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A LARGE PHOTOREACTIVE PARTICLE FROM *CHROMATIUM VINOSUM* CHROMATOPHORES

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SUMMARY

Large photoreactive particles from *Chromatium vinosum* are obtained pure and in high yield by using a mixture of detergents at high ionic strength to dissociate the chromatophore membrane. The particles contain all of the secondary electron acceptor of the chromatophores and about half of the cytochrome. Their content of ubiquinone is greatly enriched as compared with chromatophores. The individual particles have an estimated molecular weight of between 650 000 and 810 000.

Gel electrophoresis of the preparation in sodium dodecylsulfate shows polypeptides with molecular weights of 50–45 000, 30 000, 27 000, 22 000 and 12 000. The 50–45 000 components are cytochromes. The 30 000, 27 000 and 22 000 components may be analogous to the triad of polypeptides present in *Rhodopseudomonas spheroides* reaction centers. The non-cytochrome components are partly soluble in chloroform/methanol.

Aggregates of particles appear in these preparations. Electron microscopy of the aggregates demonstrates rectilinear lattices of isodiametric particles, 120 Å in diameter. These sheet-like structures are one unit thick and typically contain 9–16 members. They appear to arise by aggregation during isolation but are probably similar to native aggregates apparent within chromatophores after treatment with detergents at low salt concentration.

INTRODUCTION

Photosynthetic bacteria have provided suitable material for the isolation of subcellular systems responsible for photosynthesis [1]. From the chromatophores of *Athiorhodaceae*, relatively simple photochemical reaction center particles have been prepared. They contain only the bacteriochlorophyll complex (P_{870}) which is photoactive and the primary electron acceptor. They lack light-harvesting (or "bulk") bacteriochlorophyll and cytochromes and are deficient in secondary electron acceptors [2]. These reaction centers are composed of three polypeptide subunits centering

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on 25000 mol. wt [3]. Large photoreactive particles isolated from the *Thiorhodaceae* and *Chlorobacteriaceae* are more complex; they contain light-harvesting bacteriochlorophyll and cytochromes [4]. These are therefore of potential utility in the examination of the photosynthetic reactions which follow the initial loss of an electron from the reactive chlorophyll. The question of the mode of integration of reaction center particles into the native large subunit remains to be elucidated.

Thornber [5] has described a preparation ("fraction A") from *Chromatium vinosum* (*Thiorhodaceae*) which contains bacteriochlorophyll and cytochromes but lacks the secondary acceptor (termed "Y") that is found in intact chromatophores [6]. The polypeptide composition of fraction A has not been described. In the present study, the preparation of fraction A has been modified so that the overall yield is markedly improved and the secondary electron acceptor is retained. A high ubiquinone content for preparations with secondary electron acceptor activity is reported in this paper in agreement with the demonstration that ubiquinone is necessary for this activity in *C. vinosum* [7]. We have investigated the cytochrome content and the polypeptide composition of our preparation and have found a characteristic set of three polypeptides similar to those in reaction centers from *Athiorhodaceae*, thus enlarging the association of a set of polypeptides of this type with photochemical activity. A similar triad has also been found in algal chloroplasts [8, 9]. A triad of polypeptides centering on a mol. wt of 35000 has been reported in spinach and algal subchloroplast fractions [10]. The apparent higher molecular weight reported may be the result of the use of only one protein marker in the 30000 mol.wt range.

The structural properties of subchromatophore preparations may be determined by electron microscopy. Subfragments prepared by treating the chromatophores of *Rhodopseudomonas* sp. NHTC 133 with Triton X-100 reveal a hexagonal array of particles 130 Å in diameter [11]. Chromatophores of *Rhodopseudomonas spheroides* contain 120–130 Å particles which are liberated by Triton X-100 [12]. A photoactive fraction, prepared with Triton, from *C. vinosum* has also been shown to consist of large particles [13] and plant chloroplast Photosystem I activity has been identified with 110 Å particles [14]. In the present study we have found that our preparations are composed of 175×110 Å particles, and also that they are arranged in rectilinear arrays under certain conditions. Because we suspected that these arrays might represent an arrangement present in the original chromatophores, we have investigated their occurrence during dissolution of the chromatophores as well as the aggregation of purified particles in our preparations.

MATERIALS AND METHODS

Photochemical cytochrome oxidation. Cytochrome oxidation upon flash excitation was measured by the absorption change at 422 nm, as previously described [15], in the presence of 5–7 μM phenazine methosulfate and 190 μM $Na_2S_2O_4$. Two saturating laser flashes, 4 ms apart, were used. The cytochrome oxidation occurring on the second flash depends on the presence of secondary acceptor [16].

Electron microscopy. Samples were applied to carbon-coated formvar films which had been treated by glow discharge to improve their hydrophilicity. These were negatively stained with 1 % potassium phosphotungstate adjusted to pH 7.0 with KOH or with unadjusted 0.5 % uranyl acetate and viewed in a Philips EM 300

electron microscope. Electron micrographs of rectilinear arrays were tested for detailed substructure by optical diffraction but were consistently found to lack higher orders of data than their fundamental periodicities.

Detergent. Sodium dodecylsulfate was obtained from Sigma and Gallard-Schlesinger. Brij-58 (polyoxyethylene (20) cetyl ether) was a sample kindly furnished by ICI America. Triton X-100 was obtained from Rohm and Haas.

Protein, ubiquinone and bacteriochlorophyll determination. The concentration of large photoreactive particles and chromatophores was determined from 890 nm absorption. In order to convert these figures to bacteriochlorophyll concentration, we determined $\varepsilon = 127\ mM^{-1} \cdot cm^{-1}$ for particles and $\varepsilon = 42\ mM^{-1} \cdot cm^{-1}$ for chromatophores. Bacteriochlorophyll was determined spectrophotometrically at 767 nm after extraction into 7 : 2 acetone : methanol [17]. Protein was determined by absorption at 280 nm and also by the method of Lowry et al. [18]. Ubiquinone was determined at 275 nm as previously described [7].

Cytochrome and heme determinations. The content of cytochromes was determined by chemical difference spectroscopy using sodium ascorbate and $Na_2S_2O_4$ [5]. Heme was determined as the pyridine hemochrome in 25 % pyridine and 0.2 M NaOH after reduction with $Na_2S_2O_4$. The difference spectrum against a sample lacking $Na_2S_2O_4$ was measured and the peak at 548 nm was used with $\varepsilon = 34.7\ mM^{-1} \cdot cm^{-1}$ [19]. Heme was also determined by using benzidine as follows. To 1 vol. of solution containing 10–50 pmol of heme was added one volume of a reagent made by mixing 50 ml of methanol containing 0.2 g benzidine dihydrochloride, 10 ml glacial acetic acid, and 3 ml of fresh 3 % H_2O_2. The unstable green color was read at 590 nm after 3 min at 23 °C. Cytochrome *C* (horse heart, Sigma) was used as standard. The method cannot be used with solutions containing more than 0.05 % sodium dodecylsulfate because the detergent precipitates the benzidine.

Gel chromatography. Agarose (Biogel) was standardized by using chromatophores (breakthrough volume for A5m), bovine thyroglobulin (Sigma), pyruvate kinase (Sigma), bovine serum albumin (Nutritional Biochemicals), egg albumin (Sigma), myoglobin (Sigma), and cytochrome *c* (Sigma). 0.05 M Tris/HCl, pH 8.1, was used as solvent.

Gel electrophoresis in sodium dodecylsulfate. We used the system described by Laemmli [20] except that the electrode buffer had $\frac{1}{4}$ the concentration of tris and glycine. The protein samples were dissociated in 7.5 % sodium dodecylsulfate containing 20 % glycerol or 4 M urea, 0.01 M dithiothreitol, and 0.05 M pH 7.0 spacer buffer, by heating for 2–4 min at 80 °C. 0.01 M sodium iodoacetate was then added. Slab gels containing 10 % polyacrylamide were employed unless noted otherwise. They were run at 230 V $1\frac{1}{2}$–2 h and washed in 68 : 25 : 7 water/methanol/acetic acid. They were then stained with 0.05 % Coomassie Brilliant Blue R (Sigma) in the same solvent. Heme was stained in parallel gels by the method of Haut et al. [21]. Proteins used for heme staining cannot be heated with dithiothreitol as we observed that the reaction of sulfhydryl with heme [22] destroys the peroxidatic activity of the heme. Absorbance scans of the stained gels were obtained with a Joyce-Loebel microdensitometer. Gels were calibrated with bovine serum albumin (Sigma) (66 000 mol.wt), beef liver catalase (Sigma) (57 500), rabbit immunoglobulin (Miles Labs) (50 000 and 23 500), egg albumin (Sigma) (45 000), aldolase (Worthington) (40 000), glyceraldehyde-3-phosphate dehydrogenase (Sigma) (37 000), deoxyribonuclease I (Sigma)

(31 000), chymotrypsinogen (Worthington) (25 000), casein (Nutritional Biochemicals) (24 000), soybean trypsin inhibitor (Kunitz, Worthington) (21 000), myglobin (Sigma) (17 000), and cytochrome *c* (Sigma) (13 000). The acrylamide and *N,N'*-methylene bisacrylamide were Eastman products and their solution was decolorized with Norite before using. Molecular weight was calculated from the distance migrated relative to egg albumin using the regression line established with the above standard proteins.

Hydroxyapatite preparation. The method of Tiselius et al. [23] was used. Hydroxyapatite may also be prepared by following this method to the point of putting the adsorbent in pH 6.8 buffer and omitting the subsequent boilings in this buffer. If this procedure is used the adsorbent should be used within two months; otherwise it yields preparations having low secondary acceptor activity. The hydroxyapatite may be re-used after washing with 0.5 M sodium phosphate (pH 7) followed by a mixture of 0.2 % sodium dodecylsulfate and 0.2 % Brij-58 in water. Hydroxyapatite which gives preparations having low secondary acceptor activity because of long storage yields normal preparations upon re-use after washing.

Cell culture and chromatophore preparation. C. vinosum was grown as previously described [15]. Cells were washed once with iced 0.05 M Tris-Cl (pH 8.1–8.2) before disruption. (Unless otherwise noted the same buffer was used in all procedures.) Iced cells were disrupted in a Branson Sonifier operated at 2 A for 10 min. The sonicate was centrifuged for 10 min at 20 000 $\times g$ and the supernatant centrifuged two or more times at 105 000 $\times g$ for $1\frac{1}{2}$ h. Material from 8–10 l of cell culture was diluted to a volume of 60 ml or more for the latter centrifugations. The pellet of washed chromatophores retain full secondary acceptor activity for at least one month if resuspended in buffer and stored at 4 °C.

Standard large photoreactive particle preparation. A 4.5 $\times$ 10–12 cm column of hydroxyapatite equilibrated with 0.01 M sodium phosphate (pH 6.9–7.0) containing 0.25 M $(NH_4)_2SO_4$ (this concentration of $(NH_4)_2SO_4$ was present in all buffers used on hydroxyapatite) was loaded with 15–20 ml of a suspension of chromatophores ($A_{890\,nm}$ = 25–30) in 1.33 % sodium dodecylsulfate, 1.33 % Brij-58, and 0.25 M $(NH_4)_2SO_4$. The column was washed with the starting buffer and eluted with 0.09–0.12 M sodium phosphate. The optimum salt concentration for elution of large photoreactive particles was determined beforehand by running a small preliminary column. On fresh hydroxyapatite which had not been boiled at pH 6.8, and on re-used hydroxyapatite, the bulk of the large photoreactive particles eluted just after the detergents in 0.01 M phosphate. From hydroxyapatite prepared according to Tiselius et al. [23], the particles are eluted in 0.09–0.12 M phosphate. The eluted fraction was iced and precipitated by adding 1.2 g $(NH_4)_2SO_4$ per 10 ml. The precipitate was redissolved in a small amount of buffer and chromatographed on A5m agarose to remove an orange-brown material. The preparation is extremely stable to storage at this point, either at 4 or −20 °C. It may also be desalted and lyophilized.

Sodium dodecylsulfate-only preparations of large photoreactive particles. These were made exactly as described above except that Brij-58 was replaced by an equal volume of buffer. These particles elute from hydroxyapatite at 0.17–0.25 M phosphate.

RESULTS

Purification of large photoreactive particles containing secondary electron acceptor.

Chromatography on hydroxyapatite of the chromatophore/detergent/$(NH_4)_2SO_4$ mixture described in Methods for the standard preparation readily separates large photoreactive particles from the light-harvesting bacteriochlorophyll-protein complex. The peak of particles (Fig. 1), identified by $A_{890\,nm}$, begins to elute with 0.1 M phosphate, whereas the light-harvesting complex, identified by $A_{800\,nm}$, begins to elute with about 0.2 M phosphate. Table I (lines 1–3) gives data on the purification and yield during the course of a standard preparation carried out by stepwise elution as described in Methods. The secondary electron-acceptor activity is well maintained throughout the purification. Chromatography of the purified particles (Table I, line 3) on DEAE-sephadex gave no further purification.

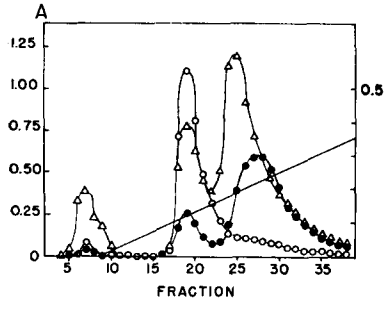


Fig. 1. Gradient elution of 6 mg chromatophore protein in 1.3 % sodium dodecylsulfate 1.3 % Brij-58 and 0.25 M $(NH_4)_2SO_4$. The material was applied in a volume of 1 ml to a 0.8 × 12 cm column on hydroxyapatite in 0.01 M phosphate containing 0.25 M $(NH_4)_2SO_4$. Fractions were 0.9 ml each. The left ordinate is absorbance at the indicated wavelength: ○, at 890 nm; ●, at 800 nm; △, at 280 nm. The right ordinate gives the molar phosphate concentration for the gradient line.

The importance of Brij-58 in this procedure is clear from the results shown in Table I. If Brij-58 is omitted (preparation on line 4) or reduced in amount (preparation on line 5), the secondary acceptor activity is practically lost and the overall yield of particles is reduced. Some of the improved yield obtained when Brij-58 is included may result from better elution of the proteins from hydroxyapatite. The standard preparation elutes at a lower phosphate concentration than does the sodium dodecylsulfate-only preparation (see above, Methods). (Brij-58 also allows the elution of chromatophores from hydroxyapatite. Chromatophores are irreversibly adsorbed in the absence of detergent.) Beyond this effect, Brij-58 appears to protect the photochemical apparatus against deterioration caused by sodium dodecylsulfate (Fig. 2). Photochemical cytochrome oxidation is markedly unstable to this detergent, particularly the oxidation occurring after the second flash, which is dependent on the presence of secondary acceptor (Fig. 2B). The instability to sodium dodecylsulfate is enhanced in the presence of 0.25 M $(NH_4)_2SO_4$. If Brij-58 is included, the photochemical activities become quite stable.

TABLE I

PURIFICATION AND YIELD OF LARGE PHOTOREACTIVE PARTICLES

	% of $A_{890\ nm}$	$\frac{A_{890\ nm}}{A_{800\ nm}}$	$\frac{A_{890\ nm}}{A_{280\ nm}}$	Activity $\frac{\Delta A_{422\ nm}}{[\text{Chlorophyll}]^{\star}}$		% of starting activity recovered	
				1st flash	2nd flash	1st flash	2nd flash
1. Chromatophores	100	0.62	0.48	0.70	0.59	100	100
2. Hydroxyapatite eluate reprecipitated with $(NH_4)_2SO_4$	48	4.2	1.3	3.8	3.0	80	73
3. As in line 2, but after agarose purification	38	4.8	1.6***	3.8	3.1	64	58
4. Sodium dodecylsulfate-only preparation after agarose purification	19	4.9	1.4	3.1	0.60	27	5
5. 1/4 Brij-58** preparation after agarose purification	21	4.0	—	3.2	0.58	28	6

* mM.

** Prepared with 1/4 the amount of Brij-58 used in the standard preparation.

*** The ratio of $A_{890\ nm}$/mg protein by the method of Lowry et al. [18] is about 8 using bovine serum albumin as standard. This ratio is about 2 for chromatophores.

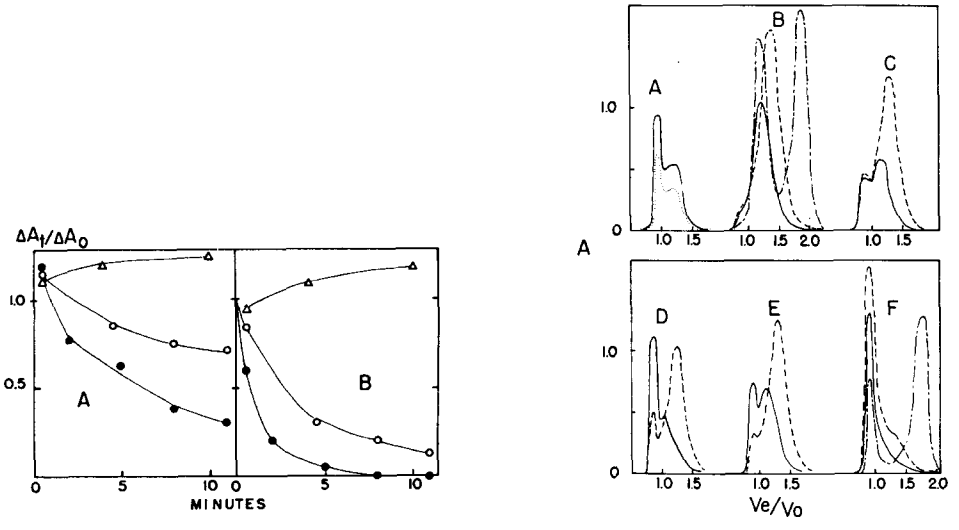


Fig. 2. The decrease of photochemical cytochrome oxidation as a function of time of exposure to detergents and salts. In A the ordinate is the fraction of absorbance change at 422 nm remaining on the first flash. In B the ordinate is the same quantity, but after the second flash. The incubation mixtures contained 12 mg/ml chromatophore protein and: ○, 1.6 % sodium dodecylsulfate; ●, 1.6 % sodium dodecylsulfate and 0.3 M $(NH_4)_2SO_4$; △, 1.6 % sodium dodecylsulfate, 1.6 % Brij-58 and 0.3 M $(NH_4)_2SO_4$. The abscissa is time in minutes. The mixture was diluted × 60 in buffer and 5 μM phenazine methosulfate was added for the assay of photochemical activity. The fraction of original $A_{890\ nm}$ remaining at 10 min was 0.55 for sodium dodecylsulfate only, 0.51 for sodium dodecylsulfate and $(NH_4)_2SO_4$, and 0.75 for sodium dodecylsulfate, Brij-58 and $(NH_4)_2SO_4$.

Fig. 3. Chromatograms on A5m agarose in buffer. Ordinate is absorbance at the indicated wavelength. Abscissa is elution volume divided by the void volume. —, 890 nm; - - -, 800 nm; . . . . ., 280 nm; — - — -, 590 nm, following reaction with benzidine. (A) Large photoreactive particles previously purified on agarose. (B) 6 mg/ml chromatophore protein treated with 1.3 % (each) sodium dodecylsulfate and Brij-58 and 0.25 M $(NH_4)_2SO_4$ and chromatographed immediately. (C) 8 mg/ml chromatophore protein treated with 1.85 % sodium dodecyl sulfate only and chromatographed immediately. (D) 13 mg/ml chromatophore protein treated with 2.2 % sodium dodecylsulfate and 0.5 % Brij-58 and chromatographed immediately. (E) Same as D except that 2 min elapsed before the mixture was applied to the column. (F) 6 mg/ml of chromatophore protein treated with 2.2 % (each) sodium dodecylsulfate and Brij-58 and chromatographed immediately.

Aggregation of large photoreactive particles

Chromatography of purified particles on A5m agarose (Fig. 3A) usually shows two components. One, with an elution ratio $V_e/V_0 = 0.9$ (V_e, elution volume; V_0, void volume) appears to be an aggregate of the material in the second peak at $V_e/V_0 = 1.19$ (see below). We have termed the latter "monomer". If the chromatophore, detergent, and salt mixture used in the first step of the standard preparation is chromatographed directly on agarose, essentially all of the $A_{890\,nm}$ elutes in a peak at $V_e/V_0 = 1.15$ (Fig. 3B). The material is, therefore, monomeric at this stage. Aggregates are not seen in the eluate from hydroxyapatite either, but they do appear if the eluate is concentrated either by vacuum dialysis in a collodion bag (70 000 mol. wt escape) or by precipitation with $(NH_4)_2SO_4$. They also appear after precipitation of potassium dodecylsulfate from the eluate by addition of K_2SO_4 to 0.25 M followed by storage at 4 °C. The last procedure results in complete aggregation within 4 days. Thus, in a

standard preparation, aggregates first appear after $(NH_4)_2SO_4$ precipitation. Usually 20–30 % of the preparation is in the form of aggregates. The proportion of aggregates in a fresh preparation is increased to 70 % if the sodium dodecylsulfate content of the chromatophore/detergent/salt mixture is slightly reduced, to 1.2 % from the standard 1.3 %. In this case, part of the material absorbing at 890 nm is still aggregated when it is applied to hydroxyapatite, unlike the situation in Fig. 3B. Evidently the amount of sodium dodecylsulfate used in the standard preparation is just sufficient to cause complete disaggregation. (Although we have not purified the light-harvesting bacteriochlorophyll protein complexes, we have observed that after the 0.2 M phosphate peak of Fig. 1 is precipitated with $(NH_4)_2SO_4$, it elutes from A50m agarose essentially like chromatophores and is, therefore, aggregated. This material is not aggregated in the original detergent mixture applied to hydroxyapatite (Fig. 3B, the 800 nm-absorbing material elutes at $V_e/V_0 = 1.4$).)

Further aggregation occurs on storage of standard preparations at 4 °C; aggregation goes to completion upon freezing and thawing in buffer. The aggregation is associated with the appearance of a slow band during electrophoresis on cellulose acetate as is to be expected for large aggregates [28]. Dissociation of the aggregates can be effected by brief exposure to 2 % sodium dodecylsulfate. They are unaffected by 6 M urea. The photochemical cytochrome oxidation per chlorophyll is the same in aggregates as in monomers in any given preparation.

Disaggregation of chromatophores

Although the large photoreactive particles are completely disaggregated in the first step of the standard preparation, studies of the effects of detergents under slightly different conditions suggest that large aggregates of the particles exist as such in the chromatophore membrane. In the absence of other salts, a combination of 2.0 % sodium dodecylsulfate and 2.0 % Brij-58 does not disaggregate the chromatophores although it removes some protein; the absorbances at 890 and 800 nm still coincide on agarose after this treatment (Fig. 3F). 1.85 % sodium dodecylsulfate alone, in the absence of other salts, causes disaggregation of the photoreactive part of the chromatophore, as represented by material absorbing at 890 nm, as well as disaggregation of the light harvesting material (absorbance at 800 nm) (Fig. 3C). Under the same condition of no added salts, a mixture of 2.1 % sodium dodecylsulfate and 0.5 % Brij-58 yields little disaggregation of the material absorbing at 890 nm but complete disaggregation of the 800 nm absorbing material; in Fig. 3D the 800 nm absorbance is dissociated while the bulk of the 890 nm absorbance still elutes at $V_e/V_0 = 0.9$. The disaggregation of the 890 nm-absorbing material increases with time of exposure to the detergents under these conditions (Fig. 3E). The fact that disaggregation of the light-harvesting complexes and of the large photoreactive particles does not go hand-in-hand indicates that the two complexes are independently aggregated in the chromatophores.

Electron microscopy

The chromatophores from which the particles were isolated may be seen in a thin section of *C. vinosum* (Fig. 4A) and by negative staining of isolated organelles (Fig. 4B). The monomer fraction of purified particles (Fig. 4C) is seen by negative staining to consist largely of asymmetric globular structures with dimensions of about

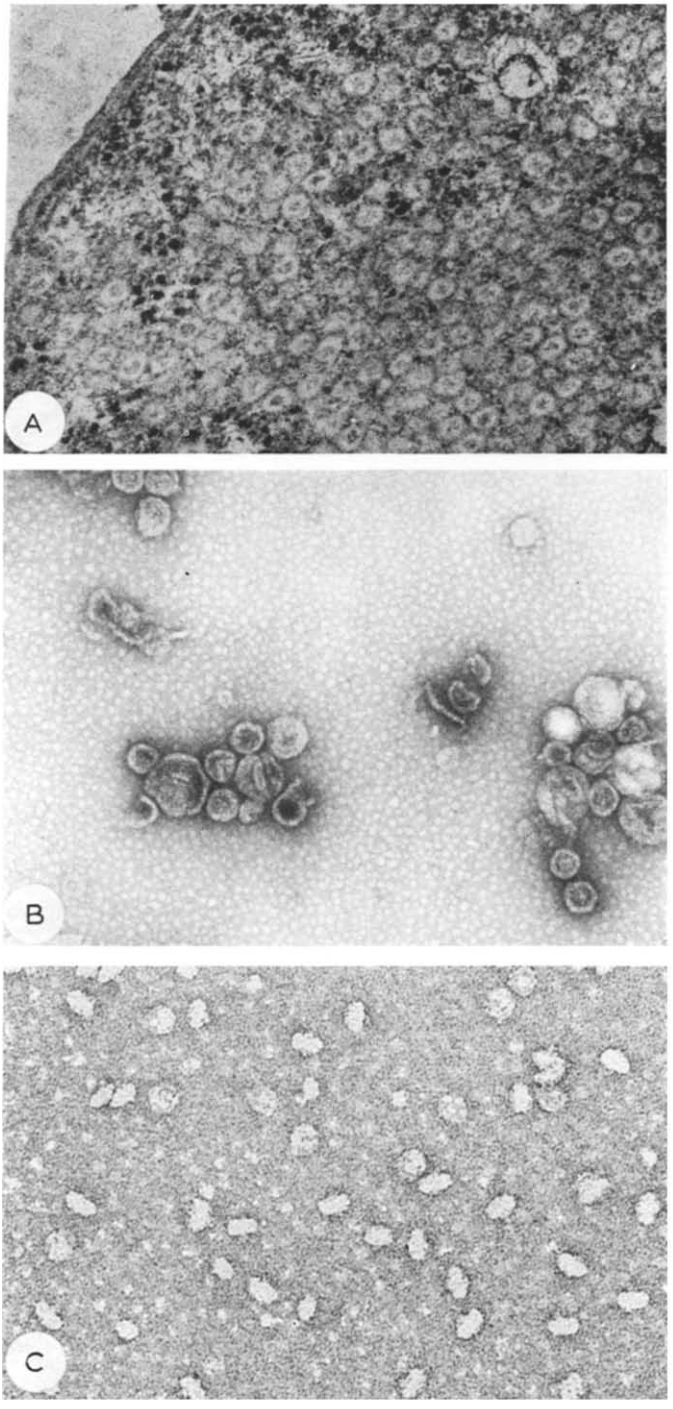


Fig. 4. (A) Thin-section electron micrograph of a *C. vinosum* cell revealing the densely packed chromatophores (× 80 000). (B) Negatively stained chromatophores isolated by sonification in buffer (× 80 000). (C) Negatively stained large photoreactive particles purified by the standard procedure and eluting from A5m agarose at $V_e/V_o = 1.19$ (× 240 000).

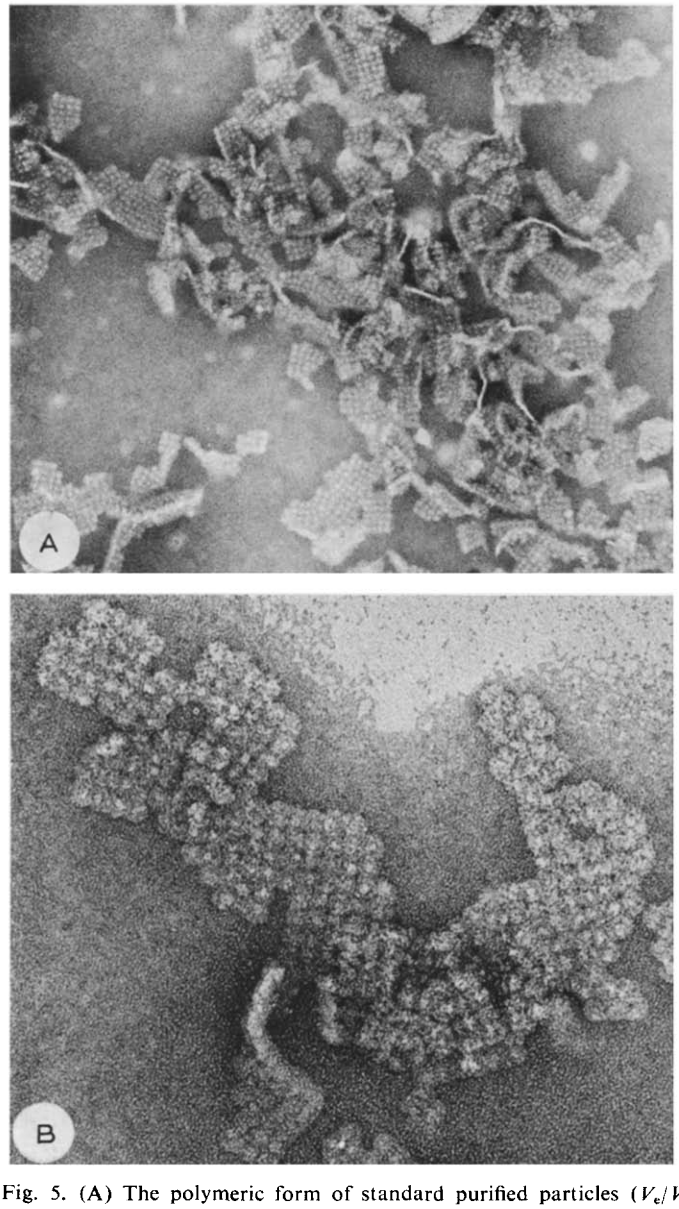


Fig. 5. (A) The polymeric form of standard purified particles ($V_e/V_o = 0.9$ from A5m agarose) negatively stained with uranyl acetate. Most are seen in frontal view but occasional sheets are seen from the edge (× 80 000). (B) Same as A but × 240 000.

175 Å × 110 Å. Less distinctly defined are nearly circular particles of about 175 Å diameter. The aggregate fraction (Fig. 5A and 5B) is composed of regular rectilinear arrays of particles about 120 Å in diameter. It is unclear how the particles seen in the monomer fraction are oriented in these lattices; nor do we know whether the two sorts of images among monomers represent alternative views, different conformational

states, or particles bearing different amounts of detergent. In any case, the sheet-like aggregates seem to be one particle in thickness (note edge views of curled sheets) and composed of nearly square domains. Their margins generally follow the perpendicular axes of the lattice. Attempts were made to resolve the subunit structure by optical enhancement methods dependent upon regularity in crystalline packing. This approach was frustrated by the finding that periodicity along one of the two perpendicular axes was invariably imprecise. This feature was readily appreciated by viewing along rows from the two perpendicular directions and finding substantial linearity of packing only in one direction. We conclude that the lattice probably does not represent a lattice with local 4-fold symmetry, but instead is composed of a parallel arrange-

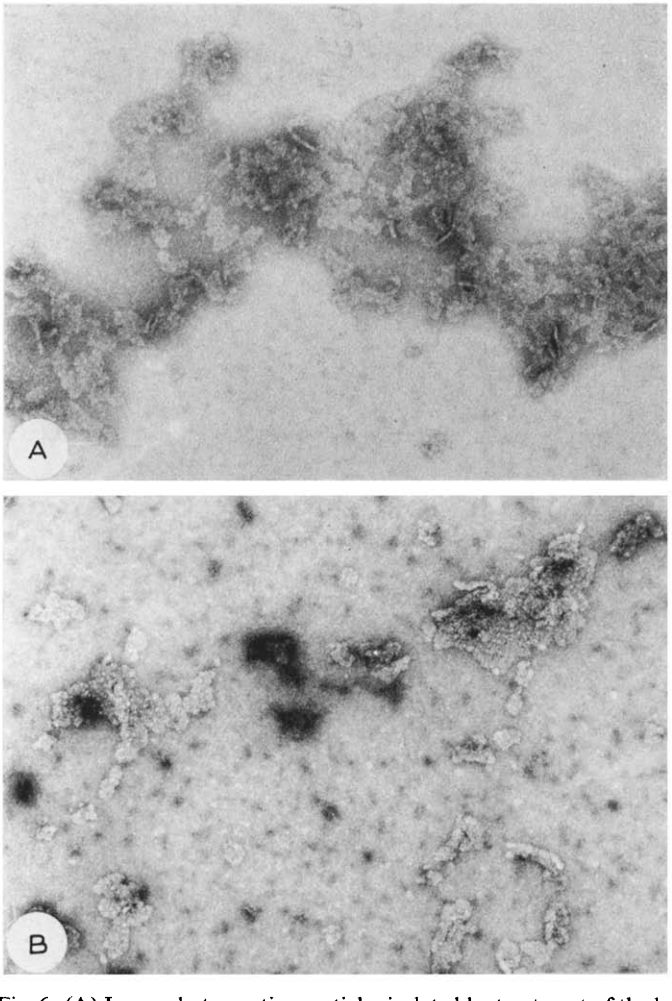


Fig. 6. (A) Large photoreactive particles isolated by treatment of the hydroxyapatite eluate of a standard preparation with K_2SO_4 followed by chromatography of the solution on A5m agarose, V_e/V_o = 0.9, negatively stained with uranyl acetate. Note rudimentary sheets (× 80 000). (B) Material from the V_e/V_o = 0.9 peak of Fig. 3D (2.1 % sodium dodecylsulfate, 0.5 % Brij-58, no salts) negatively stained with uranyl acetate (× 80 000). Irregular lattices are seen.

TABLE II

ESTIMATES OF MOLECULAR WEIGHT OF PARTICLE MONOMER

Method	Mol. wt
Agarose A5m	650 000*
Agarose A50m	800 000
Ultracentrifuge**	810 000
Electron microscope***	770 000

* The same figure is obtained for standard preparations or sodium dodecylsulfate-only preparations.

** Sedimentation velocity determination (by Dr D. C. Teller) in 0.075 M NaCl and 0.025 M Tris · Cl, pH 8.1. Two species with $s_{20,w}$ of 9.77 and 15.50 were found. The molecular weight was calculated from $s_{20,w} = 9.77$ assuming $\bar{\nu} = 0.86$. The component at $s_{20,w}$ appears to be a dimer with mol. wt $\simeq 1.78 \times 10^6$. The reaction center particles used had been twice purified on A1.5m agarose.

*** Calculated assuming $\bar{\nu} = 0.86$ for an ellipsoid of $110 \times 110 \times 175$ Å.

ment of polarized linear polymers held together by relatively aspecific lateral interactions. We have not observed single chains of large photoreactive particles.

Aggregates derived by alternative procedures were also examined by electron microscopy. Those formed by addition of 0.25 M K_2SO_4 appear similar (Fig. 6A) to the usual rectilinear aggregates, but were of smaller size. Aggregates found after treatment of chromatophores with 2.2 % sodium dodecylsulfate and 0.5 % Brij-58 (without added salt; this is the peak at $V_e/V_0 = 0.9$ in Fig. 3D) showed some sheets of regular periodicity but most were of irregular outline and showed an indistinct packing (Fig. 6B). It is possible that these aggregates preserve the arrangement of subunits originally present in the chromatophores.

Molecular weight of large photoreactive particles

Estimates of the molecular weight of purified particles by agarose chromatography, sedimentation velocity, and electron microscopy are given in Table II. They fall between 650 000 and 810 000 for the monomeric material. Chromatography on A5m agarose of fragmented chromatophores invariably indicated that the material absorbing at 890 nm had a particle weight of about 650 000, even if the chromatophores were treated with 5.9 M urea and 1.4 % Triton X-100 at pH 12 [29], or treated with 5.9 M urea and 0.7 % sodium dodecylsulfate at pH 12. From the data of Table III (see next section), an integral content of 5 cytochromes implies a molecular weight of 760 000 for the standard preparation. This figure (not corrected for the amount of water in the lyophilized preparation) falls within the estimates of Table II.

Chlorophyll, ubiquinone, and cytochrome content of purified particles

The ubiquinone contents shown in Table III confirm previous indications [24] that ubiquinone is concentrated in the reaction center portion of the chromatophore. The sodium dodecylsulfate-only preparation contains much less ubiquinone than does the standard preparation. We attempted to reconstitute secondary acceptor activity in the former preparation by adding ubiquinone in petroleum ether to the lyophilized particles [7]. No increase in secondary acceptor activity was obtained. This procedure was successful, however, in improving the secondary acceptor activity in

TABLE III

ACTIVITY AND COMPOSITION OF LARGE PHOTOREACTIVE PARTICLE PREPARATIONS

$\Delta A_2/\Delta A_1$ is the ratio of cytochrome oxidation on the second laser flash to that on the first flash. The cytochrome values were determined according to Thornber [5] using $\varepsilon = 20\ mM^{-1} \cdot cm^{-1}$ for each cytochrome. Bchl, bacteriochlorophyll. Lines 2 and 3 are independent standard preparations.

	$\frac{\Delta A_2}{\Delta A_1}$	Bchl $\left(\frac{nmol}{mg}\right)$	Ubiquinone $\left(\frac{nmol}{mg}\right)$	Cytochromes: high potential $\left(\frac{mol}{mol\ Bchl}\right)$	Cytochromes: low potential $\left(\frac{mol}{mol\ Bchl}\right)$	Cytochromes: low potential / high potential	Benzidine reaction $\frac{mol\ heme}{mol\ Bchl}$	Pyridine reaction $\frac{mol\ heme}{mol\ Bchl}$
1. Chromatophores	0.75	37	9.3	0.027	0.052	1.93	0.030	0.073
2. Purified particles	0.75	—	39.4	0.052	0.077	1.48	0.13	0.11
3. Purified particles	0.67	50	39.7	—	—	—	0.11	0.12
4. Purified particles*	0.36	52	26.9	—	—	—	—	—
5. Particles; sodium dodecylsulfate-only preparation	0.16	27	7.5	0.079	0.096	1.20	0.050	0.15

* Prepared with aged hydroxyapatite.

the preparation of line 4, Table III, which had been prepared with Brij-58, but on aged hydroxyapatite. The sodium dodecylsulfate-only preparation perhaps lacks phospholipids which may be necessary to rebind ubiquinone [25].

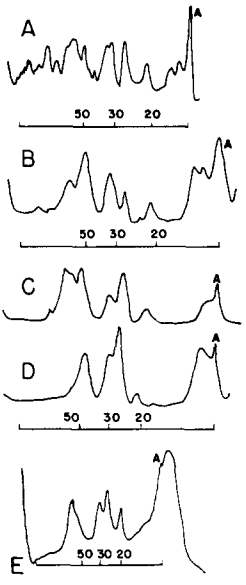
Low-potential and high-potential cytochromes occur in the standard particles in a ratio of about 1.5 : 1 (Table III, line 2; this estimate assumed the two cytochromes have similar $\Delta\varepsilon$ values at 552 nm upon reduction [5]). Because the smallest integral numbers consonant with this ratio are 3 : 2, a particle containing 39 bacteriochlorophyll molecules is required, assuming all particles are uniform in cytochrome content ($39 \times 0.077 = 3.0$ and $39 \times 0.052 = 2.0$). The sum of the two cytochromes in standard particles is about 0.12 mole/mole bacteriochlorophyll whether determined by chemical difference spectroscopy, benzidine reaction or pyridine hemochrome (Table III).

In chromatophores, the ratio of low to high potential cytochromes appears to be higher than it is in large photoreactive particles (Table III, column 6). The total cytochromes of chromatophores determined by chemical difference spectroscopy agrees well with heme determined as pyridine hemochrome (0.079 and 0.073). Figures of 0.05 [26] and 0.07 [27] heme/bacteriochlorophyll have been published for chromatophores. The figure from the benzidine reaction, on the other hand, gives a low value (0.03) not only here but also for total heme in sodium dodecylsulfate-only particles (Table III). It appears that both chromatophores and the sodium dodecylsulfate-only particles contain heme groups which have very little peroxidatic activity.

Protein subunits of large photoreactive particles

The major protein bands of the chromatophores are shown in Fig. 7A. Chromatophores modified by treatment with 2.2 % (each) sodium dodecylsulfate and Brij-58 (material of peak at $V_e/V_0 = 0.9$, as in Fig. 3F) still appear vesicular in the electron microscope but yield a simplified protein pattern (Fig. 7B). The purified particle preparations (Figs 7C and 7D) show bands with apparent mol. wts of about 45–50 000, 30 000, 27 000, 22 000, and 15 000 on 10 % gels. The smallest of these polypeptides appears to have a mol.wt of 12 000 on 15 % gels; however, electrophoresis in the presence of sodium dodecylsulfate does not afford an accurate measure of molecular weight for so small a protein [30]. The three bands with molecular weights of about 30 000, 27 000 and 22 000 are similar to those seen in preparations of reaction centers from *R. spheroides* and *Rhodospirillum rubrum* [3, 4]. Their apparent relative proportions vary depending on whether or not lipids have been removed prior to electrophoresis. Unextracted particles (Fig. 7E) show nearly equal intensity of all three bands in contrast to the small amount of the lowest molecular weight component seen in Figs 7C and 7D in extracted preparations. If purified particles are delipidated with one-fifth the volume of solvent used in Fig. 7, the contribution of the middle component of the triplet is also markedly reduced (Fig. 8A). In material extracted in this way the triplet mol. wts are 28 000, 25 000, and 22 000. The two smaller of these polypeptides also appear in the lipid solvent after such an extraction (Figs. 8B and 8C). The bulk of the 12 000 mol. wt protein is present in the extracts together with a small amount of 28 000 mol. wt component. In unextracted particles, the bulk of the chlorophyll moves with the 12 000 mol. wt protein although we have not attempted to determine if it is, in fact, bound to this protein.

It appears that the 12 000 mol. wt component is very soluble in chloroform/methanol. Of the polypeptides in the 30 000–20 000 mol. wt range, the smallest



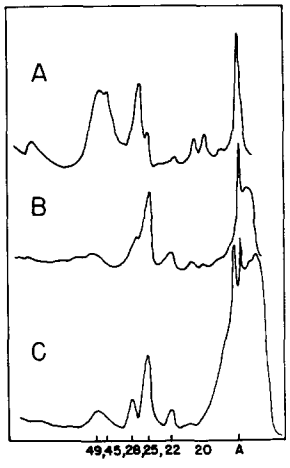


Fig. 7. Densitometer scans of gels (10 %) stained with Coomassie Blue. All samples were dissociated in the presence of dithiothreitol. The direction of migration is to the right. The band marked A on each trace is an artifact line present on the gels. Mol. wt $\times 10^{-3}$ is indicated by the horizontal scales. For all samples except E, 2.0 mg were extracted with 4 ml of chloroform/methanol, 2 : 1. (A) Chromatophores; (B) Chromatophores treated with 2.2 % (each) sodium dodecylsulfate and Brij-58 and recovered after chromatography on A5m agarose; (C) Sodium dodecylsulfate-only preparation of large photoreactive particles; (D) Standard preparation of particles; (E) Same as D except no extraction with chloroform/methanol.

Fig. 8. Densitometer scans of gels stained with Coomassie Blue. All samples were dissociated in the presence of dithiothreitol. The direction of migration is to the right. Mol. wt $\times 10^{-3}$ is indicated on the abscissa. A on the abscissa indicates the artifact line. (A) 1.9 mg standard particles extracted two times with 0.8 ml of chloroform/methanol. The insoluble residue was used for this trace. (B) Chloroform/methanol extracts from A (above) evaporated and re-extracted with 0.4 ml of the same solvent mixture. The trace is for the insoluble residue at this step. (C) Chloroform/methanol extract from B (above) diluted with 6 vol. of ether. The trace is for the precipitate.

(22 000) is largely removed in the extraction and may even be dissociated by delipidation inasmuch as there is no large peak at 22 000 mol. wt in Figs 8B or 8C. The middle component (25 000) is prominent in the chloroform/methanol extracts. The extraction of these proteins into the lipid solvents may reflect the presence of residual detergent in the preparation, as well as the intrinsic solubility properties of the polypeptide chains, because their extraction is enhanced by using a higher ratio of protein to solvent. The proteins with molecular weights of 45–50 000 (or 60 000 in the unextracted preparation of Fig. 7E) are discussed below.

Heme-containing proteins of large photoreactive particles

Delipidated chromatophores contain hemoproteins (cytochromes), visualized by staining with benzidine, with the following molecular weights: 63 000; 48 000; 46 000; 22 000; and 20 000 (doublet) (Fig. 9). Unextracted chromatophores reveal very little of the 22 000 and 20 000 mol. wt hemoproteins. No heme-containing pro-

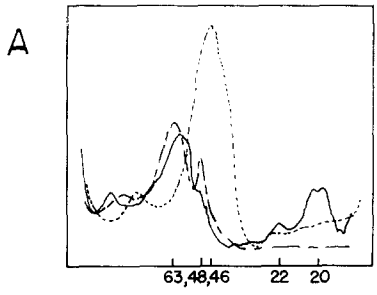


Fig. 9. Densitometer scans of gels stained for heme. For all samples, 2 mg were extracted with 4 ml chloroform/methanol and dissociated in the absence of dithiothreitol. The direction of migration is to the right. Molecular weight $\times 10^{-3}$ is indicated on the abscissa. —, chromatophores; — – —, chromatophores treated as in Fig. 3F (2.2 % each sodium dodecylsulfate and Brij-58, no salts); - - -, standard particle preparation.

teins appear to be extracted by the lipid solvents. These observations suggest that the presence of lipids may hinder the dissociation of the large hemoproteins by sodium dodecylsulfate. Alternatively, the hemoproteins may be rather firmly associated with other proteins that are extracted by the chloroform/methanol (see above).

Chromatophores treated with 2.2 % (each) sodium dodecylsulfate and Brij-58 and re-isolated on agarose (material of $V_e/V_0 = 0.9$, as in Fig. 3F) do not show any low molecular weight hemoproteins after delipidation and sodium dodecylsulfate-gel electrophoresis. Part of the 63 000 mol. wt hemoprotein is also lost in this case; this allows the 48 000 mol. wt component to become more apparent (Fig. 9). This detergent treatment removes about half of the hemoproteins of the chromatophore as determined by the benzidine reaction (Fig. 3F). A similar proportion of the total hemoprotein is removed during the standard preparation of particles (Fig. 3B). The amount of heme in the 890 nm-absorbing peak of Fig. 3F appears artifactually low because the limited peroxidatic activity of the chromatophores (Table III) persists after this detergent treatment at low ionic strength. The molecular complexity of the cytochromes of the chromatophore, as evidenced by the number of heme-containing polypeptides, appears greater than the two categories determined in chemical difference spectroscopy (Table III). This may reflect the association of cytochromes with different protein components of the chromatophore or aggregation of cytochrome polypeptides.

Purified particles show one diffuse heme-staining band in sodium dodecylsulfate-gel electrophoresis (Fig. 9) with a peak at 46 000 mol. wt. Since dithiothreitol cannot be used when dissociating proteins for gels which are to be stained for heme, the number of polypeptides in this region is more reliably shown when the proteins are dissociated for Coomassie Blue staining. In this case at least two polypeptides are revealed (Fig. 8A) at 49 000 and 45 000 mol. wts. In preparations extracted as in Fig. 8, but dissociated in the absence of dithiothreitol, the only heme-staining bands are in the original chloroform-methanol residue (as in Fig. 8A) and they occur at 49 000, 45 000 and 20 000 (doublet) mol. wts. The protein at about 45 000 mol. wt in Figs 8B and 8C may represent a multimer of one or more of the non-heme-containing lower molecular weight components. Further work is clearly necessary to clarify the number of different heme-containing polypeptides.

DISCUSSION

Thornber found about 45 bacteriochlorophyll molecules per fraction A particle [5]. Our estimate of 39 per particle agrees reasonably well with this. He reported 7 low-potential cytochromes per molecule while we find 3; the material removed by agarose contains heme and this may be the source of the discrepancy. The molecular weight estimates we present are considerably larger than the 400 000 given for fraction A [5], which is equivalent in activity to our sodium dodecylsulfate-only preparation. The difference may result from stripping off lipid and detergent by repeated precipitation by salt in the preparation of fraction A. Alternatively, the method of molecular weight estimation (by entry into a polyacrylamide gel [28]) used by Thornber may not be reliable for proteins associated with lipids and/or detergents. Our estimates agree more closely with the molecular weight of 650 000 for particles containing ubiquinone and cytochromes from *R. spheroides* [31].

The size of the monomer observed in the electron micrographs of purified particles is similar to the size reported for fractions from bacterial chromatophores [11, 12, 13] and Photosystem I preparations [34]. The fact that aggregates which we observe after purification are similar to those present in chromatophores from which only the light-harvesting components have been removed suggests that large photoreactive particles may exist in the chromatophore membrane as an aggregate lying beside a layer of antenna bacteriochlorophyll-protein complex. The observed increase in disaggregation of the 890 nm-absorbing material with time of exposure to detergents when no salts are added makes it unlikely that we are observing an aggregation of originally separate particles. Reed and Raveed [12] found their large particles to lie inside the *R. spheroides* chromatophore. Reed et al. [35] observed independent reaggregation of large photoreactive particles and antenna bacteriochlorophyll-protein complex after their separation by Triton X-100. It is of interest that two independently aggregated protein layers have been proposed to exist in the mitochondrial inner membrane as well [36].

The nature of the purified particle array, whether "square" or not, may depend on the redox state of some component(s) of the complex as was observed for cytochrome oxidase [37], or it may depend on the phospholipid components present. The electron micrographs of Fig. 5 indicate that there is slippage between rows of particles. The lack of specificity of bonding on this surface of the particles would permit the collapse of this planar surface into the smaller dimensions of the chromatophores.

The content of cytochromes and other polypeptides in these particles similarly shows parallels to the photosynthetic systems of other bacteria. The set of three polypeptides with molecular weights of about 30 000, 27 000 and 22 000 we have observed in particles from *Chromatium* are similar to those observed in reaction center particles from *Athiorhodaceae*. We do not wish to assert that the polypeptides we observe are identical with those from *R. spheroides*. There may well be differences in the subunits from these two classes of bacteria. When *R. spheroides* reaction-center particles (kindly furnished by Dr R. K. Clayton) were dissociated (not delipidated) and run in our system we observed 27 000, 23 000 and 22 000 mol. wt subunits. These polypeptides, therefore, do not run identically with the *Chromatium* subunits.

With regard to the cytochrome polypeptides in our large particle preparations,

we find molecular weights consonant with the 45 000 mol. wt cytochrome Kennel and Kamen [32] report in fraction A. That some or all of the cytochromes of chromatophores and large photoreactive particles can dissociate to smaller sizes is shown (a) by the smaller molecular weights found by Kennel and Kamen after purifying the fraction A cytochrome, (b) by the smaller cytochrome isolated by Cusanovitch and Bartsch [33] from chromatophores, and (c) by our observations of lower molecular weight cytochromes in delipidated chromatophores (Fig. 9) and in some delipidated purified particles (see discussion of Fig. 8 above). We were not able to dissociate the cytochromes of large photoreactive particles any further by 10 min heating at 100 °C than by 2 min at 80 °C. Further work is necessary on the exact manner of delipidation required to assure complete dissociation of heme-containing polypeptides by sodium dodecylsulfate. It is likely that chromatophore and large photoreactive-particle cytochromes exist as aggregates of one or more small heme-containing polypeptides. Our work suggests that there are at least two such aggregates with molecular weights of 49 000 and 45 000.

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REFERENCES

1 Frenkel, A. W. and Nelson, R. A. (1971) in Methods in Enzymology (San Pietro, A., ed.), Vol. XXIII, pp. 256–268, Academic Press, New York
2 Clayton, R. K. and Wang, R. T. (1971) in Methods in Enzymology (San Pietro, A., ed.), Vol. XXIII, pp. 696–704, Academic Press, New York
3 Okamura, M. Y., Steiner, L. A. and Feher, G. (1974) Biochemistry 13, 1394–1403
4 Parson, W. W. (1974) Annu. Rev. Biochem. 43, 115–133
5 Thornber, J. P. (1970) Biochemistry 9, 2688–2698
6 Case, G., Parson, W. W. and Thornber, J. P. (1970) Biochim. Biophys. Acta 223, 122–128
7 Halsey, Y. D. and Parson, W. W., (1974) Biochim. Biophys. Acta 347, 404–416
8 Hoober, J. K. (1970) J. Biol. Chem. 245, 4327–4337
9 Hoober, J. K. (1972) J. Cell Biol. 52, 84–96
10 Levine, R. P., Burton, W. G. and Duram, H. A. (1972) Nat. New Biol. 237, 176–177
11 Garcia, A., Vernon, L. P., Ke, B. and Mollenhauer, H. M. (1968) Biochemistry 7, 326–332
12 Reed, D. W. and Raveed, D. (1972) Biochim. Biophys. Acta 283, 79–91
13 Garcia, A., Vernon, L. P. and Mollenhauer, H. (1966) Biochemistry 5, 2399–2407
14 Arntzen, C. J., Dilley, R. A. and Crane, F. L. (1969) J. Cell Biol. 43, 16–31
15 Case, G. D. and Parson, W. W. (1971) Biochim. Biophys. Acta 253, 187–202
16 Parson, W. W. (1969) Biochim. Biophys. Acta 189, 384–396
17 Clayton, R. K. (1963) in Bacterial Photosynthesis (Gent, H., San Pietro, A. and Vernon, L. P., eds), p. 498, Antioch Press, Ohio
18 Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275
19 Paul, K. G., Theorell, H. and Åkeson, A. (1953) Acta Chem. Skand. 7, 1284–1287
20 Laemmli, U. K. (1970) Nature 227, 680–685

21 Haut, A., Tudhope, G. R., Cartwright, G. E. and Wintrobe, M. M. (1962) J. Clin. Invest. 41, 579–587
22 Haurowitz, F. and Hardin, R. L. (1954) in The proteins (Neurath, H. and Bailey, K., eds), pp. 322–323, Academic Press, New York
23 Tiselius, A., Hjertén, S. and Levin, Ö. (1956) Arch. Biochem. Biophys. 65, 132–155
24 Ke, B., Vernon, L. P., Garcia, A. and Ngo, E. (1968) Biochemistry 7, 311–318
25 Singer, T. P. and Gutman, M. (1971) Advances Enzymol. 34, 79–153
26 Cusanovich, M. A. (1967) Ph. D. Thesis, p. 57, University of California, San Diego
27 Newton, J. W. and Newton, G. A. (1957) Arch. Biochem. 71, 250–265
28 Blattler, D. P. and Reithel, F. J. (1970) J. Chromatogr. 46, 286–292
29 Loach, P. A., Sekura, D. L., Hadsell, R. M. and Stemer, A. (1970) Biochemistry 9, 724–733
30 Fish, W. W., Reynolds, J. A. and Tanford, C. (1970) J. Biol. Chem. 245, 5166–5168
31 Reed, D. W. (1969) J. Biol. Chem. 244, 4936–4941
32 Kennel, S. J. and Kamen, M. D. (1971) Biochim. Biophys. Acta 253, 153–166
33 Cusanovich, M. A. and Bartsch, R. G. (1969) Biochim. Biophys. Acta 189, 245–255
34 Vernon, L. P. and Shaw, E. R. (1971) in Methods in Enzymology (San Pietro, A., ed.), Vol. XXIII, p. 288, Academic Press, New York
35 Reed, D. W., Raveed, D. and Israel, H. W. (1970) Biochim. Biophys. Acta 223, 281–291
36 Crane, F. L. and Hall, J. D. (1972) Ann. N.Y. Acad. Sci. 195, 24–34
37 Wakabayashi, T., Senior, A. E., Hatase, O., Hayashi, H. and Green, D. E. (1972) Bioenergetics 3, 339–344