Structure of Chromatophores of *Rhodopseudomonas spheroides*. Removal of a Nonpigmented Outer Layer of Lipid*

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ABSTRACT: When chromatophores of *Rhodopseudomonas spheroides* were treated with cholate and deoxycholate their sedimentation coefficient dropped from 160 S to 120 S. This change was accompanied by the loss of some material of low molecular weight but no pigment. The 120 S particles were very unstable and appeared to disintegrate within a few hours. Chemical analysis showed that the change from 160 to 120 S particles was accompanied by the loss of 70% of the extractable lipid and less than 20% of the protein and carbohydrate. The 120 S particles retained the ability to catalyze photophosphorylation for a short period. Addition of salts disrupted the particles into at least two pigmented fractions. The results indicate that the chromatophore consists of a pigmented photoactive core surrounded by a thin shell of nonpigmented lipid. The implications for the interpretation of electron micrographs are discussed.

In previous reports in this series evidence was presented which showed that in *Rhodopseudomonas spheroides* the insoluble structure containing the photosynthetic pigments consists almost entirely of a single rather homogeneous particulate fraction whose sedimentation coefficient is about 160 S and mean diameter 570 A (Gibson, 1965a,b,c). These particles were identified with the chromatophores of Schachman *et al.* (1952). This report is concerned with an investigation into the architecture of chromatophores, and presents evidence to show that they consist of a central core particle containing all the pigment and the photosynthetic systems surrounded by a thin pigment-free layer consisting mostly of lipid.

Experimental Section

The strain of organism and the methods of growth and preparation of extracts are described by Gibson (1965a). The isolation of chromatophores by zone centrifugation in a gradient of CsCl is described by Gibson (1965b). Methods for carrying out moving-boundary sedimentation, for assaying photophosphorylation, and for estimating protein and bacteriochlorophyll are also described in those two papers.

Other Analytical Methods. Total P was measured by the method of Bartlett (1959) and total N according to Green and Melamed (1963). Carbohydrate was assayed by the orcinol reaction (Johansen *et al.*, 1960) using a heating period of 30 min at 80°. A standard of D-mannose was employed, and results are expressed in terms of this. Spectra were recorded with a Unicam SP700 recording spectrophotometer (Cambridge, England).

Preparation of Core Particles. To suspensions of chromatophores (10–30 mg of protein/ml) in 0.01 M tris(hydroxymethyl)aminomethane (Tris) buffer, pH 7.5, at 5° solutions of sodium cholate (20 mg/ml) and sodium deoxycholate (10 mg/ml) were added to give final concentrations of 1–2 mg of each/mg of protein. After standing for 10 min the suspension was diluted about fivefold with 0.01 M Tris buffer, pH 7.5, and centrifuged for at least 2.5 hr at 39,000 rpm in the SW39L rotor of the Spinco Model L ultracentrifuge. The supernatant, which was essentially free of pigment, was discarded and the pellet was suspended in 0.01 M Tris buffer.

Extraction of Phospholipids from Chromatophores and Core Particles. Lipids were extracted essentially according to Artom (1957). Suspensions of particles (about 20–30 mg of protein/ml) were extracted with approximately 50 volumes of acetone for 20 min at room temperature. They were collected by centrifugation and the precipitate was extracted with the same volume of ethanol for 20 min at 50–60°; this was repeated twice. The precipitate was extracted four times at 50–60° with the same volume of ethanol–ether (1:1, v/v). The combined extracts were evaporated to dryness, and the residue was dissolved in chloroform to give a solution containing all of the pigments and the extractable lipids. Bound P was extracted from the precipitate remaining after solvent extraction by adding 1–2 ml of 1 N ethanolic HCl and keeping the suspension at 37° for 4 hr. After centrifuging, the precipitate was extracted again with 1–2 ml of ethanol and the combined ethanolic extracts were evaporated to dryness. The residues were dissolved in ethanol.

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Results

When purified chromatophores were centrifuged by the moving-boundary technique described previously in which the distribution of pigment is determined in an autoanalyzer the resulting profile showed only one boundary (Figure 1). When sodium cholate and sodium deoxycholate (1.2 mg/mg of protein) were added to the suspension beforehand, the resulting profile still showed only one boundary, but it had not moved so far from the meniscus (Figure 1). The sedimentation coefficients calculated from these two boundaries are given in Table I. The treatment with cholate and deoxycholate had apparently lowered the s value of the chromatophores to three-quarters of its original value. Increasing the concentration of bile salts to 5 mg/mg of protein led to a profile which was very nearly identical with that obtained at the lower concentration.

TABLE I: Sedimentation Coefficient of Chromatophores before and after Treatment with Bile Salts.

Concn. of cholate and Deoxycholate[a] (mg/mg of protein)	Sedimentation Coefficient (S) at 6°[b]	Sedimentation Coefficient (S) at 20°[c]
...	106	155
1.2	81	119

[a] Bile salts were added 30 min before centrifuging. [b] Calculated from the boundaries in Figure 1. [c] Calculated from the s values at 6°.

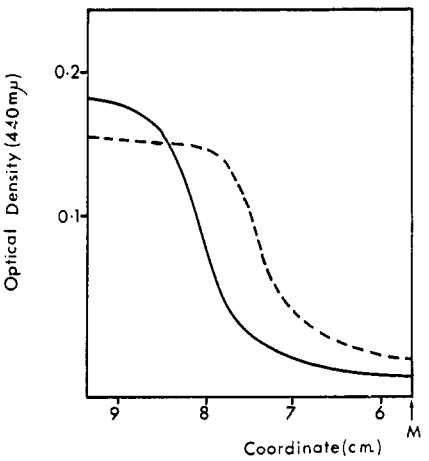

FIGURE 1: Moving boundary sedimentation of chromatophores before (solid line) and after (broken line) addition of bile salts. Contents of the tube were sampled from the bottom; M = meniscus; linear KCl gradient (0–3.8% w/w), 35,400 rpm, 39.0 min, 6°.

The two boundaries in Figure 1 lie almost exactly parallel to one another. This shows that the degree of polydispersity of the particles released by bile salts is very much the same as that of untreated chromatophores; the question of polydispersity of the latter was discussed previously (Gibson, 1965a,c). However, the particles released by bile salts do not have the marked tendency to aggregate shown by chromatophores, so that even if the preparation of chromatophores from which they were prepared contained aggregates, the addition of bile salts released material which was not aggregated. A simple test for aggregation is the equality of the s values calculated with the first and second methods of Gibson (1965d) for locating a boundary. The second formula involves the initial concentration, whereas the first involves only the concentration in the region just below the boundary. If any large aggregates are present they will be removed quantitatively during centrifugation and will therefore exercise little or no effect on the calculation of s by the first method; on the other hand, they will be included in the initial concentration and will tend to increase the s value calculated by the second method. Thus, if the values for s calculated by the two methods do not agree, this is evidence for the presence of aggregates, but if they are equal it is good evidence that there is no aggregation. The result of such calculations, performed with a different preparation of chromatophores from the one in Figure 1, is shown in Table II. Clearly these chromatophores were aggregated, but the particles released from them by bile salts showed no sign of the presence of aggregates.

TABLE II: Sedimentation Coefficients Calculated by Two Methods.

Concn. of Cholate and Deoxycholate[a] (mg/mg protein)	Sedimentation Coefficient[b] (S) calculated by Method 1[c]	Sedimentation Coefficient[b] (S) calculated by Method 2[c]
...	158	186
2.5	113	113
5.0	105	104

[a] Bile salts were added 15 min before centrifuging. [b] Corrected to 20°. [c] Gibson (1965d).

The technique of moving-boundary centrifugation in the preparative ultracentrifuge which was used in Figure 1 only measures the distribution of pigment. In order to determine whether any nonpigmented material

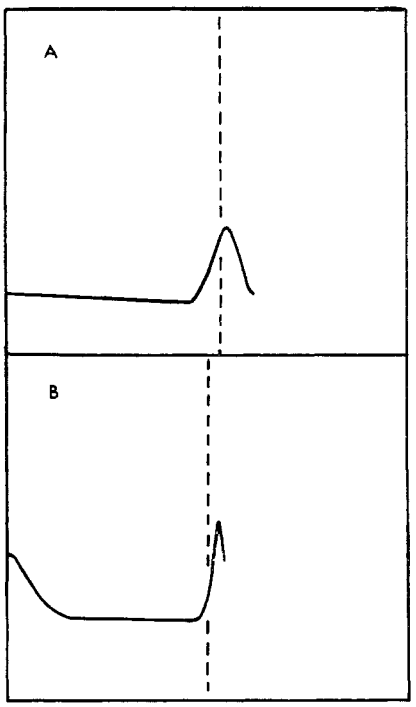

FIGURE 2: Schlieren pattern of chromatophores before (A) and after (B) addition of bile salts. Broken line shows position of pigment boundary; 29,500 rpm, 20°, photographed 10 min (A) and 20 min (B) after start.

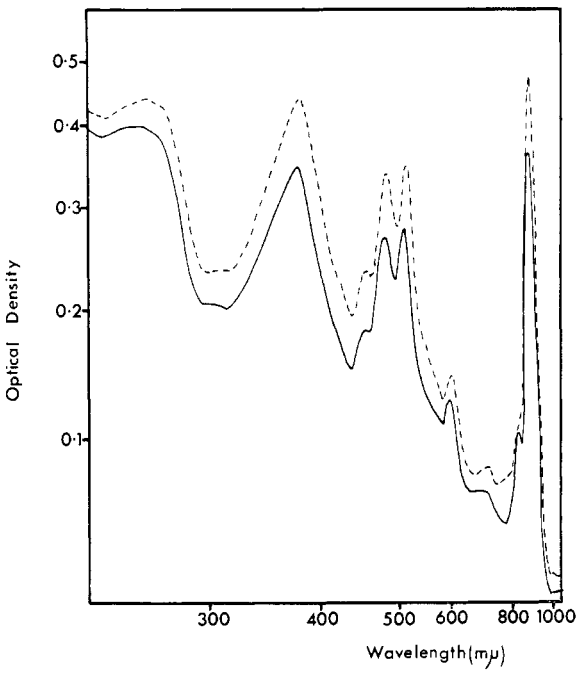

FIGURE 3: Spectra of chromatophores (solid line) and core particles (broken line) from 240 to 1100 mμ.

was released by the treatment with bile salts, a run was performed in an analytical ultracentrifuge (Figure 2). The preparation of chromatophores had only a single boundary (Figure 2a), corresponding to an $s_{20,w}$ value of 168 S. The concentration of chromatophores was 0.7 mg of protein/ml, and so the $s_{20,w}$ value ought to have been smaller (Gibson, 1965b); thus the preparation was aggregated. When cholate and deoxycholate were added at a concentration of 1.2 mg/mg of protein and the mixture was centrifuged the pattern in Figure 2b was obtained. This shows only one boundary in the middle of the cell with an s_{20} value of 77 S. No allowance has been made for the change in viscosity due to the presence of the bile salts, which in this experiment were present at a concentration of nearly 1%; hence too much significance cannot be attached to this figure. However, it does show that the pigmented particle released by bile salts is smaller than the original chromatophore and confirms that it is not aggregated. The only other boundary that could be distinguished was one at the meniscus, which did not move even when the angular velocity was increased to 60,000 rpm for 1.5 hr. This boundary therefore corresponds to low molecular weight material.

Chromatophores do not release smaller pigmented particles when treated with 0.01 M mercaptoethanol nor does the presence of this substance cause any change in the size of the particles released by bile salts (Table III). Substitution of more potent detergents, such as sodium dodecyl sulfate (10^{-3} M) or Tween 20 (1% v/v) for cholate and deoxycholate, led to further breakdown of chromatophores and the formation of aggregated material.

TABLE III: Effect of Bile Salts and Mercaptoethanol on the Sedimentation Coefficient.

Additions to Chromatophores[a]	Sedimentation Coefficient[b] (S)
Cholate + deoxycholate (1.2 mg/mg of protein)	114
Mercaptoethanol (0.01 M)	156
Cholate + deoxycholate + mercaptoethanol	120

[a] Added 30 min before centrifuging. [b] Corrected to 20°.

Stability of Core Particles

Unlike the chromatophores from which they are derived the 120 S particles (core particles) are very unstable and change their structure within 1–2 hr even at 5°. This process is greatly accelerated by the presence of salts in excess of about 0.5 M. The particles appear to lose their rather uniform size and become extremely polydisperse, probably by combining to form a range of

TABLE IV: Analysis of Chromatophores and Core Particles.

	Total[a]			Lipid[a]		
	N	P	Carbohydrate	N	P	Carbohydrate
Preparation 1						
Chromatophores	0.97	0.140	1.10	0.17	0.105	0.25
Core particles	0.83	0.075	0.75	0.06	0.045	0.20
Preparation 2						
Chromatophores	1.01	0.135	1.25	0.21	0.095	...[b]
Core particles	0.81	0.065	0.95	0.05	0.025	...[b]

[a] Expressed as mg/mg of bacteriochlorophyll. [b] Not determined.

aggregates or by breaking into smaller units which then reaggregate (see below). For this reason it was impossible to carry out moving-boundary sedimentation with concentrations of KCl higher than 4% (w/w). Attempts to substitute gradients of sucrose for KCl gradients were not very successful, perhaps because the time required for centrifugation at high concentrations of sucrose was longer than the lifetime of the particles. Alternatively the particles may be unstable in hypertonic solutions. Whatever the reason, moving-boundary experiments carried out in the presence of high concentrations of sucrose or KCl or both showed no sharp boundaries but a continuously increasing concentration all down the tube, indicating the presence of material of many sizes. The instability of core particles showed itself in other ways, *e.g.*, in a loss of photophosphorylating activity with time (see below), and as a tendency to disrupt when dried down on electron microscope grids (Gibson, 1965c). By contrast, core particles showed no sign of increased turbidity when they were exposed to dilute solutions of polyvalent cations or anions or dialyzed against distilled water, under conditions which led the chromatophores from which they were derived to aggregate strongly.

Constitution of Core Particles

Core particles were collected by centrifugation as described in the Experimental Section. The spectrum of this material was identical with that of the original chromatophores apart from two small differences (Figure 3). One occurred in the region of the absorption band near 850 mμ, which is due to bacteriochlorophyll. It is doubtful whether the difference noted in Figure 3 has any great significance as it was not always observed. The absorption in this region is very sensitive to small changes in the surrounding medium (Cohen-Bazire *et al.*, 1957; Worden and Sistrom, 1964). The other difference, which was observed consistently, was a slight drop in the absorption at 280 mμ relative to that at 370 mμ due to the Soret band of bacteriochlorophyll. The ratio (absorption at 280 mμ/absorption at 370 mμ) fell from 1.15 in these chromatophores to 1.00 in their core particles. This may have been partly due to the loss of a small amount of protein which was removed by bile salts; however, only about 20% of the absorption at 280 mμ of chromatophores or core particles can be due to protein.

The composition of two batches of chromatophores and their core particles is given in Table IV. The preparations of both chromatophores and core particles were exhaustively dialyzed against 0.01 M KCl before analysis. The conversion of one particle to the other was accompanied by a loss of 10–20% of the total N and 25–30% of the carbohydrate. However the total P dropped by about 50%. The change in total P was found to be due entirely to the loss of 60–70% of the lipid P, the bound P being unchanged (Table IV, columns 2 and 3). This was confirmed directly with another batch of chromatophores by extracting the bound P by treatment with ethanolic HCl at 37°; the result suggests that this P may represent lipid which is covalently attached to the core particles (Table V). The nature of the lipid that is lost was not investigated, but a preliminary examination of the bases by paper chromatography suggested that there was a drop in several components. The fall in the total N was mostly due to the removal of lipid (Table IV) but partly to the removal of a small amount

TABLE V: Phosphorus Content of Chromatophores and Core Particles.

	Total P[a]	Lipid P[a]	Acid Extractible P[a,b]
Chromatophores	0.235	0.210	0.020
Core particles	0.095	0.075	0.015

[a] Expressed as mg/mg of bacteriochlorophyll. [b] Released by treatment with ethanolic HCl.

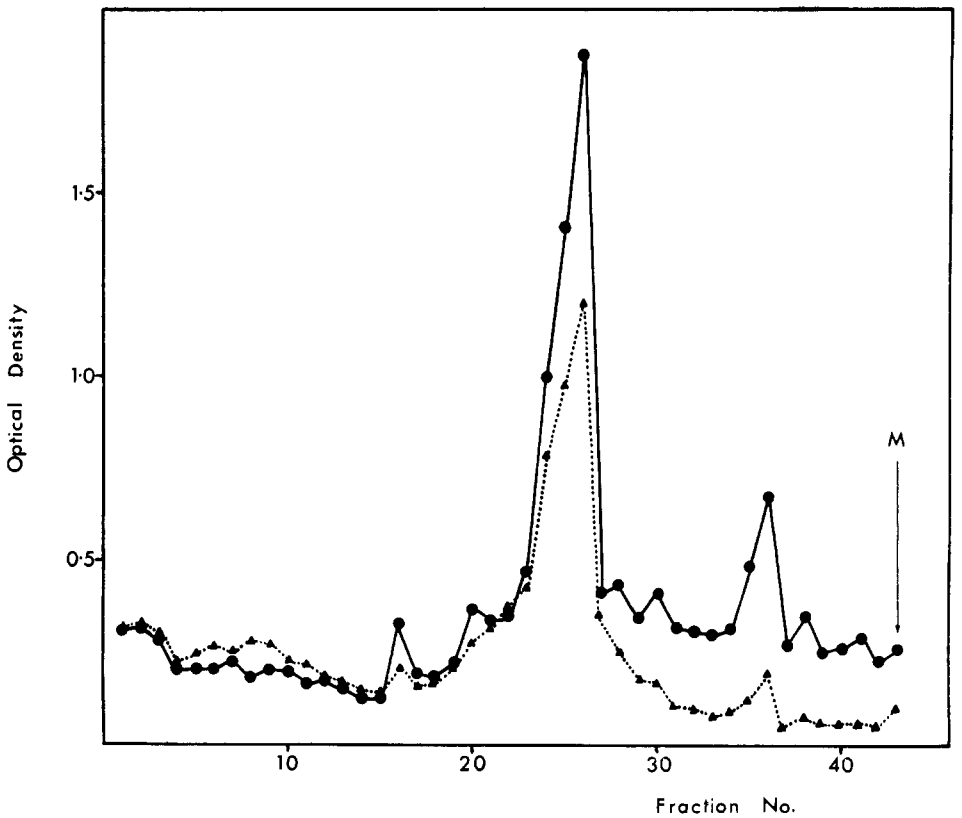

FIGURE 4: Zone centrifugation of KCl-treated core particles in linear gradient of sucrose. Fractions collected through capillary inserted near bottom of tube. Optical densities read at 280 (●) and 370 mμ (▲).

TABLE VI: Approximate Composition of Chromatophores and Core Particles.

	Protein[a]	Carbohydrate[a]	Lipid[a,b]
Chromatophores	5.0	1.2	3.6
Core particles	4.7	0.9	1.2
Difference	0.3	0.3	2.4

[a] Expressed as mg/mg of bacteriochlorophyll. [b] Calculated from the lipid P with a factor obtained from the data of Bull and Lascelles (1963).

TABLE VII: Photophosphorylation by Core Particles

Enzyme Prepn	Phosphate Esterified[a] (μmole/hr/mg of bacteriochlorophyll)
Chromatophores	1.5
Core particles[b]	1.7
Core particles[c]	0.1

[a] Assayed with ^{32}P; results based on a 30-min incubation. [b] Assayed 5 min after addition of bile salts. [c] Assayed after being centrifuged for 3 hr at 39,000 rpm and suspended in 0.01 M Tris buffer.

of protein, which may have been derived from the trace of impurities present in these preparations of chromatophores (Gibson, 1965b). The approximate composition of chromatophores and core particles, calculated from the data in Tables IV and V, is shown in Table VI. The material removed by bile salts consists mainly of phospholipid, together with traces of protein and carbohydrate.

Photophosphorylation. Core particles were tested for their ability to catalyze cyclic photophosphorylation. It was found that the cores were as active as chromatophores provided that they were tested as soon as possible after the addition of bile salts (Table VII). However the activity was unstable and disappeared rapidly on standing.

Further Breakdown of Chromatophores. Core particles could be broken into smaller units by exploiting their instability in the presence of salts. The conditions which bring about this change could be varied considerably, the only requirement being for salt in excess of about

1 M. Typically, core particles were prepared by the addition of bile salts to chromatophores (1 mg or more/mg of protein) and after a few minutes solid KCl was added to give a final concentration of 1–2 M. After 30–60 min the solution was dialyzed for at least 4 hr against 0.01 M Tris buffer, pH 7.5. Alternatively, after the addition of bile salts saturated $(NH_4)_2SO_4$ (adjusted to pH 7.5 with 5 N KOH) was added to give a final saturation of 40%. Low-speed centrifugation for 20 min caused all the pigmented material to float to the top of the tube, from which it was removed, dissolved in 0.01 M Tris buffer, and dialyzed.

The spectrum of these preparations was much the same as that of the original chromatophores. However, when they were layered on a linear gradient of sucrose (0.15–0.60 M) and centrifuged for 15 hr at 30,000 rpm in the SW39L rotor of the Spinco Model L ultracentrifuge at 5° they separated into two or more pigmented bands. The uppermost band, which under these conditions migrated 1.0–1.5 cm from the meniscus, was brown in colo. Below this, at about 2.0 and 2.5 cm from the meniscus, came two narrow red bands, and below these a trail of red material led to a red pellet at the bottom of the tube. A tube which had been centrifuged in this way was fractionated through a bent capillary as described previously (Gibson, 1965a), and the absorption at 280 and 370 mμ (Soret band of bacteriochlorophyll) is shown in Figure 4. The large peak near the center of the tube corresponds to the band of brown material; the spectrum of the latter is shown in Figure 5. The peaks due to bacteriochlorophyll in the region of the Soret band and near 850 mμ were unchanged in their shape and relative height, but the peaks between 400 and 550 mμ due to carotenoid were lower than they were in unbroken chromatophores. There was also a much greater relative amount of absorption in the ultraviolet; most of this is probably due to coenzyme Q and derivatives (Clayton, 1962).

The spectrum of the red bands was not very different from that of chromatophores. It is probable that all these bands including the pellet represent aggregates of the same fraction. Examination of the centrifuge tube after short intervals of centrifugation showed that all the red material moved down the tube as a single band, becoming broader as it approached the bottom. This suggests that it is initially rather homogeneous and becomes more aggregated as time goes on. The tendency of all the fractions to form aggregates was very marked, and frustrated all attempts to separate them on columns of Sephadex or polyacrylamide beads. The fractions also aggregated irreversibly when attempts were made to concentrate them by precipitation with $(NH_4)_2SO_4$, pervaporation, or pressure dialysis, although some success was obtained with the third technique. Addition of 5×10^{-4} M sodium dodecyl sulfate and 1 M urea to all solutions delayed aggregation somewhat but could not prevent it altogether or reverse it.

The separation of core particles into these fractions occurred equally well if the concentration of bile salts was as high as 7 mg/mg of protein provided that salt was present. However substitution of 6 M urea for KCl released only a trace of pigment, the bulk of it being still in a form which passed right through the sucrose gradient. When core particles were dialyzed against 8 M urea, then against 0.01 M Tris buffer, and centrifuged, the same result was obtained, but if KCl were added after the urea the same pattern of pigmented bands was obtained as with KCl alone. Thus the bonds which hold the brown and the red fractions together are clearly mainly polar in nature, and neither fraction is apparently affected by urea. The relative quantities of protein in each fraction have not been determined accurately as it is clear that the optimum conditions for their separation have not been reached, but preliminary data show that there is about the same amount in each.

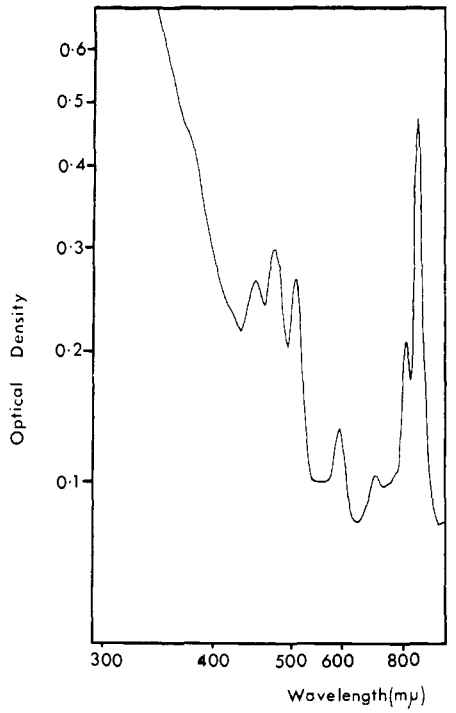

FIGURE 5: Spectrum of brown fraction from core particles from 300 to 1000 mμ.

Discussion

Structure of the Chromatophore

The chromatophores of *Rps. spheroides* are spherical or near-spherical particles whose diameters are approximately normally distributed with mean near 570 A and standard deviation about 40 A. They contain much water of hydration (55% by volume), and the density of the remainder is 1.175 g/cm³ (Gibson, 1965a,b,c). The question arises what alteration they undergo when they are treated with bile salts. The drop in sedimentation coefficient from 160 to 120 S is too small to be caused by a change from dimer to monomer, and the most reasonable explanation is that some nonpigmented material comes away from the chromatophore. This is borne out by the investigation into the gross chemical

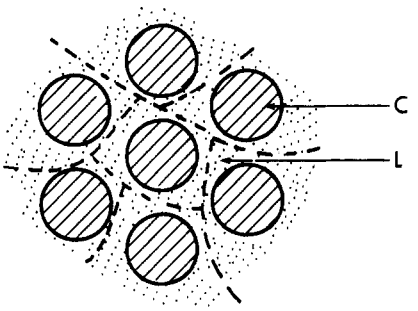

FIGURE 6: Hypothetical intracellular structure giving rise to chromatophores. Broken lines indicate fracture by shearing forces; C = core particle; L = lipid matrix.

composition of the cores and the chromatophores, which showed that a large fraction of the extractable lipid, and not much else, was removed by cholate and deoxycholate. It remains to decide what part of the structure the lipid detaches from, and here all the evidence suggests that it comes from the surface and that normally the greater part if not the whole of the surface is covered by lipid. The large difference in stability between chromatophores and their cores indicates that the lipid plays a major part in holding the chromatophore together. This does not mean that the lipid must cover the surface, but it must be on the surface as its removal is not accompanied by any immediate change in the rest of the particle. However, the reaction of core particles to the presence or absence of salts, which is totally different from that of chromatophores, suggests that the lipid does cover most of the surface in fact. The strong tendency to aggregate which was observed with whole chromatophores is almost certainly a property of their surface layer, and removal of this layer would be expected to alter completely the behavior of the remaining structure. On the other hand, if some of the surface layer were left, the core particles ought to show some of the behavior of the original particles, and this they do not. Examination in the electron microscope provided confirmatory evidence. When chromatophores were dried down in the presence of phosphotungstate they collapsed but did not disintegrate, but when core particles were dried down they not only collapsed but showed very definite signs of coming apart as well (Gibson, 1965c), as would be expected if they had lost an outer stabilizing shell.

From these considerations it is concluded that the lipid is distributed as a thin layer on the surface of the chromatophores. A thickness was calculated for this layer from the data presented in Table VI; the value lay between 25 and 55 A, depending on the degree of hydration assumed for the lipid layer. The sedimentation coefficient of core particles was calculated from the data in Table VI to be between 125 and 135 S, depending on the density and degree of hydration assumed for the lipid layer. The agreement with experiment is satisfactory.

Interpretation of Electron Micrographs

The observations reported in this paper have a profound significance for the interpretation of electron micrographs of chromatophores. In sections of fixed and stained preparations of whole cells or purified chromatophores from many organisms (Vatter and Wolfe, 1958; Hickman and Frenkel, 1959; Cohen-Bazire and Kunisawa, 1963; Gibson, 1965c) these particles appear as circular or slightly elliptical structures with a thin electron-dense outer layer and an electron-lucent interior. This appearance is part of the evidence suggesting that there are no separate chromatophores but only a continuous membranous structure whose appearance in cross section fortuitously resembles clumps of vesicles (Karunairatnam *et al.*, 1958; Cohen-Bazire and Kunisawa, 1963). The thickness of the membrane has been estimated to be 75–80 A in *Rhodospirillum rubrum*.

However, the picture of the chromatophore which has been drawn here leads to quite a different interpretation of the membrane-bound vesicles in *Rps. spheroides*. In fixed and stained preparations isolated chromatophores and intracellular membrane-bound vesicles have the same distribution of diameters and the same surrounding shell of electron-dense material (Gibson, 1965c). The thickness of this shell is between 30 and 70 A, which is close to the value calculated for the layer of lipid surrounding the core particle. The agreement between the various figures suggests strongly that the electron-dense shell seen in fixed particles, whether intracellular or not, is the same as the lipid shell removed by bile salts. If this interpretation is correct, it has the following important consequence: that the part of the chromatophore which contains all the bacteriochlorophyll and is responsible for its specific biological activity appears almost totally blank and featureless when viewed in the electron microscope. The layer which can be observed clearly and distinctly with this instrument might play a fundamental role in holding chromatophores together and in regulating the flux of metabolites in and out, but it can hardly be considered directly responsible for the photosynthetic reactions which are their essential contribution to the economy of the anaerobic cell. Likewise, any structure which is observed on the surface of chromatophores can play no direct part in light-catalyzed reactions; this would apply to the small particles observed on the surface of negatively stained chromatophores of *R. rubrum* (Löw and Afzelius, 1964) and to similar structures seen in *Rps. spheroides* (K. D. Gibson, unpublished experiments).

State of Chromatophores in Vivo

In *Rps. spheroides*, as in *R. rubrum*, chromatophores appear in sections of whole cells as clumps of membrane-bound vesicles which tend to lie near the surface of the cell. However, isolated chromatophores behave like almost homogeneous preparations of separate particles. It was suggested in the previous paper that the clumping observed *in vivo* might be brought about by the presence of various ions inside the cell (Gibson, 1965b). An alternative view of the structure *in vivo* can now be put

forward; this is that it consists of a number of pigmented core particles embedded in a matrix of lipid, in the manner illustrated in Figure 6. If this structure were exposed to shearing forces, as in the French press, it might be expected to develop fissures in the lipid matrix rather than through the core particles and would thus break up in such a way as to leave each core particle surrounded by a thin layer of the matrix. Such particles would be virtually impossible to distinguish by available methods from particles which are formed separately and distinctly. There is at present no way of distinguishing absolutely between the two hypotheses, although the evidence on the whole favors the view that the structures are separate particles which tend to aggregate together inside the cell.

Acknowledgments

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