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LIGHT-INDUCED ELECTRON TRANSPORT IN *CHROMATIUM* STRAIN D

I. ISOLATION AND CHARACTERIZATION OF *CHROMATIUM* CHROMATOPHORES

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SUMMARY

Fractionation of *Chromatium* Strain D chromatophores by centrifugation in sucrose density gradients results in two fractions, designated 'light' and 'heavy'. The light particles are enriched in photosynthetic pigments, are relatively homogeneous and can be prepared reproducibly. They do not scatter light appreciably, contain 41 % of the bacteriochlorophyll, 26 % of the carotenoid, 25 % of the heme and 4 % of the protein found in intact cells, and are approx. 75 % lipid, and 25 % protein, 30 % of which can be ascribed to cytochromes. The molecular weight is $12.9 \cdot 10^6$.

In contrast, the heavy particles are heterogeneous, can not be prepared reproducibly, scatter light and contain approx. 14 % of the bacteriochlorophyll, 9 % of the carotenoid, 8 % of the heme and 6 % of the protein present in intact cells.

Three meso-heme proteins were identified in the light particles: cytochrome *c*-552, cytochrome *cc'*, and cytochrome *c*-555. Both cytochrome *c*-552 and cytochrome *cc'* bound CO when solubilized but not when bound in the light particles. No protoheme (*b*-type cytochrome) was found, even in whole cells.

These results permit characterization of the chemical and physical properties of a subcellular fraction of *Chromatium* Strain D, which does not appear to be an artifact of preparation.

INTRODUCTION

Particulate fractions, termed 'chromatophores', have been isolated from several of the photosynthetic bacteria[1–3]. These particles appear spherical in the electron microscope and are similar to spherical vesicles observed in intact cells[4–6]. The absence of these particles in the photosynthetic bacteria grown in the dark in the

Abbreviations: BChl, bacteriochlorophyll; DCIP, 2,6-dichlorophenolindophenol; PS buffer, 100 mM potassium phosphate–10% sucrose (pH 7.5); σ, standard deviation = $\left(\frac{\Sigma|\bar{x} - x_i|^2}{n - 1}\right)^{1/2}$

presence of oxygen and their ability to carry on photophosphorylation[7,8] and pyridine nucleotide photo-reduction[9,10], have suggested that they are the site of photosynthesis in the intact cell. Contrary findings are the absence of chromatophores from some photosynthetic bacteria[6,11], failure of enzymatic lysis[11] to release particles in appreciable amounts, more rapid rate of appearance of soluble proteins than chromatophores during sonication[12] and evidence from electron micrographs which indicates that what appear to be spherical vesicles are, in fact, an extension[13] of the cell membrane.

Chromatium chromatophores, which we may term 'classical', have been heterogeneous and poorly defined as to molecular composition. Thus, interpretation of light-induced absorption changes could not be undertaken in a precise manner.

In this, and succeeding Papers II and III in this series, we describe the preparation of chromatophores which do not appear to be artifacts of preparation, and which possess properties which make possible characterization in terms of known chemical constituents and biochemical systems. In addition, light-induced reactions in these chromatophores are amenable to physico-chemical analysis, in that their responses to light activation can be studied under a variety of controlled conditions.

MATERIALS AND METHODS

Chromatium Strain D was grown heterotrophically with succinic acid as the carbon source[14] in 1-l prescription bottles placed in a water bath thermostated at $35° \pm 2°$. Uniform illumination (approx. 50 ft-candles) was provided by 40-W showcase lamps immersed parallel to the culture bottles in the water bath. The cells were harvested just before the termination of exponential growth using a Sharples continuous flow centrifuge. The unwashed collected cell paste was stored at $-10°$ until required.

Bacteriochlorophyll (BChl) was determined as described by VAN NIEL AND ARNOLD[15] ($\varepsilon_{mM} = 9.89$ at 668 mμ), and carotenoid by measurement of the absorbance at 487 mμ ($\varepsilon_{mM} = 150$) in an aqueous suspension of particles. Protein was assayed by a modified biuret reaction[16], and dry weight by precipitation with 10 % trichloroacetic acid, followed by a distilled-water wash, and drying to constant weight at 110°.

Lipid was determined by precipitation of a sample with cold 10 % trichloroacetic acid, and sequentially washed with cold 5 % trichloroacetic acid and distilled water. The pellet was suspended in absolute methanol, 2 vols. of chloroform added, and the suspension was allowed to stand 4 h at room temperature. Then a quarter volume of water was added, and the chloroform layer removed, filtered, and dried to constant weight at 80°.

Heme content was assayed by extraction of a suitable pigmented aliquot with 7:2 (v/v) acetone–methanol. The pigment-free residue was suspended in 0.2 M NaOH–25 % pyridine and the heme present was determined from a reduced ($Na_2S_2O_4$) *minus* oxidized difference spectrum by use of the difference in absorbance between the peak at 550 mμ and the trough at 570 mμ ($\varepsilon_{mM} = 23$)*. To determine the iron content, samples were first washed with concentrated H_2SO_4 (0.1 ml) *plus* HNO_3 added dropwise until no color remained, then the iron was assayed colorimetrically as the

* Determined from the pyridine hemochrome of horse-heart cytochrome *c* using $\Delta\varepsilon_{mM} = 19.1$ at 550 mμ (ref. 17) in the reduced *minus* oxidized difference spectrum.

ferrous-*o*-phenanthroline complex, as described by SANDELL[18], using β-mercaptoethanol as the reductant. Ubiquinone was determined by the method of PUMPHREY AND REDFEARN[19]. Carbohydrate was measured, as described by ASHWELL[20], after exhaustive extraction of lipids with 7:2 (v/v) acetone–methanol.

Assays

NADH-dye reductase, NADH-heme reductase and NADH oxidase were determined, as described by HORIO AND KAMEN[21]. Dye reductase was measured using 2,6-dichlorophenolindophenol (DCIP), and heme reductase with horse-heart cytochrome *c*, or *Rhodospirillum rubrum* cytochrome c_2, as electron acceptors. Succinic dehydrogenase was determined as described by WOODY AND LINDSTROM[22], with *Rhodospirillum rubrum* cytochrome c_2 as the electron acceptor.

Physical measurements

All absorption spectra were obtained with the Cary 14R recording spectrophotometer using 1-cm pathlength cells. Spectra of scattering suspensions were determined using a modification of the SHIBATA[23] technique, with Lucalox disks of sintered alumina (diameter 24 mm, thickness 2 mm) purchased from the General Electric Co. These disks which have no absorption bands between 200 and 2000 mμ were used to scatter both the sample and reference beams. Difference spectra were obtained using either the 0–2 or 0–0.2 slidewires, as needed.

Analytical ultracentrifugation was performed, using a Spinco Model E ultracentrifuge equipped with a rotor temperature-indicating and control system. Absorption photographs were taken using a monochromator accessory set at 375 mμ. An AN-D rotor with 12 mm Kel-F 4° single sector cell was used. The samples were always suspended in 10 mM Tris (pH 7.5) and the temperature maintained at 20°.

Partial specific volume and intrinsic viscosity were measured as described by SCHACHMAN[24]. Electron micrographs of negatively stained preparations were made using a Siemens Elmiskop-2. A small amount of chromatophores was suspended in 3% phosphotungstic acid buffered with 0.1 M $(NH_4)_2CO_3$ (pH 8.0). A drop of the suspension was placed on a 200-mesh Formvar-coated copper grid. The excess liquid was removed, the grid was then air-dried and viewed.

Preparation of chromatophores

In Fig. 1, a flow sheet summarizing the procedure used to prepare purified particles is presented. Succinate-grown *Chromatium* Strain D was suspended in 100 mM potassium phosphate–10% sucrose (pH 7.5) (PS buffer) in the proportion of 4 vols. of buffer to 1 vol. of packed wet cells.

A cell-free extract was prepared with a Ribi cell fractionator (Ivan Sorvall, Inc., Newark, Conn.), operated at 20000 lb/inch^2 ram pressure with the temperature of the effluent broken-cell stream carefully maintained not to exceed 20°. Alternatively, the packed cells were ground with alumina (Alcoa A-305). All subsequent operations were carried out at 0–5°. The following method of fractionation is patterned after that of WORDEN AND SISTROM[3].

The cell-free extract was centrifuged at 30000 × *g* for 1 h. The pellet (large particles) was washed once with PS buffer (one-fourth starting volume) and the combined supernatants centrifuged at 144000 × *g* for 2 h. The supernatant solution

(soluble proteins) was decanted and the residue (classical chromatophores) was suspended in 4 vols. of PS buffer and then made 35 % by weight in RbCl (97 % purity, Matheson Co.). This suspension was then centrifuged at 144000 × *g* for 2 h, causing the chromatophores to rise to the top, and the ribosomes to fall to the bottom, of the tube. (The 144000 × *g* centrifugation prior to the addition of RbCl could be omitted if classical chromatophores were not desired.) The chromatophore suspension was removed by aspiration, diluted with 10 vols. of PS buffer and then centrifuged again at 144000 × *g* for 2 h.

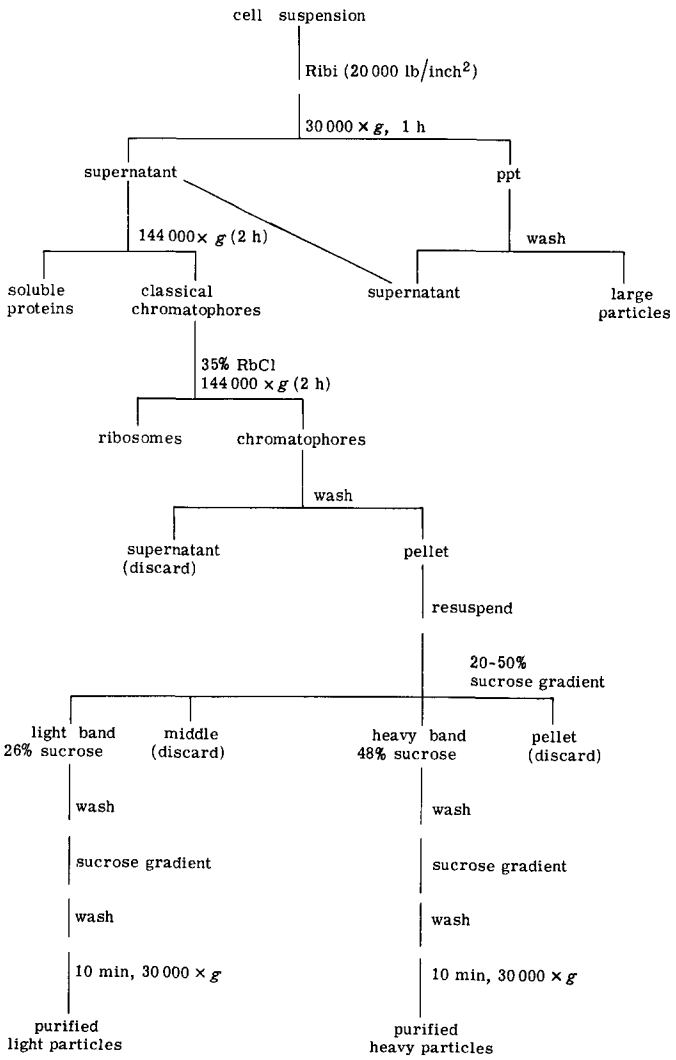


Fig. 1. Flow diagram for preparation of purified *Chromatium* chromatophores.

The resultant pellet was resuspended in PS buffer, layered on a linear sucrose density gradient (10–50 % sucrose), and then centrifuged in a Spinco SW 25.1 swinging bucket rotor at 25000 rev./min for 90 min. Two bands were present at the end of this

time. The upper band centered at 26 % sucrose was designated the 'light' fraction and the lower band, at 48 % sucrose, the 'heavy' fraction (consistent with the WORDEN–SISTROM notation). In addition, there were two other fractions—a small amount of pigmented material uniformly distributed between the bands and a very small amount of material in a pellet on the bottom of the centrifuge tube—which were usually discarded.

The light and heavy fractions were removed by suction from the top of the centrifuge tube, diluted (3-fold) and centrifuged at 144000 × *g* for 2 h. The pellets were resuspended in PS buffer (2 vols.), layered on a sucrose density gradient as before, centrifuged for 90 min, diluted, sedimented and resuspended in PS buffer. This resuspended material was then centrifuged at 30000 × *g* for 10 min to remove aggregated material.

The suspensions thus obtained were the preparations of purified light and heavy particles used in this investigation.

RESULTS

General properties

The most obvious difference between the light and heavy particles was in light-scattering properties. The light fraction was deep red and optically clear. The heavy fraction was reddish-pink and quite turbid. These results recall previous observations with *R. rubrum*[25] and *Rhodopseudomonas spheroides*[3].

The heavy fraction will form aggregates on standing at 0–5° in air for a short time (3–5 days) so that most of the bacteriochlorophyll can be removed from the suspension by low-speed centrifugation (30000 × *g* for 10 min). The light fraction is much more stable, remaining in suspension for as long as 2 weeks under these conditions.

Both the light and heavy particles can be stored for 4–6 weeks at 0–5° without any settling, provided they are kept anaerobic.

As previously noted, the heavy particles band at 48 % sucrose (density, 1.22) for a 90-min centrifugation. When RbCl-treated classical chromatophores are centrifuged 20–30 h, the heavy particles still band at 48 % sucrose, but the light particles are observed to concentrate at 37 % sucrose (density, 1.17). Longer centrifugation causes no further change.

In an attempt to understand the origin of the purified particles, various fractions were subjected to sucrose density-gradient centrifugation, as described for the RbCl-treated classical chromatophores. Table I shows the distribution of BChl for a number of fractions after such centrifugation. The values given are the percentages of total pigment applied to the gradient which were recovered in a particular fraction.

The light particles are relatively pure after one treatment while the middle and heavy particles apparently aggregate, as evidenced by the large amount of precipitate found in the second centrifugation. The amount of the broken cell suspension found in the region of the light band is probably due to the presence of the ribosomes, which seem to interfere with fractionation. It would also appear that both the middle and heavy fractions contain a large number of light particles. This suggests that the heavy particles attach some light particles. The middle band may consist in part of light particles released from association with heavy particles during centrifugation.

Table II shows the distribution of the major photosynthetic pigments and protein during the course of fractionation. The data presented are average values for five preparations isolated from the various fractions, as prepared in the Ribi cell fractionator. The values presented for the light and heavy particles are for the best yields obtained, that is, when the most care was taken to prevent losses. Some losses which occurred during washing were unavoidable.

TABLE I

RECOVERY OF BACTERIOCHLOROPHYLL FROM SUCROSE DENSITY GRADIENTS

Fraction applied	*% BChl recovered*			
	Light band	*Middle region*	*Heavy band*	*Pellet*
Broken cell suspension	60	15	0	25
Large particles	9	9	0	82
Classical chromatophores	51	14	17	18
Classical chromatophores after RbCl	64	11	22	3
Light particles after one gradient	90	9	0	1
Middle fraction after one gradient	17	35	32	16
Heavy particles after one gradient	26	21	40	13

TABLE II

DISTRIBUTION OF VARIOUS COMPONENTS

The total amounts of the various components do not add up to 100% (that is, B ≠ D + E) because of differential losses incidental to multiple required operations.

Fraction	*% Found (whole cells equal 100%)*			
	BChl	*Carotenoid*	*Heme*	*Protein*
A. Large particles	24	26	23	50
B. Classical chromatophores	64	66	50	19
C. Soluble proteins	0	0	18	22
D. Light particles	41	26	25	4
E. Heavy particles	14	9	8	6

Very large percentages of pigments and heme are in the light particles. Only small amounts of heme are solubilized during fractionation.

It has been previously reported that, in *Rhodopseudomonas spheroides*[26], the yields of light particles relative to heavy particles are greatly reduced when the particles are prepared from cells which had been frozen. This is not true in *Chromatium*. The yield of light particles is independent of the length of storage (up to 10 months) and of the method of storage (at −10° or 4°).

In addition, four preparations of particles were made from cell-free extracts by grinding whole cells with alumina. When the particles were prepared in this fashion, the same fractions were found although the yields were reduced because of the lower efficiency of cell breakage. On the basis of BChl content, the alumina-prepared particles contained on the average 4.2 times as many light particles as heavy particles, com-

pared to 3.4 times as many light particles as heavy particles from preparations made with the Ribi cell fractionator.

Because of the lower efficiency of breakage by alumina treatment, it is suggested that this is a gentler method of cell breakage than Ribi fractionation. The greater yield of light particles from alumina grinding supports the notion that the light particles are not the result of comminution of the cytoplasmic membrane, but that the heavy particles may be so derived from the cell membrane, with their relative yield increased the more vigorous the method of cell disruption.

Three photosynthetic bacteria—*R. rubrum*[25], *Rhodopseudomonas spheroides*[3] and *Chromatium* Strain D—have now been shown to yield light and heavy particles. Electron micrographs of thin sections of these three bacteria[4–6] show electron-transparent vesicles. If light particles are a result of comminution of the cytoplasmic membrane, then it might be expected that organisms which do not contain vesicles would also yield light particles. *Rhodopseudomonas palustris*[27] has been shown to contain an extensive system of closely packed lamellae. When *Rhodopseudomonas palustris* (VAN NIEL'S Strain 2.1.37) was subjected to the treatment described for *Chromatium*, only one band was found for a 90-min centrifugation. This band was centered about 40 % sucrose and suggests that only heavy particles are derived from *Rhodopseudomonas palustris*.

Spectral properties

The absorption spectra of the light and heavy particles are shown in Fig. 2. The wavelengths of the absorption maxima are the same for both fractions. The most probable assignments of the various absorption maxima are as follows: (1) Maxima at 888, 845, 805, 590 and 370 mμ result from BChl[28]; (2) the maximum at 487 mμ and the shoulders at 510 and 460 mμ result from carotenoid[29]; (3) the peak at 310 mμ has been associated with BChl[30]; (4) the peak at 270 mμ results primarily from BChl with small contributions from aromatic amino acids, quinones and nucleic acids.

Table III shows the ratios of the various absorption maxima in the light particles

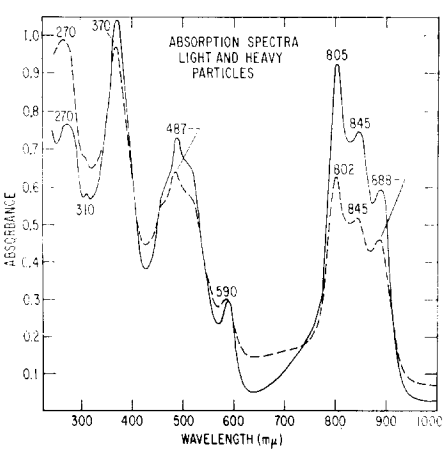


Fig. 2. ———, absorption spectrum of light particles; – – – –, absorption spectrum of heavy particles. Both spectra with Lucalox disks, at pH 7.5 (PS buffer).

relative to the 888 mμ peak. Also presented are the standard deviations for these ratios, based upon nine preparations.

It is seen that, in terms of constancy of BChl and carotenoid composition, the light particles can be prepared reproducibly. The lack of variation in the amount of 270 mμ-absorbing material suggests that the light particles have been purified to a relatively low protein content.

Bacteriochlorophyll, carotenoid, heme and protein content

Table IV presents the contents of the major pigments for purified particles. The results given are average values for nine preparations of each fraction. The light and heavy particles are quite different and the light fraction can be prepared more reproducibly than the heavy fraction.

If the data are presented as mμmoles of pigment per mg of protein as in Table V (seven preparations), it is seen that the light particles are the fraction richest in the photosynthetic pigments, which implies the removal of extraneous protein from the light particles as compared to the heavy particles.

TABLE III

ABSORBANCE CHARACTERISTICS OF LIGHT PARTICLES

	Absorption peak ratios (mμ/mμ)					
	888/845	*888/805*	*888/590*	*888/487*	*888/370*	*888/270*
Light particles	0.79	0.62	1.93	0.78	0.49	0.69
σ	0.05	0.04	0.11	0.05	0.04	0.06

TABLE IV

BACTERIOCHLOROPHYLL:CAROTENOID RATIO FOR LIGHT AND HEAVY PARTICLES

Fraction	*Moles BChl:mole carotenoid*	*Moles BChl:carotenoid:heme**
Light particles	3.98 (σ = 0.18)	23:6:1 (22.8:5.8:1)
Heavy particles	3.34 (σ = 0.28)	20:6:1 (20.0:5.8:1)

* Rounded off to integral values (actual data in parentheses).

TABLE V

PIGMENT AND HEME CONTENTS OF LIGHT AND HEAVY PARTICLES

Fraction	*Content (mμmoles/mg protein)*		
	BChl	*Carotenoid*	*Heme*
Light particles	340.0	85.7	14.9
σ	19.5	5.7	1.5
Heavy particles	79.0	24.4	4.5
σ	16.0	9.6	2.4

Cytochrome

Two heme proteins have been solubilized and characterized from *Chromatium* Strain D. These are cytochrome *c-552*[31] ('C-552') and cytochrome *cc'*[31]. C-552 is a diheme protein with typical *c*-type cytochrome spectra, with the α-peak in the reduced form at 552 mμ. C-552 has a molecular weight of 74000 (ref. 32) with one flavin (FMN) per molecule and $E_0' \cong 10$ mV. Cytochrome *cc'* is an atypical diheme *c*-type cytochrome with a molecular weight of 27000 and $E_0' \cong -5$ mV. In solution both of these heme proteins bind CO (ref. 33).

Autotrophically grown *Chromatium* has been shown to contain a cytochrome with an α-peak at 556 mμ (ref. 34). This protein ($E_0' = 330$ mV) has been observed in light-induced difference spectra of whole cells and chromatophores (R. G. BARTSCH AND J. M. OLSON, personal communication). A similar protein has been found in heterotrophically grown *Chromatium*, with an α-peak at 555 mμ ('C-555') and $E_0' = 319$ mV (ref. 35). No evidence of such a protein has been detected in soluble form. Extensive efforts to solubilize C-555 with detergents (Triton X-100, sodium salts of cholate, dodecyl sulfate and deoxycholate) and organic solvents (acetone, ethanol, chloroform, butanol) have failed. It is concluded that C-555 is a particle-bound heme protein.

No protoheme was detected in *Chromatium* Strain D, either in pyridine hemochromes or in acid methyl ethyl ketone extracts of cells or particles which had been washed free of chlorophyll and carotenoid pigments. It is concluded that no *b*-type cytochromes are present in *Chromatium*.

Table VI gives the yields of various heme proteins extracted from whole cells and classical chromatophores. Also included are the two non-heme iron proteins which have been identified in *Chromatium* Strain D, high potential iron protein[36] and ferredoxin[37].

The purified particles have not been extracted, as were the classical chromatophores, because of lack of sufficient material. However, the quantities of the heme proteins in the light particles could be estimated; *i.e.*, in mμmoles/μmole BChl, the values were approximately: 7.4 ($\sigma = 1.3$) cytochrome *cc'*; 8.4 ($\sigma = 0.6$) C-552 and 11.5 ($\sigma = 0.9$) C-555.

It was not possible to satisfactorily quantitate the heme protein composition of heavy particles because of large fluctuations in data obtained.

Cytochrome *c-552* was determined from reduced ($Na_2S_2O_4$) *minus* oxidized

TABLE VI

YIELDS OF SOLUBLE IRON-CONTAINING PROTEINS FROM *Chromatium* STRAIN D

Classical chromatophores from 100 g of heterotrophic packed cells were extracted for 24 h with 0.5% Trition X-100 (protein concentration during extraction 10 mg/ml).

Protein	*Yield (μmoles/100 g packed cells)*		
	Autotrophic cells	*Heterotrophic cells*	*Classical chromatophores*
Ferrodoxin	+	+	?
High potential iron protein	4.4	2.7	1.5
Cytochrome *c-552*	0.8	1.0	0.5
Cytochrome *cc'*	2.0	0.9	0.3

(air) difference spectra, using the peak to valley difference in absorption in the α region (552/575). Cytochrome *cc'* does not contribute to difference spectra in this region, whereas C-555 was reduced in both cuvettes under these conditions.

C-555 was determined from light-induced difference spectra in the α region (555/575)[35]. For this purpose, freshly prepared chromatophores were used. Identical samples were placed in the sample and reference cuvette under an atmosphere of argon. The sample cuvette was then illuminated with an intensity of 888 mμ light sufficient to elicit the maximum change in absorbance at 555 mμ ($2.2 \cdot 10^4$ ergs$\cdot$ cm$^{-2}\cdot$sec^{-1}) (ref. 35). C-555 was assumed to have a molecular weight of 13500 and one heme per molecule and the absorptivity of *R. rubrum* cytochrome c_2 (ref. 32) was used for quantitation.

The amount of di-heme cytochrome *cc'* was determined by subtracting the contribution of C-552 and C-555 from the total heme, as given by pyridine hemochrome assay, and dividing by two.

The ability of soluble C-552 and soluble cytochrome *cc'* to bind CO (ref. 33) was compared to that for CO binding in the light and heavy particles. Reduced-CO *minus* reduced difference spectra were determined by reducing two identical samples, suspended in PS buffer, with an excess of dithionite and tracing a baseline with the spectrophotometer using the 0–0.1 absorbance slidewire. CO was then bubbled through the sample cuvette for 2 min and the difference spectrum in the Soret region determined under an atmosphere of CO. The addition of more dithionite, or longer bubbling with CO, caused no further changes in the difference spectrum.

The absorptivity of the heme–CO complex was taken as $\Delta\varepsilon_{mM} = 200$, per heme. This value is generally valid for bacterial heme–CO complexes[33] of *c*-type cytochromes. In the soluble form, reduced-CO *minus* reduced cytochrome *cc'* has a Soret peak at 418 mμ and $\Delta\varepsilon_{mM} = 202$ per heme[33]. Reduced-CO *minus* reduced C-552 has a Soret peak at 414 mμ and $\Delta\varepsilon_{mM} = 110$ (ref. 32).

It was found that both the light and heavy particles exhibited Soret peaks at 416 mμ when treated with CO. With light particles, the reduced-CO *minus* reduced difference spectrum gave a Soret peak at 416 mμ, equivalent to 0.80 mμmole of heme binding CO per μmole BChl (determinations on four preparations). Since 43.8 mμmoles of heme were present per μmole BChl (see Table V), only 1.8 % of the heme present in the light particles bound CO under conditions tested. From the cytochrome composition (determined as above) for the light particles, 48 % of the heme present should have bound CO. Thus, the CO-binding capacity of the heme proteins found in the light particles was approx. 4 % of that expected. Similar determinations with the heavy particles showed 3–5 % of the heme present bound CO.

The position of the Soret peak at 416 mμ in the CO-treated particles suggests that a mixture of reduced cytochrome *cc'* and C-552 complexes with the reagent. It is possible that these two cytochromes are not accessible to CO when bound in the particles. Although oxygen, dithionite, ferricyanide and other redox reagents apparently can interact with the heme proteins present[35], this fact does not prove accessibility to CO because it has been shown that reactions between heme of cytochrome *cc'* and redox reagents may not require contact[38] with the central iron.

A further possibility is the cytochrome *cc'* and C-552 on extraction by aqueous solvents are so modified that they attain the capacity to bind CO. This possibility is of considerable significance, if true. If these heme proteins are modified to an

extent that new ligand-binding characteristics emerge in the soluble state, it is possible that other properties of these proteins are modified when bound. Thus, absorption spectra, redox potential and specificities for electron acceptors and donors could vary greatly, depending on binding conditions.

Iron

Non-heme iron, because of its redox properties and the implication of non-heme iron proteins in green plant[39] and mitochondrial electron transport[40], may play an important role in bacterial photosynthesis. NEWTON AND NEWTON[2] have found a 10-fold excess of iron over heme iron in *Chromatium* classical chromatophores. Preliminary Mössbauer spectral studies (A. J. BEARDEN, T. H. MOSS, R. G. BARTSCH AND M. A. CUSANOVICH, unpublished observations) indicates that the classical chromatophores contain one dominant species of iron.

Iron analysis of the light and heavy particles revealed that in the light particles 8.9 ($\sigma = 3.4$) μmoles of iron per μmole heme were present, whereas in the heavy particles, a variable set of data was obtained, with values ranging from 5–20 μmoles Fe/μmole heme. In the light particles, heme accounted for only 11 % of the iron. Some of the remaining iron was in the form of non-heme iron proteins (*e.g.*, high potential iron protein, and possibly ferredoxin and succinate dehydrogenase) which could not be estimated precisely. The amount of high potential iron protein present in classical chromatophores was as given in Table VI. Applying this result to the light particles, 20–30 % of the iron could be accounted for as high potential iron protein, leaving 60–70 % of the iron unidentified.

Quinones

Quinones have been observed and implicated[43] in electron transport in *Chromatium*. Identified so far are vitamin K_2 (ref. 44) in classical chromatophores (approx. 75 mμmoles/μmole BChl) and CoQ_7 (ref. 44) (approx. 0.22 μmole/μmole BChl). Using procedures described in METHODS, we found 26 mμmoles of CoQ_7 per μmole BChl or 8.7 mμmoles/mg protein. These values, which are below those previously reported, are still at a relatively high level (over half that of heme).

Enzymology

Enzymology of the particles was studied by assaying for various enzymes which might catalyze electron transport; included were NADH oxidase, succinate dehydrogenase, heme protein reductase and DCIP reductase. Table VII gives the distribution of the various enzymes during fractionation. The recoveries do not total 100 % because of the omission of minor fractions and inactivation of the enzymes.

Table VII shows that there is essentially no dye reductase, heme reductase or NADH oxidase in the purified particles. It would appear that these are soluble and removed in washing procedures.

Succinate dehydrogenase is found in large amounts in both the light and heavy particles, as expected for a particle-bound enzyme. The specific activities of succinate dehydrogenase (unit activities per mg of protein) in the two particles are similar—0.35 for the light particles and 0.43 for the heavy particles. Relative to BChl, the heavy particles are approx. 6 times richer in succinate dehydrogenase than are the light particles.

Protein–lipid content

In Table VIII, we present the percentage of protein relative to the dry weight of the various fractions. Also shown is the percent of dry weight extractable with organic solvents.

TABLE VII

ENZYME CONTENT OF VARIOUS FRACTIONS

Concentrations of the reagents were as follows: DCIP reductase-DCIP 100 μM, NADH 600 μM; horse-heart cytochrome *c* reductase-horse-heart cytochrome *c* (Fe^{3+}) 33 μM, NADH 600 μM; c_2 reductase-*R. rubrum* cytochrome c_2 (Fe^{3+}) 33 μM, NADH 600 μM; NADH oxidase NADH 600 μM; succinate dehydrogenase-*R. rubrum* cytochrome c_2 (Fe^{3+}) 33 μM, sodium succinate 10 mM. All reactions occurred in 0.1 M Tris buffer (pH 8.0). The temperature was 22° ± 2° and the total reaction volume was 1.0 ml. DCIP reductase was monitored as the decrease in $A_{600\ m\mu}$/min, horse-heart cytochrome *c* and c_2 reductase as the increase in $A_{550\ m\mu}$/min, NADH oxidase as the decrease in $A_{340\ m\mu}$/min and succinate dehydrogenase as the increase in $A_{550\ m\mu}$/h.

Fraction	*Enzyme assayed* (%)				
	DCIP reductase	*Horse-heart reductase*	*c_2 reductase*	*NADH oxidase*	*Succinate dehydrogenase*
Broken cell suspension	100	100	100	100	100
Large particles	6	10	7	18	10
Soluble proteins	70	41	37	3	0
Classical chromatophores	19	13	5	20	71
Classical chromatophores after RbCl	7	4	4	6	56
Light particles	(0.1)	(0.1)	1	0	17
Heavy particles	(0.1)	(0.1)	2	0	43

TABLE VIII

PROTEIN AND LIPID CONTENT OF VARIOUS FRACTIONS

Fraction	% *dry weight*	
	As protein	*Organic soluble*
Whole cells	79	—
Large particles	80	—
Soluble proteins	84	—
Classical chromatophores	33	—
Light particles	26–28	62–72
Heavy particles	62–68	22–30

The classical chromatophores and the light particles are very rich in non-proteinaceous material. The heavy particles have a composition quite similar to the large particle fraction which contains large pieces of the cell membrane.

The light particles are composed, on the average, of 67 % lipid and 27 % protein. The lipid content is unusually high for electron transport tissue, *e.g.*, quantasomes have been found to be approx. 50 % lipid and 50 % protein[45] and mitochondria 25 % lipid and 75 % protein[46].

Carbohydrate

Less than 0.3 % of the dry weight of the light particles could be attributed to carbohydrate. The heavy particles were found to contain up to 1 % carbohydrate by weight.

These results indicate that the fractionation method used removes essentially all of the carbohydrate and appears to exclude carbohydrate as an essential component in the structure or function of our chromatophore preparations.

Triton X-100 extraction

A recent report[47] described fractionation of chromatophores when treated with Triton X-100, a non-ionic detergent. This work was done on particles sedimenting between 30000 and 100000 $\times$ *g* in 1 h. This method of fractionation left much of the BChl in the supernatant fraction. We tested this procedure with the light and heavy particles.

The particles were diluted to 10 mg of protein per ml; then, the suspension was made 0.5 % in Triton X-100, and allowed to stand for 2 h at 4°. The suspensions were then pelleted by centrifugation at 144000 $\times$ *g* for 2 h.

In the case of light particles, after centrifugation, all of the BChl was found in the pellet. On resuspension, these particles exhibited an absorption spectrum essentially identical to the particles before treatment with the detergent, except for a decrease (approx. 10 %) of the 270 mμ peak. Residual Triton X-100 was removed by passing the treated particles over Sephadex G-25.

The supernatant of the Triton-treated particles contained about 15 % of the heme present in the untreated particles. Although it was not determined how much of the CO-binding capacity should be ascribed to cytochrome *cc'*, or C-552, it was clear that a large percentage of the solubilized heme bound CO. From measurements of the absorbance of the CO complex, it was found that approximately 35 % of the solubilized heme bound CO. Since 15 % of the heme present in the untreated particles had been solubilized, this meant that over 5 % of the total heme bound CO after treatment with Triton X-100. This was 2.5 times the CO-binding capacity found in the untreated particles and verified the previous conclusion that the CO-binding capacity of *Chromatium* heme proteins was increased by solubilization.

The cytochrome composition of the Triton-treated particles could not be determined because the addition of dithionite caused precipitation even after passing the particle suspension through a Sephadex G-25 column to remove remaining Triton X-100.

In the case of heavy particles, centrifugation resulted in two BChl-containing fractions, a precipitate which could not be resuspended and a supernatant fluid rich in pigment. The supernatant contained 66 % of the BChl and 44 % of the heme present prior to treatment with Triton X-100. The absorption spectra of the supernatant fluid were quite similar to those found by GARCIA, VERNON AND MOLLENHAUER[47] after a second treatment of their heavy particles with Triton X-100. The fractionation by GARCIA, VERNON AND MOLLENHAUER resulted in two fractions, one consisting apparently of large pieces of membrane, and the other small spherical particles. These results indicate that the fraction studied by GARCIA, VERNON AND MOLLENHAUER may correspond to the heavy particles described in our work.

Weight composition

Table IX shows the composition of the purified particles. Of special interest is the high percentage of protein accounted for as cytochrome—over 30 % of the protein present in the light particles.

TABLE IX

MOLECULAR COMPOSITION OF LIGHT PARTICLES

Component	% *dry weight*	
	Light particles	*Heavy particles*
Cytochrome	8.6	—
Unidentified protein	18.4	65.0
Ubiquinone	0.2	—
BChl	8.3	5.2
Carotenoid	1.3	1.0
Unidentified lipid	57.0	19.5
Iron	0.2	—

The contribution of each cytochrome to protein content (and thus dry weight) was calculated from Eqn. 1.

$$\frac{\text{mg cytochrome}}{\text{mg protein}} = (\text{mol. wt.}) \frac{(\mu\text{mole cytochrome})}{(\mu\text{mole BChl})} \frac{(\mu\text{mole BChl})}{(\text{mg protein})} \qquad (1)$$

where μmole cytochrome/μmole BChl was obtained from data given above and μmole BChl/mg protein from data of Table V.

Partial specific volume

The partial specific volume of the light particles was determined at different concentrations. No concentration dependence was found over the range 500 μM to 3 mM in BChl.

The partial specific volume of the classical chromatophores was found to be 0.803, in good agreement with the value (0.801) given by BERGERON[5]. The partial specific volume of the light particles was 0.667. This value, while quite low for such a lipid-rich fraction, was determined 5 times on three different preparations with a standard deviation of $\pm$0.030.

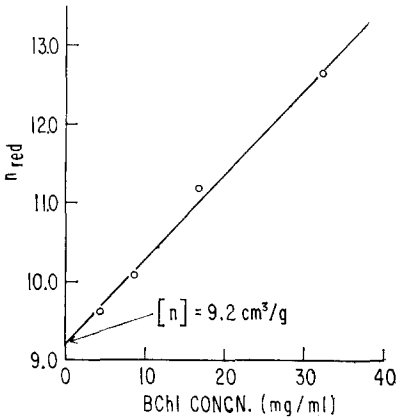
Intrinsic viscosity

The specific viscosity of the light particles, determined at different concentrations and extrapolated to infinite dilution, as shown in Fig. 3, gave an intrinsic viscosity ($[\eta]$) of 9.2 cm^3/g.

Sedimentation constant

Ultracentrifugation of the light particles yielded a sedimentation constant of 145 S at 20°, extrapolated to infinite dilution, as shown in Fig. 4. This value agrees well with that reported by BERGERON[5]. The particle concentration was varied from 4 to 20 μM in BChl. The speed was 20410 rev./min. Both schlieren and absorption optics were used, giving identical results.

The heavy particles were found to be grossly heterogeneous in the ultracentrifuge with much high molecular weight material present, even at high dilution (2 μM in BChl).



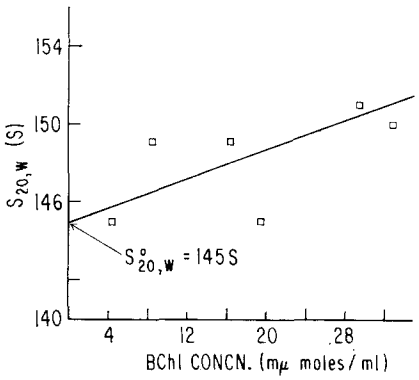


Fig. 3. Extrapolation of the reduced viscosity of the light particles to infinite dilution, at pH 7.5 (10 mM potassium phosphate).

Fig. 4. Extrapolation of the sedimentation constant of the light particles to infinite dilution, at pH 7.5 (10 mM potassium phosphate). Rotor speed 20410 rev./min, temp. 20°.

Molecular weight

The molecular weight of the light particles was determined from Eqn. 2

$$M = \frac{4690\,(s^0_{20,w})^{3/2}\,[\eta]^{1/2}}{(1 - \bar{v}\rho)^{3/2}} \tag{2}$$

where, $s^0_{20,w}$ is the sedimentation constant at 20° extrapolated to infinite dilution; $[\eta]$, intrinsic viscosity in $(g/100\ ml)^{-1}$; $\bar{v}$, partial specific volume; ρ, density of the solution.

Eqn. 2 was derived from Eqn. 3 as given by SCHERAGA AND MANDELKERN[48]:

$$M^{2/3} = \frac{Ns[\eta]^{1/3}\eta}{\beta(1 - \bar{v}\rho)} \tag{3}$$

where, η, the viscosity of the solution; N, Avogadro's number and β is a constant which is independent of axial ratio for oblate ellipsoids and varies only slowly for prolate ellipsoids. An average value for β of $2.16 \cdot 10^6$ was used[49].

Eqn. 2 gives a molecular weight of $12.9 \cdot 10^6$ for the light particles. This value is consistent with BERGERON's previous estimates[5] for the molecular weight of chromatophores ($13 \cdot 10^6$).

Radius

The radius of a spherical particle was calculated by combining Eqns. 4 and 5 (see ref. 49 for derivation of the equations).

$$f = 6\pi\eta r \tag{4}$$

$$M = \frac{Nfs}{(1 - \bar{v}\rho)} \tag{5}$$

Thus,

$$r = \frac{M(1 - \bar{v}\rho)}{sN6\pi\eta} \qquad (6)$$

On substitution of the experimental values, the radius of the light particles was found to be 262 Å.

Electron microscopy

An electron micrograph of the negatively stained light particles is shown in Fig. 5.

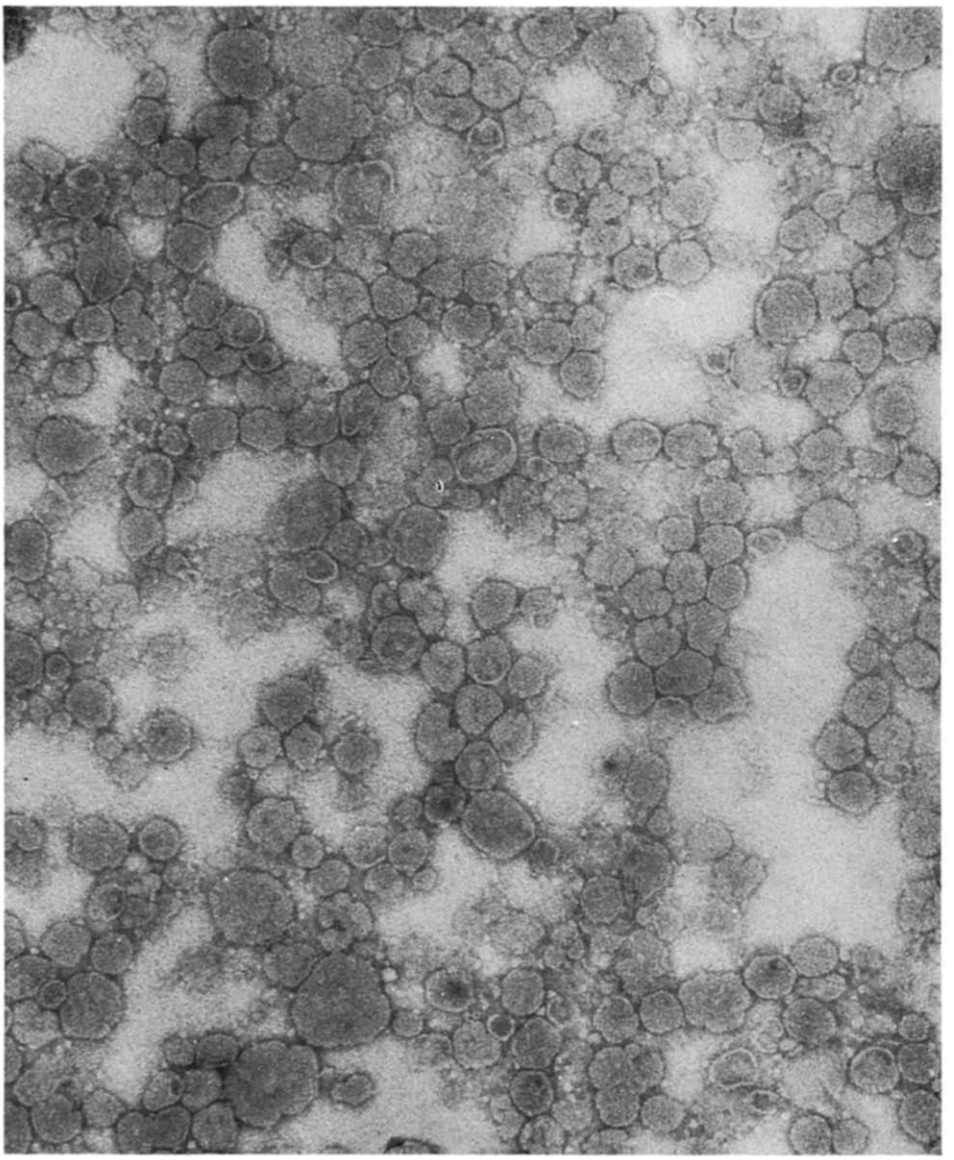


Fig. 5. Electron micrograph of light particles negatively stained with 3% phosphotungstate. Magnification 90000 ×.

The light particles appeared to be spheres of a fairly uniform size. Statistical analysis of 1000 of the particle images, assuming they were spheres, yielded an average image radius of 327 Å. This value held for the hydrated chromatophore which is the active system in our researches. Calculations indicate that for the anhydrous system the radius would be approx. 150 Å, a value in good agreement with that of BERGERON[5]. 85 % of the particles lay within 100 Å of the mean radius. Thus, there was some heterogeneity. Remembering that the particles were exposed to a high vacuum during observation in the electron microscope, and hence subject to some distortion,

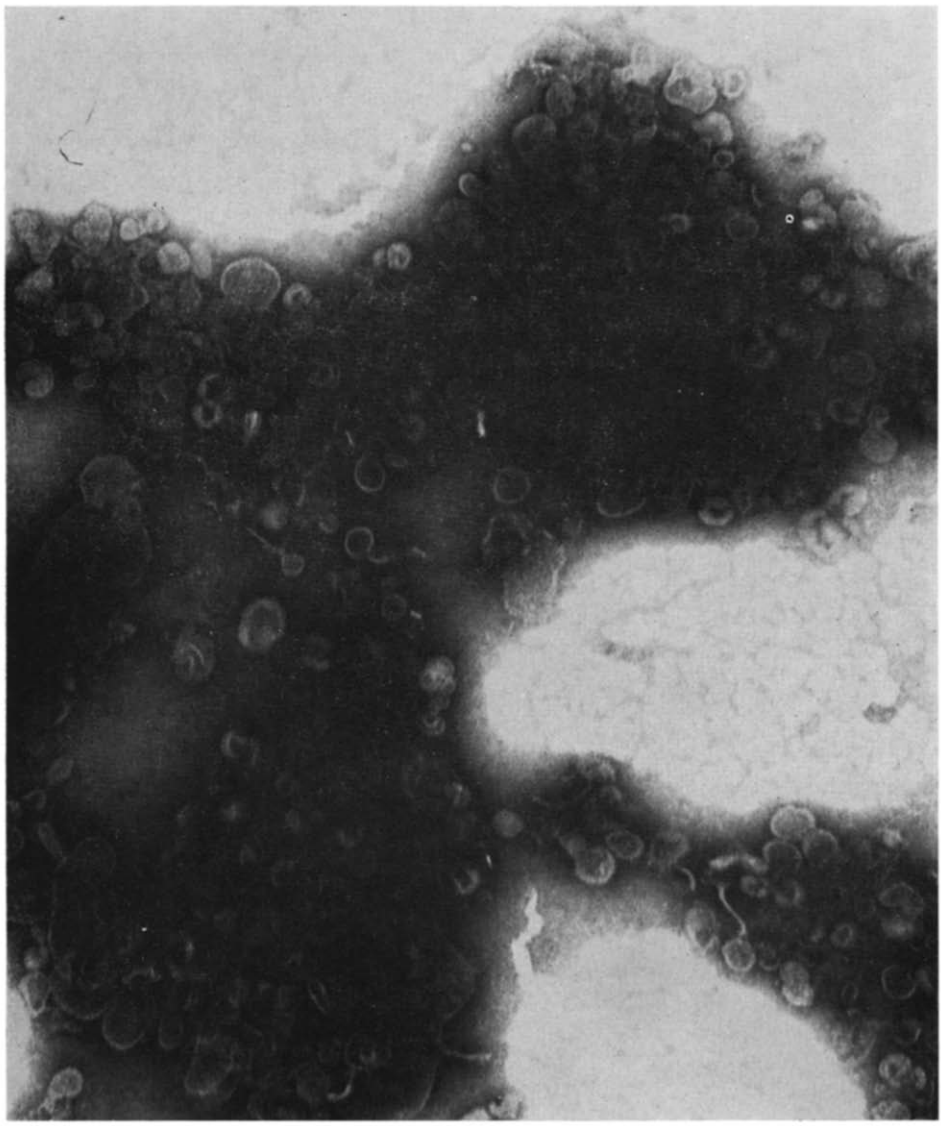


Fig. 6. Electron micrograph of heavy particles negatively stained with 3% phosphotungstate. Magnification 90000 ×.

the agreement between the average radius obtained by visual inspection of the electron micrographs and that calculated from the ultra centrifuge was satisfactory.

An electron micrograph of the heavy particles, shown in Fig. 6, indicates that heavy particles were quite heterogeneous with much non-spherical material present. Further, what appeared to be 'tails', that is, short pieces of membrane attached to the particles, were present on a number of the heavy particles.

Molecular composition

From the molecular weight and the percent dry weight contribution of the various components, the molecular composition of the light particles could be estimated for some of the components identified. The results of these calculations are presented in Table X.

TABLE X

DISTRIBUTION OF SUBSTANCES IN LIGHT PARTICLES (ON BASIS OF A $12.9 \cdot 10^6$ MOL. WT.)

Lipid composition (molecules/particle)		
1170	Bacteriochlorophylls	1 070 000
302	Carotenoids	168 000
30	Ubiquinones	22 000
—	Unidentified lipids	7 400 000
		8 660 000
Protein		
9.9	Cytochrome *c*-552	728 000
13.2	Cytochrome *c*-555	179 000
8.4	Cytochrome *cc'*	207 000
—	Unidentified protein	2 350 000
		3 464 000
Other		
466	Iron	25 800
	Unidentified components	750 000
		775 800
	Total	12 899 800

The values, normalized to the content of various heme proteins, may be considered as reflecting the size of a hypothetical photosynthetic unit involving only a single primary cytochrome (Table XI).

TABLE XI

MOLECULAR CONTENT OF LIGHT PARTICLES BASED ON SINGLE HEME CONTENT

Component	*Molecules/molecule cytochrome*		
	c-555	*c*-552	*cc'*
BChl	90	117	146
Carotenoid	23	30	38
CoQ_7	2	3	4
Iron	37	47	58
Cytochrome *c*-555	1.0	1.3	1.6
Cytochrome *c*-552	0.7	1.0	1.2
Cytochrome *cc'*	0.6	0.8	1.0

DISCUSSION

Except for the work of WORDEN AND SISTROM[3], little has been done previously to refine the heterogeneous mixtures of subcellular particles obtained from photosynthetic bacteria by differential centrifugation, or by other procedures. The lack of reproducibility of such preparations, called indiscriminately 'chromatophores' (our term 'classical'), has been a hindrance in advancing knowledge of structure and function in the bacterial photosynthetic apparatus. Uncertainties also exist as to the source of chromatophores, their relation to the internal structure of the bacteria, and even their relevance to the actual *in vivo* machinery of the intact photochemical system.

However, it is idle to speculate on the degree to which chromatophores, or 'chromatophore fragments' may be artifacts. The question is rather whether functional units can be obtained which exhibit in some degree homogeneity in shape, size and composition, which are relatively stable and which can be prepared in a reproducible fashion. Such particles should be well adapted to precise measurement of photochemical functions, such as photophosphorylation, light-induced electron transport, *etc.* In particular, they should permit experiments permitting classification of these processes by measurements of responses under carefully controlled conditions. In the researches discussed in this and succeeding papers, this expectation has been realized in large measure. Accordingly, we have applied the term 'chromatophore' to the fraction we have termed 'light particles', in distinction to the usual heterogeneous mixtures, which we have called 'classical chromatophores'.

We have described the fractionation of classical chromatophores into two components, very different in chemical composition and properties. One component—light particles—can be prepared in large yields containing as much as 40 % of the total photoactive pigment in the whole cells. In addition, they can be prepared reproducibly and are satisfactorily homogeneous. In contrast, the other component—heavy particles—contains only a small part of the total pigment, is heterogeneous and can not be prepared reproducibly.

The small but reproducible amount of protein in the light particles suggests that these particles have been stripped of much non-functional protein. Cytochrome accounts for 30 % of the protein present. Succinic dehydrogenase—generally a high molecular weight protein—is present. Thus, cytochromes and succinic dehydrogenase alone could account for much of the protein present.

The heavy particles differ from the light particles mainly with respect to protein content. The protein–lipid content of the heavy particles is quite similar to that of fractions containing large pieces of the cell membrane. Moreover, the more vigorous methods of fractionation of whole cells result in greater yields of heavy particles, and electron microscopy reveals that pieces of membrane appear to occur still attached to the heavy particles. These facts support the suggestion that the heavy particles are derived from the cytoplasmic membrane.

The data presented indicate but do not prove that our *Chromatium* chromatophores exist *in vivo* and are not the result of the method of preparation.

It should be stressed that our conclusions are not meant to apply in general to all photosynthetic bacteria. There is ample evidence that many of the photosynthetic bacteria do not contain bodies recognizable as chromatophores. Our results are valid for one strain of *Chromatium* cultured under one particular set of growth conditions.

Thus, it has been shown[50] that *Chromatium* Strain D, when grown under certain conditions—particularly under much higher light intensity than we used—contains what appears to be lamellar structures. This observation is not inconsistent with the results of the present research, because it is not unreasonable to expect *Chromatium* to reorganize its internal structure when its environment is modified.

The major finding may be summarized thus: chromatophores, in the form of very small, lipid-rich particles which can be obtained reproducibly from *Chromatium*, exhibit a fixed shape and size under a variety of extraction procedures and are homogeneous in their content of many important biochemical substances—photoactive pigments and electron transport components.

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