porphyrins excreted in the growth medium were measured spectroscopically as coproporphyrin III (see ref. 3), after centrifugation of the cells and appropriate acidification of the medium ($\varepsilon_{mM} = 489$ in HCl 1.4 M).

Ubiquinone was determined by the method of PUMPHREY AND REDFEARN[12].

Physical measurements

Spectra were recorded with a Cary model 14R. Opal glass plates were used to record the spectra of whole cells suspensions.

Electron microscopy was kindly performed for us by Madame A. Cassant.

RESULTS

Growth of R. spheroides strain Y as a function of iron, and composition of whole cells

Iron is a limiting factor when *R. spheroides* strain Y is grown in the L medium, as exemplified by two experiments described in Tables I and II. In the first, cells are adapted to the L medium by several transfers, then inoculated into this medium to which various amounts of iron have been added. They are grown at 33°, and 4000 lux.

TABLE I

GROWTH CHARACTERISTICS OF *R. spheroides* IN THE L MEDIUM OF VARYING IRON CONTENT

Expt. 1: growth at 33°, 4000 lux; inoculum: cells adapted to the L medium (see text). *Expt. 2*: growth at 33°, 8000 lux; inoculum: cells adapted in A to low iron, in B to 17 μM iron.

	Expt. 1					*Expt. 2*	
						A	*B*
Iron content of the medium (μM)	1	2.2	4.3	6.5	8.6	1	17
Division time (h)	7.5	5	5	5	5	4.0	2.5
Yield (g wet wt. cells/l) after 44-h culture	4.4	6.3	7.4	8.5	8.7	4.5	8.1
Coproporphyrin excreted in the medium (μmoles/l) after 44-h culture	59	1.2	0.4	0.4	0.4	51	1.2

In the second experiment, cells are adapted either to the L medium or to an L medium containing 17 μM iron/l, and grown at 33° and 8000 lux. In both cases, the division time is the longer in the low iron medium, and the yield of cells is less (Table I).

In agreement with LASCELLES[4], we have confirmed that the excretion of porphyrins is enhanced in stationary phase, as measured by the amount of coproporphyrin III excreted by 44-h cultures. As shown in Table I, this excretion is greatly enhanced in the low-iron medium. During the various stages of growth the pigmentation of the cells is markedly lower in the low-iron medium. This is confirmed by the bacteriochlorophyll content of stationary phase cells given in Table II. There is indeed an inhibition of the bacteriochlorophyll synthesis in this medium; yet it may be noted that the bacteriochlorophyll synthesis still depends on the light intensity (compare Expts. 1 and 2). One may note also in the first experiment that the critical range leading to iron deficiency is below 10 μM which is in good agreement with the obser-

TABLE II

COMPOSITION OF STATIONARY PHASE CELLS (44-h CULTURE) GROWN IN THE L MEDIUM AT VARIOUS IRON CONTENTS

Conditions as indicated in Table I.

	Expt. 1					*Expt. 2*	
Iron content of the medium (μM)	1	2.2	4.3	6.5	8.6	1	17
Bacteriochlorophyll (μmoles/g protein)	27	72	76	81	80	22	71
Poly-β-hydroxybutyrate (% of dry wt.)	25	—	12	7	6	25	7
Protein (% of dry wt.)	34	32	40	36	38	32	42
Total iron (μmoles/g protein)	—	—	—	—	—	1.8	5.2
Heme iron (μmoles/g protein)	—	—	—	—	—	0.04	0.8

vations of LASCELLES[3] and WIESSNER[13] on another strain of this species. Consequently we have used throughout the L, 17 μM iron medium as a control for growth in presence of an excess of iron.

We observed that, after breakage, the cells grown in the low-iron medium released a large amount of a white waxy material. This prompted us to measure the amount of the reserve polymer, poly-β-hydroxybutyrate, in stationary-phase cells. This polymer was found to accumulate in the low-iron cells. No such accumulation was noted when iron was added to the L medium. One may see that the protein content (expressed on a dry weight basis) was slightly lower in the low-iron cells (Table II).

Cells grown in the low-iron L medium, and in the L, 17 μM iron medium and harvested in stationary phase, were analysed for their bacteriochlorophyll, total iron and total heme iron contents; results are given in Table II. It is striking that the total content of iron decreased only by a factor of 2.5 in the low-iron cells, whereas that of the heme iron decreased by about 20. As judged from the position of the hemochrome maximum, most if not all the hemoproteins were of the *c* type in the two culture conditions. Their nature is presently under investigation.

General properties of the light and heavy fractions

The fractionation of the bacteriochlorophyll-containing membranes was done on cells grown either in the low-iron L medium, or in the L, 17 μM iron one. These

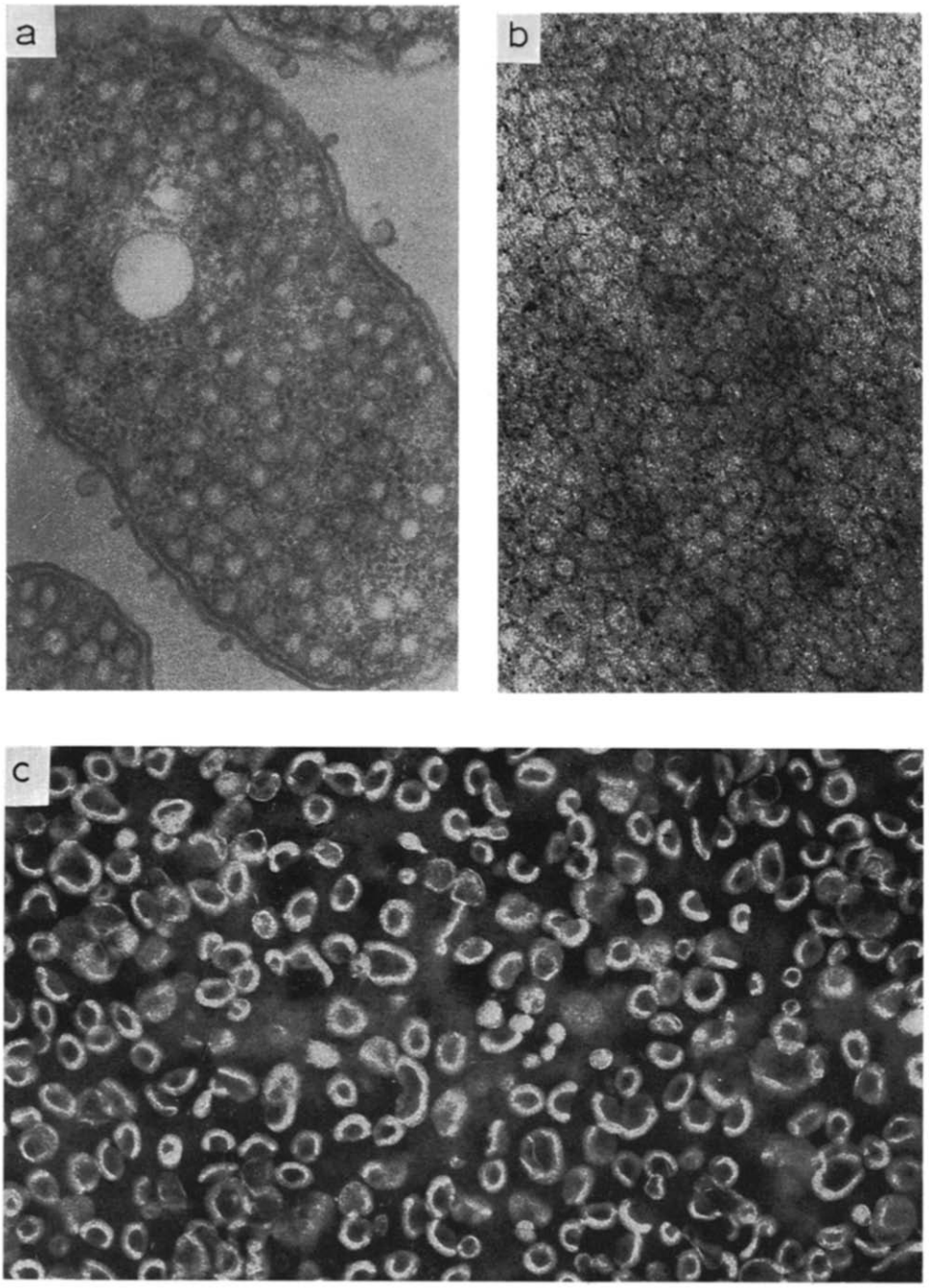

Fig. 1. (a) Ultrathin section of bacterial cell grown at 8000 lux in the L, 17 μM iron medium; fixation by osmium tetroxide according to Ryter's and Kellenberger's procedure. Direct magnification: × 60000. (b) Ultrathin section of the light fraction derived from the cells shown in (a); fixation by osmium tetroxide as in (a). Direct magnification: × 60000. (c) Same light fraction after negative staining by 2% phosphotungstic acid.

membranes separated into two fractions on the final sucrose gradient. The upper fraction (called "light") was devoid of appreciable turbidity and did not aggregate over weeks if kept in the dark at 5°; the lower one (called "heavy") was turbid and floculated after a few days. The heavy fraction was at equilibrium in the gradient at a density of 1.15 g/cm^3 after 90 min centrifugation; the light required 15 h to equilibrate at a buoyant density of 1.12 g/cm^3. The light fraction contained about 70 % of the total bacteriochlorophyll initially layered on the gradient. All these values were the same for preparations derived from either medium.

In both cases, the electron microscopic observations on ultrathin sections of isolated particles revealed that the light fraction was composed of spherical vesicles about 700 Å in diameter (Fig. 1b). Their morphological features corresponded perfectly to those of the chromatophores observed in situ in fixed bacterial cells (Fig. 1a). As shown also on negatively stained preparations (Fig. 1c), it was remarkably devoid of contaminating material, such as ribosomes or larger pieces of membranes. The heavy fraction (not shown) also contained such small vesicles, mostly clumped; in addition, larger and irregular pieces of membrane or cell debris were found.

Composition of the light particles

It was observed in a number of experiments that the specific bacteriochlorophyll content (bacteriochlorophyll/protein ratio) was about twice as high in the light as in the heavy fraction prepared from the same cells, an observation which recalled results with another strain of *R. spheroides*[14] and with other organisms[6,15]. On the basis of the greater purity and stability of the light fraction, the analyses were performed on this fraction only. Cells harvested in stationary phase (44-h cultures) grown at 8000 lux either in the low-iron L medium, or in the L, 17 μM iron medium, were used.

We determined the contents of bacteriochlorophyll, heme iron and total iron, as well as of ubiquinone, a possible electron-transport component which might be expected to be independent of the iron supply. Results are expressed on a protein basis, and as molar ratios to bacteriochlorophyll (Table III). As anticipated, the iron supply did not markedly affect the ubiquinone level, whereas the bacteriochlorophyll, the heme iron and the non-heme iron levels (calculated by difference) were depressed in the low-iron particles.

The nature of the bound hemoproteins is under investigation; most of them are of the *c* type as indicated by the hemochrome spectra. As in the whole cells, it is

TABLE III

COMPOSITION OF LIGHT PARTICLES (FROM CELLS GROWN FOR 44 h AT 8000 LUX)

Values expressed as μmoles/g protein.

Iron content of the L medium (μM)	1	17
Bacteriochlorophyll	127	162
Ubiquinone	32	25
Heme	0.15	2.5
Total iron	3.3	6.8
Molar ratio		
Bacteriochlorophyll : ubiquinone : heme : iron	100 : 25 : 0.1 : 2.6	100 : 15 : 1.5 : 4.2

quite obvious that the level of heme iron bound to the membrane is much more depressed than that of the non-heme iron.

Absorption spectra of whole cells and particles

During this work it became evident that iron deficiency was affecting the infrared spectra of whole cells and particles. Fig. 2 shows the absorption spectra of whole cells grown 44 h at 8000 lux and 33° either in the low-iron L medium, or in the L, 17 μM iron one, in the range 700–1000 nm. These spectra were taken just after harvesting the cells and resuspending them in buffer (pH 7.5), after centrifugation. In the low-iron cells, the 855-nm main maximum was broadened by a strong secondary maximum located at about 870 nm. In the L, 17 μM iron cells, this secondary maximum was much weaker. We observed that this last type of spectrum was also obtained when low-iron cells were readapted to an L, 2.2 μM iron medium.

In Fig. 3, the absorption spectrum of light particles prepared from the low-iron cells is given, together with that of the heavy particles, in the infrared region. In the visible range (not shown) the light and the heavy fractions had the same spectrum

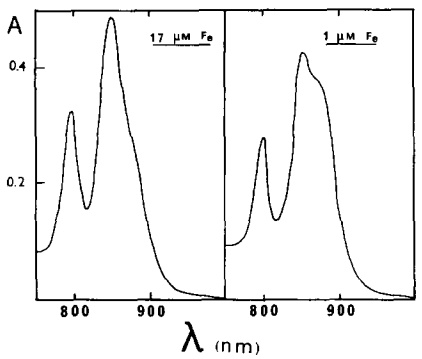

Fig. 2. Absorption spectra of cell grown 44 h at 33° and 8000 lux in either the L medium (on the right) or the L, 17 μM iron medium (on the left).

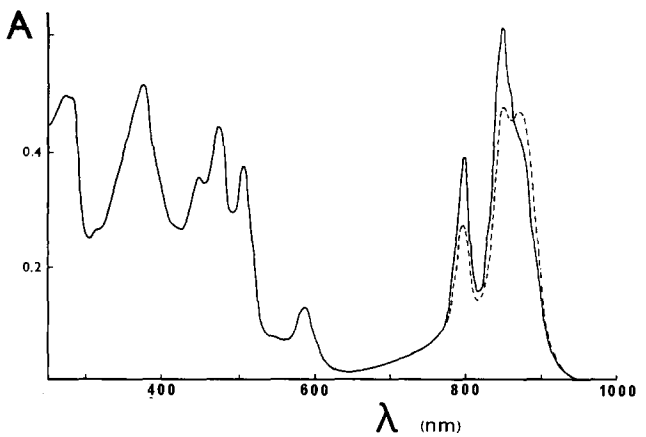

Fig. 3. Absorption spectra of the light (———) and the heavy (– – –) particles from cells grown 44 h at 33° and 8000 lux in the L medium. Bacteriochlorophyll concentration in both cases: 7.2 nmoles/ml.

apart from variations owing to the turbidity of the heavy fraction, and to its lower specific bacteriochlorophyll content, which increased its absorption in the 280-nm range. In the infrared region, differences were evident: the secondary maximum at 870 nm was much higher in the heavy fraction spectrum (Fig. 3). No such large difference was found when light and heavy fraction were derived from the L, 17 μM iron grown cells (Fig. 4). If corresponding fractions were compared for the two iron conditions, one noted that iron deficiency correlated with increase of the 870-nm maximum, as observed on the whole cells.

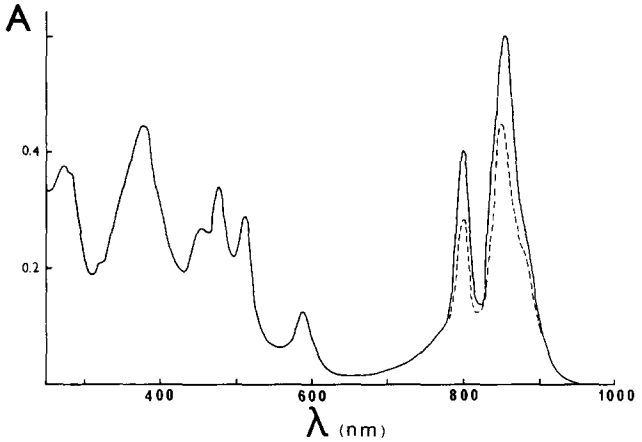

Fig. 4. Absorption spectra of the light (———) and the heavy (– – –) particles from cells grown 44 h at 33° and 8000 lux in the L, 17 μM iron medium. Bacteriochlorophyll concentration: light: 6.1 nmoles/ml. Heavy: 4.1 nmoles/ml.

All these spectra were very reproducible, once the growth conditions (temperature, intensity, medium), were controlled.

DISCUSSION

From the data presented here, large variations in the composition and properties of *R. spheroides* strain Y are seen to be linked to iron deficiency.

The total heme iron, as well as the membrane-bound heme iron show greatly depressed levels in low-iron cells. Expressed on a protein basis, the total heme iron decreases by a factor of about 20, and the membrane bound heme iron by about 16. This may be compared with the factor of 4 given by LASCELLES[4] for the total heme iron decrease expressed per unit dry weight. In that case, however, another *R. spheroides* strain was used and residual iron in the low-iron culture was not measured, so that an attempt to compare these results with those of our experiments is not indicated. Increases in the total heme content of bacteria during transitions between dark aerobic and light anaerobic growths have been reported not to exhibit evidence for preferential variation of a particular heme type[16]; in our experiments, more detailed studies now in progress should show if this is indeed the case.

More surprising is the high proportion of iron in the non-heme state (such an observation has already been noted by CUSANOVICH AND KAMEN[6] with *Chromatium*) and its relatively minor decrease in low-iron cells. This is also found when non-heme

iron is measured either in the whole cells or as membrane-bound. Such behavior would indicate that in the non-heme state iron is part of component(s) indispensable for growth. As knowledge about these components is still scarce, more detailed investigations are needed.

The decrease in the gross bacteriochlorophyll content found for the low-iron cells confirms the previous results of LASCELLES[3]. Moreover, the specific bacteriochlorophyll content of the light particles also shows a decrease. This indicates that the pigment content of the chromatophore membrane itself is modified, and not merely the total amount of membrane. If one assumes that the whole of the bacteriochlorophyll is contained in the light particles, this amount may be roughly estimated by dividing the specific bacteriochlorophyll content of the whole cells by that of the particles (Tables II and III). Thus 17 % of the total cell protein would be in the membranes of low-iron cells (grown at 8000 lux) against 43 % in the L, 17 μM iron-grown cells. Such a variation indicates a complete reshaping of the iron-deficient cells. The accumulation of poly-β-hydroxybutyrate during growth in iron deficient media is another aspect.

The separation of the bacteriochlorophyll-containing membranes into two fractions and their characteristics confirm similar results already published for *R. spheroides*[14,17,18], and other species[6,15,19]. Here too, the light fraction may be considered as the purest chromatophore material, and could originate from the comminution of the intracellular membrane system. The heavy fraction would appear to contain pieces of the outer cellular membrane, either poorly or not pigmented[18,20]. The modifications observed in the infrared spectra of whole cells and particles remain to be explained. Differences between light and heavy particles of another *R. spheroides* strain have already been described by WORDEN AND SISTROM[14] and seen to be dependent on light intensity during growth. In our experiments, the differences may be ascribed wholly to the iron supply in the growth medium, all other culture parameters being the same. As the nature of the bacteriochlorophyll moieties giving rise to the 800-, 855- and 870-nm absorption bands is unknown, these variations for the present may be taken merely as indications of local changes in the bacteriochlorophyll environment when the iron content of the membrane is modified.

ACKNOWLEDGEMENTS

The authors wish to express their gratitude for the excellent assistance of Mrs. A. Raguideau with the culture of the organisms, and of Mrs. T. Taverny and B. Kop with the analyses. Partial support by grants from the National Institutes of Health (HD-01262, Bethesda, Md., U.S.A.) and the National Science Foundation (GB-7033, Washington, D.C., U.S.A.) is acknowledged.

REFERENCES

1 M. D. KAMEN, in H. GEST, A. SAN PIETRO AND L. P. VERNON, *Bacterial Photosynthesis*, Antioch Press, Yellow Springs, Ohio, 1963, p. 61.
2 K. DUS, H. DE KLERK, K. SLETTEN AND R. G. BARTSCH, *Biochim. Biophys. Acta*, 140 (1967) 291.
3 J. LASCELLES, in G. E. W. WOLSTENHOLME, *Biosynthesis of Porphyrins and Porphyrin Metabolism*, Ciba Foundation Conference, London, 1955, p. 265.
4 J. LASCELLES, *Biochem. J.*, 62 (1956) 78.

5 S. HUTNER, quoted by G. COHEN-BAZIRE, W. SISTROM AND R. Y. STANIER, *J. Cellular Comp. Physiol.*, 49 (1957) 25.
6 M. A. CUSANOVICH AND M. D. KAMEN, *Biochim. Biophys. Acta*, 153 (1968) 376.
7 G. COHEN-BAZIRE AND W. R. SISTROM, in L. P. VERNON AND G. R. SEELY, *The Chlorophylls*, Academic Press, New York, 1966, p. 313.
8 O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR AND R. J. RANDALL, *J. Biol. Chem.*, 193 (1951) 265.
9 P. COLLINS AND H. DIEHL, *Anal. Chim. Acta*, 22 (1960) 125.
10 R. Y. STANIER, M. DOUDOROFF, R. KUNISAWA AND R. CONTOPOULOU, *Proc. Natl. Acad. Sci. U.S.*, 45 (1959) 1246.
11 J. H. LAW AND R. A. SLEPECKI, *J. Bacteriol.*, 82 (1961) 33.
12 A. M. PUMPHREY AND E. R. REDFEARN, *Biochem. J.*, 76 (1960) 61.
13 W. WIESSNER, *Flora*, 149 (1960) 1.
14 P. B. WORDEN AND W. R. SISTROM, *J. Cell Biol.*, 23 (1964) 135.
15 G. COHEN-BAZIRE AND R. KUNISAWA, *Proc. Natl. Acad. Sci. U.S.*, 46 (1960) 1543.
16 R. J. PORRA AND J. LASCELLES, *Biochem. J.*, 94 (1965) 255.
17 A. GORCHEIN, *Proc. Roy. Soc. London, Ser. B*, 170 (1968) 255.
18 A. GORCHEIN, A. NEUBERGER AND G. H. TAIT, *Proc. Roy. Soc. London, Ser. B*, 170 (1968) 319.
19 P. A. KETCHUM AND S. C. HOLT, *Biochim. Biophys. Acta*, 196 (1970) 141.
20 J. YAMASHITA AND M. D. KAMEN, *Biochem. Biophys. Res. Commun.*, 34 (1969) 418.