Isolation and Characterisation of Chromatophores from *Rhodopseudomonas spheroides**

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ABSTRACT: Chromatophores were isolated in 75–95% yield by zone centrifugation through a cesium chloride gradient. From their content of nucleic acid they were judged to be at least 95% pure. The preparation showed a single boundary in the ultracentrifuge whose sedimentation coefficient was close to 160 S. The chromatophores had a strong tendency to aggregate under a variety of conditions, especially in the presence of divalent cations.

The aggregation was prevented in the presence of cesium chloride. Purified chromatophores catalyze cyclic photophosphorylation, but only at 10–15% of the rate found with crude particles. Further observations suggested that the drop in activity is partly due to an extreme lability to autoxidation and partly to the removal of an unidentified factor.

In the previous paper (Gibson, 1965a) evidence was presented that at least 85% of the pigment in anaerobically grown *Rhodopseudomonas spheroides* is contained in particles whose $s_{20,w}$ value is about 160 S, the remainder being in particles of about 55 S. This paper describes the isolation and some of the properties of the 160 S particles, which will henceforth be identified with the chromatophores of Schachman *et al.* (1952).

Experimental Section

Methods. The strain of organism, methods of growth, and the preparation of extracts in a French press were described in an accompanying paper (Gibson, 1965a). The methods for assaying protein and bacteriochlorophyll are given in the same paper. Nucleic acid was assayed after extraction with phenol. The method was essentially that of Luck and Reich (1964) up to the treatment with ribonuclease. Total nucleic acid was then estimated from the absorption at 260 mμ.

Zone and moving-boundary sedimentation were carried out in the same way as before (Gibson, 1965a). Moving-boundary sedimentation was also performed in a Spinco Model E analytical ultracentrifuge equipped with schlieren optics.

Assay of Photophosphorylation. Two methods were used for assaying photophosphorylation. The first was slightly modified from one used by Geller (1963). The incubation mixture contained, in a total volume of 0.4 ml, tris(hydroxymethyl)aminomethane (Tris) buffer, pH 7.5 (20 μmoles), $MgCl_2$ (2 μmoles), adenosine triphosphate (ATP)[1] (5 μmoles), mannose (40 μmoles), hexokinase (0.4 mg.), $K_2H^{32}PO_4$ (0.8 μmole, 1–2 μc/μmole), and the preparation to be tested (usually containing 2–20 μg of bacteriochlorophyll). The mixture was incubated for periods of up to 30 min at 32° either in the dark or under illumination at an intensity of 160 foot candles. The reaction was stopped by the addition of 0.4 ml of 10% trichloroacetic acid and protein was removed by centrifugation. An aliquot of 20 or 50 μl of the supernatant fluid was applied as a spot to paper and electrophoresed under the conditions described by Geller (1963). The paper was exposed to X-ray film overnight to locate the radioactivity spots, which were cut into strips, introduced into bottles containing 10 ml of a solution of 0.3% 2,5-diphenyloxazole and 0.05% 1,4-bis-2-(5-phenyloxazolyl)benzene in toluene, and counted in a Tritomat liquid scintillation counter (Isotope Developments Ltd., Reading, England). The amount of inorganic phosphate which was esterified during the incubation was calculated from the radioactivities found in the spots of inorganic phosphate and mannose 6-phosphate.

The second method of assay employed the customary technique of measuring directly the disappearance of inorganic phosphate. The assay was exactly the same as before except that nonradioactive inorganic phosphate was used. After the removal of denatured protein, inorganic phosphate was estimated in a 0.5-ml aliquot of the supernatant by the method of Fiske and Subbarow (1929).

Isolation of Chromatophores. For the preparation of chromatophores the CsCl gradient described previously was scaled up for use in the SW25 rotor of the Spinco Model L ultracentrifuge. Chromatophores were collected from extracts of cells together with unbroken

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[1] Abbreviations used: ATP = adenosine triphosphate; NAD = nicotine-adenine dinucleotide; $NADH_2$ = reduced NAD.

cells, cell walls, and some ribosomes by centrifugation for 2 hr at 40,000 rpm in the No. 40 rotor. The pellet was suspended in dilute (0.001–0.01 M) Tris buffer, pH 7.5 (about one-twentieth of the volume of the extract). Aliquots (up to 2.0 ml) of this suspension were layered onto linear gradients of CsCl, constructed from 13.0 ml of 0.60 M CsCl and 13.0 ml of 1.66 M CsCl, both in 0.01 M Tris buffer, pH 7.5, with a conventional device, and the tubes were centrifuged in the SW25 rotor at 25,000 rpm for periods of 2.5–3 hr, excluding the time required for acceleration and deceleration. The contents of the tubes were removed through a capillary and divided into fractions of about 0.5 ml by means of a drop-counting device. The material in the tubes corresponding to the pigmented band was combined, diluted with an equal volume of dilute Tris buffer, and collected by centrifugation for 2 hr at 40,000 rpm. The pellet was resuspended in dilute Tris buffer and stored at 5°. Preparations which were to be used for assaying photophosphorylation were usually stored under argon or nitrogen.

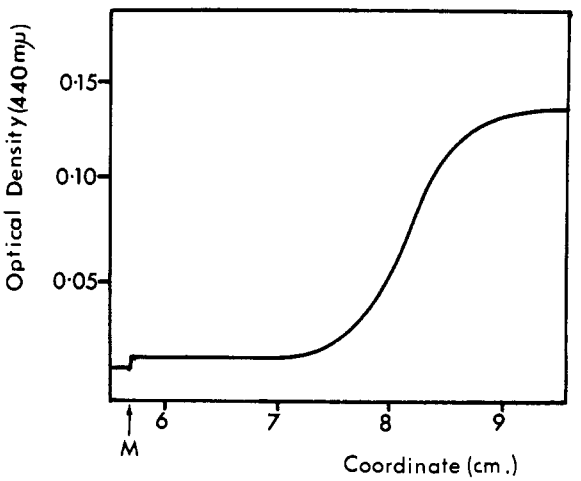

FIGURE 1: Moving boundary sedimentation of purified chromatophores. Contents of tube were sampled from the top; M = meniscus; linear KCl gradient (0–3.8% w/w), 35,400 rpm, 33.0 min, 7.5°.

Results

Through the use of a continuous CsCl gradient, chromatophores can be isolated in a high state of purity and in excellent yield. In all preparations which have been tested, the amount of bacteriochlorophyll in the purified particles, based on the absorption at 850 mμ or on estimates of acetone–methanol extracts, has been between 75 and 95% of the total bacteriochlorophyll originally present in the crude extract of cells. The isolated chromatophores behave as a single peak when centrifuged on a (small-scale) CsCl gradient (Gibson, 1965a), as regards both the distribution of pigment and the optical densities at 260 and 280 mμ. The purified material has an absorption spectrum and gross chemical composition which are very similar to published data; the experimental findings are reported elsewhere (Gibson, 1965b).

Nucleic Acid Content of Chromatophores. One criterion which has been used to estimate the purity of chromatophore preparations is the amount of RNA (Worden and Sistrom, 1964), since according to available data chromatophores contain only traces of this substance (Lascelles, 1962). Chromatophores isolated on a CsCl gradient were therefore assayed for their RNA content (Table I). The material which was obtained after a single passage through a CsCl gradient contained about twice as much nucleic acid as did the material prepared by Worden and Sistrom, but even so this represented less than 15% of the nucleic acid present in the crude 40,000 rpm precipitate. A further passage through a CsCl gradient brought the nucleic acid content well below the level observed by Worden and Sistrom (1964), while yet another passage reduced it to less than one-twentieth of the value they recorded. Thus, by the criterion of their nucleic acid content, chromatophores isolated by one passage through a CsCl gradient contained about three times as much impurity as the ones Worden and Sistrom isolated by a somewhat longer procedure, but a further application of the gradient led to a product which was purer than theirs. The third passage through the gradient reduced the nucleic acid to 1 μg/mg of protein. Treatment with ribonuclease showed that 70% of this was deoxyribonucleic acid (DNA).

TABLE I: Nucleic Acid and Protein Content of Purified Chromatophores.

No. of Times through CsCl Gradient	Protein (mg/mg of bacteriochlorophyll)	Nucleic Acid
..[a]	125	1.70
0[b]	13.5	1.35
1	5.5	0.45
2	5.1	0.047
3	5.0	0.005

[a] Supernatant after centrifugation of a crude extract of cells at 10,000 rpm for 10 min. [b] Crude particles precipitated from extract by centrifugation at 40,000 rpm for 2 hr.

Sedimentation Coefficient of Chromatophores. Purified chromatophores were examined by moving-boundary sedimentation both in an analytical ultracentrifuge (schlieren optics) and with a linear KCl gradient in the preparative ultracentrifuge, using an autoanalyzer to follow the absorption at 440 mμ. In both cases only a single boundary was seen (Figures 1 and 2). The sedimentation coefficients calculated from these boundaries are listed in Table II; the discrepancy between the

TABLE II: Sedimentation Coefficient of Purified Chromatophores.

Type of Ultracentrifuge	Method of Locating Boundary	Concn of Chromatophores (mg of protein/ml)	$s_{20,w}$ (S)
Preparative	Optical density at 440 mμ	0.04	168
Analytical	Schlieren optics	0.4	134

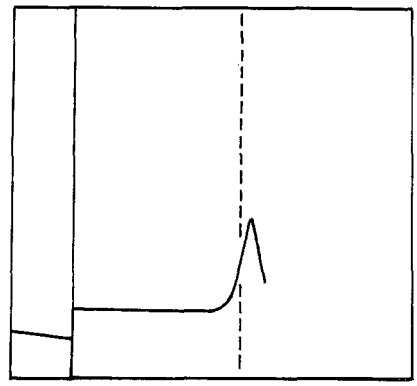

FIGURE 2: Schlieren pattern of purified chromatophores. Broken line shows position of pigment boundary; 20,400 rpm, 25°; photographed 20 min after start.

figures is due to the concentration dependence of s, which is discussed later.

However, in many preparations there was evidence for the presence of aggregated material, from experiments with the analytical and the preparative ultracentrifuge. In the former case this evidence manifested itself as a too rapid decrease in the absorption of light due to pigment in the plateau region, indicating the presence of heavier material. No second boundary could be detected and therefore each aggregate must have been present at less than 10% of the concentration of the main boundary. In the preparative ultracentrifuge the aggregation showed as an increase in the slope of the absorption curve below the boundary, so that it became considerably greater than the theoretical slope due to the deviation from a plateau (Gibson, 1965c). This suggested the presence of a continuum of aggregated particles, each present at a very small concentration and ranging from dimers to large aggregates. A further piece of evidence for the presence of aggregates in some preparations was the fact that the s values calculated by the first and second methods of Gibson (1965c) differed considerably (see Gibson, 1965b).

Aggregation of Chromatophores. It appeared from these experiments that chromatophores tend to form aggregates rather readily, and a study was therefore undertaken of the conditions which promote the process. For this purpose a rough assay was used which measured the increase in turbidity caused by the treatment under investigation. The turbidity was determined from the optical density at 645 mμ, which is an absorption minimum for the chromatophores, and the increase was used as a qualitative guide to the degree of aggregation. While this method only detects gross changes it was found adequate for the purpose in hand.

One procedure which caused marked aggregation was freezing and thawing, which gave rise to gross changes clearly visible to the naked eye. This probably accounts for the observation made in preliminary experiments that crushing cells in a Hughes press (Hughes, 1951) releases a whole range of pigmented particles of various sizes. If whole cells are frozen and thawed before being disrupted in a French press, the chromatophores become aggregated and do not give a symmetrical peak when centrifuged in a CsCl gradient; thus the pigmented peak in Figure 3 clearly shows the presence of aggregation on its lower side. When extracts of cells which had been frozen and thawed before disruption were examined by moving-boundary centrifugation, two boundaries were visible in about the same positions as with normal extracts, but the slope of the absorption curve below the lower boundary was very great, indicating the presence of considerable quantities of aggregates of all sizes.

TABLE III: Aggregation of Chromatophores in the Presence of Salts.

Solution[a]	Concn[a] (M)	OD at 645 mμ	Degree of Aggregation
. . .[b]	. . .	0.19	−
Tris-HCl, pH 7.5	0.1	0.49	++
	0.01	0.17	−
	10^{-3}	0.19	−
	10^{-4}	0.19	−
K^+ phosphate, pH 7.4	0.1	0.33	+
	0.01	0.19	−
	10^{-3}	0.17	−
$MgCl_2$[c]	10^{-2}	0.59	++
	10^{-3}	0.17	−
Na^+_3 citrate	10^{-2}	0.47	++
	10^{-3}	0.17	−
NaCl[c]	0.1	0.35	+
	0.01	0.20	−
CsCl[c]	0.1	0.20	−
	0.01	0.15	−
Sucrose[c]	0.2	0.20	−

[a] Chromatophores were dialyzed against these solutions overnight. [b] Not dialyzed. [c] In the presence of 10^{-3} M Tris buffer, pH 7.5.

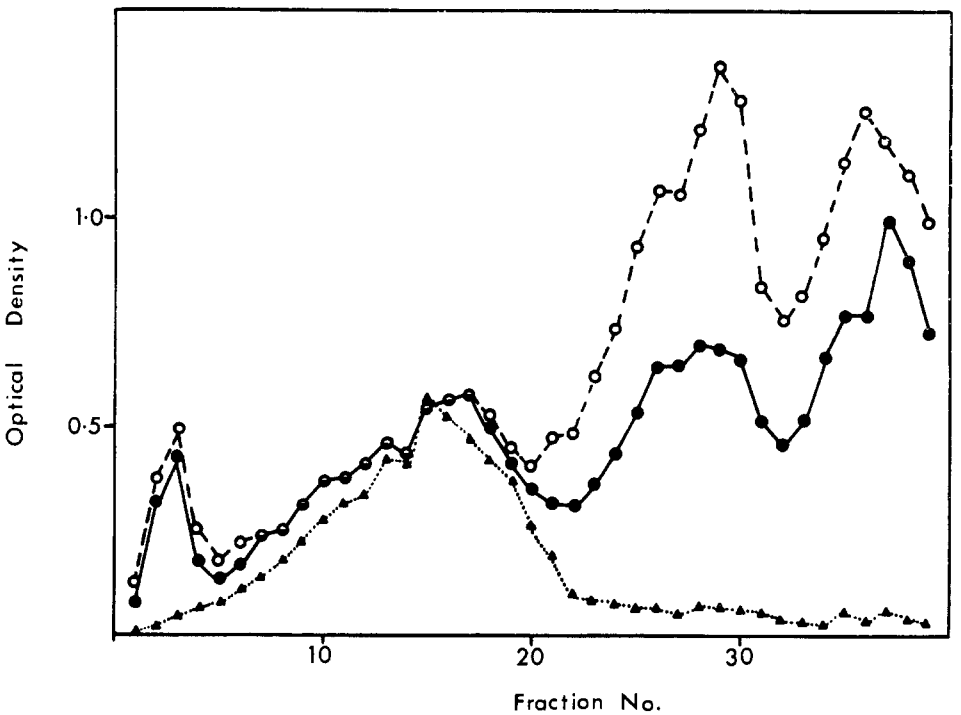

FIGURE 3: Zone centrifugation of extract of frozen cells in a linear gradient of CsCl (0.60–1.66 M). For experimental conditions see Gibson (1965a). Optical density was determined at 260 (○), 280 (●), and 850 mμ (▲).

TABLE IV: Aggregation of Chromatophores Caused by Divalent Cations.

Type of Salt[a]	OD at 645 mμ	Degree of Aggregation
...[b]	0.21	−
$MgCl_2$	0.40	+
$CaCl_2$	0.41	+
$MnCl_2$	0.92	+++
$ZnCl_2$	0.64	++
$CoCl_2$	0.65	++
$FeCl_3$	0.47	+

[a] Chromatophores were dialyzed overnight against 0.01 M solutions of these salts in the presence of 10^{-3} M Tris buffer, pH 7.5 [b] Not dialyzed.

TABLE V: Effect of Alkali Metals on the Aggregation of Chromatophores.

Type of salt[a]	OD at 645 mμ	Degree of Aggregation
...[b]	0.22	−
LiCl	0.65	++
NaCl	0.35	+
KCl	0.27	±
RbCl	0.28	±
CsCl	0.23	−

[a] Chromatophores were dialyzed overnight against 0.2 M solutions of these salts in the presence of 10^{-3} M Tris buffer, pH 7.5. [b] Not dialyzed.

Of somewhat more significance was the observation that the aggregation of chromatophores is very sensitive to the ionic environment, being influenced greatly by the ionic strength and by the nature of the ions present. Suspensions of chromatophores, with protein concentrations in the range 0.15–0.25 mg/ml, were dialyzed overnight against an excess of the solution whose effect was to be tested, and their turbidities were determined. No increase in turbidity was detected at ionic strengths between about 10^{-5} and 10^{-3} whatever ions were present. However at ionic strengths above 10^{-2} most salts showed some ability to cause aggregation. In particular, divalent and trivalent metals were extremely effective at concentrations of 10^{-2} M (Tables III and IV). Polyvalent anions also caused aggregation (Table III), but the effect did not depend on osmotic forces since 0.5 M sucrose caused no change in turbidity. The lower limit of ionic strength at which aggregation occurred was found to vary slightly from preparation to preparation, and occasional batches of chromatophores became turbid when dialyzed against 0.01 M Tris buffer.

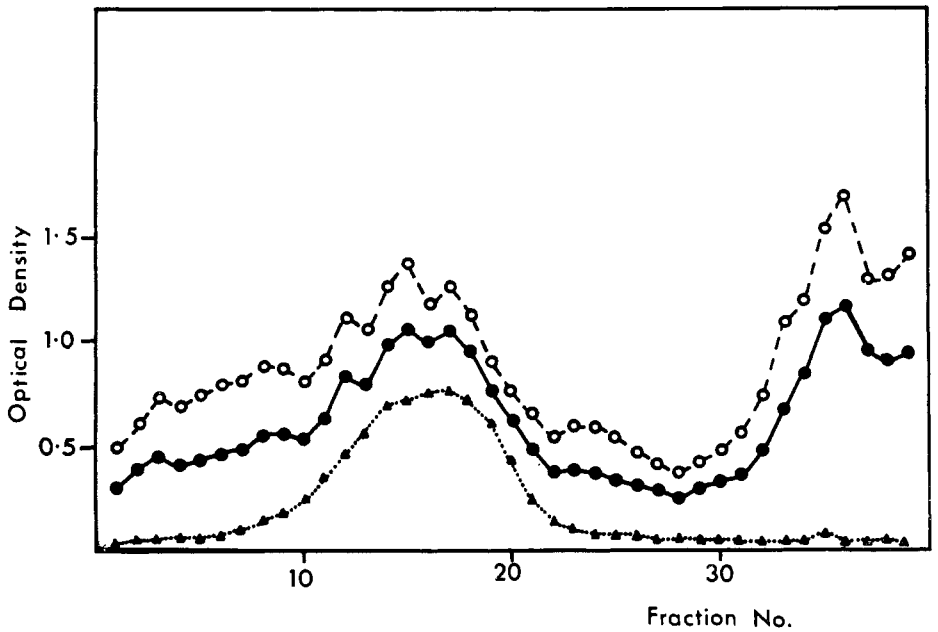

FIGURE 4: Zone centrifugation of extract of anaerobic cells in a linear gradient of CsCl, in the presence of $MgCl_2$. Experimental conditions were as in Figure 3, except that the extract and the CsCl gradient contained 0.02 M $MgCl_2$; wavelengths: 260 (○), 280 (●), and 850 mμ (▲).

However, all preparations which have been encountered so far remained clear when dialyzed against solutions whose ionic strengths lay between 10^{-5} and 10^{-3}.

The effects of some monovalent cations are shown in Table III and particularly in Table V, where the action of the alkali metals is compared. There is a marked decrease in the ability of these metals to cause aggregation as the atomic number increases, so much so that Cs^+ appears to protect against the aggregation caused by some solutions and even to reverse it. The aggregation which is caused by high concentrations of Tris buffer was also reversed on dialysis against lower concentrations of Tris or phosphate buffer. When a preparation which had been aggregated and disaggregated in this way was centrifuged in a CsCl gradient, it showed a single pigmented peak which migrated in the same position as freshly isolated chromatophores but was somewhat broader than usual. It was not found possible to reverse the aggregation caused by Mg^{2+} by dialysis against 0.01 M Tris buffer or by the addition of 0.5 M CsCl. On the other hand, when 0.02 M $MgCl_2$ was added to an extract of whole cells and it was then centrifuged in a CsCl gradient to which 0.02 M $MgCl_2$ had been added, the pigmented band migrated to the same position and showed no sign of aggregation, although the peak which contained the ribosomes had moved down the tube and was clearly aggregated (Figure 4). Thus the presence of Cs^+ had prevented the chromatophores from aggregating even in the presence of 0.02 M Mg^{2+}.

Chromatophores also aggregate at very low ionic strengths. When suspensions were dialyzed against distilled water, there was a marked increase in turbidity; for instance, the optical density at 645 mμ of one suspension rose from 0.25 to 0.80 when it was dialyzed against distilled water. The possibility that this was due to traces of heavy metal ions in the distilled water is ruled out by the observation that deionized water or doubly glass-distilled water produced exactly the same result. It was not possible to reverse the process by adding CsCl or dialyzing against 0.01 M Tris buffer. Dialysis of fresh chromatophores against 0.01 M Tris buffer containing 10^{-3} M EDTA did not cause any increase in turbidity.

Heavy and Light Chromatophores. A number of investigators have provided evidence which suggests that there are two types of pigmented structure in photosynthetic bacteria (Newton and Newton, 1957; Cohen-Bazire and Kunisawa, 1960; Worden and Sistrom, 1964). These have been called heavy and light chromatophores, and it has been suggested that the heavy particles consist of light ones attached to some other constituent of the cell. In view of the ease with which chromatophores of *Rps. spheroides* aggregate, it seemed possible that the heavy fraction is an artifact produced by aggregation of light chromatophores, possibly together with some other constituent of the cell. An attempt was therefore made to prepare a heavy fraction from extracts of *Rps. spheroides*.

A crude extract of organisms was centrifuged for 10 min at 10,000 rpm to remove large fragments and the supernatant was centrifuged for 1 hr at 15,000 rpm. This procedure is similar to that used to prepare heavy chromatophores from *Chromatium* (Newton and Newton, 1957). The precipitate was suspended in dilute Tris buffer and layered on a standard CsCl gradient. An aliquot of the original extract was layered on

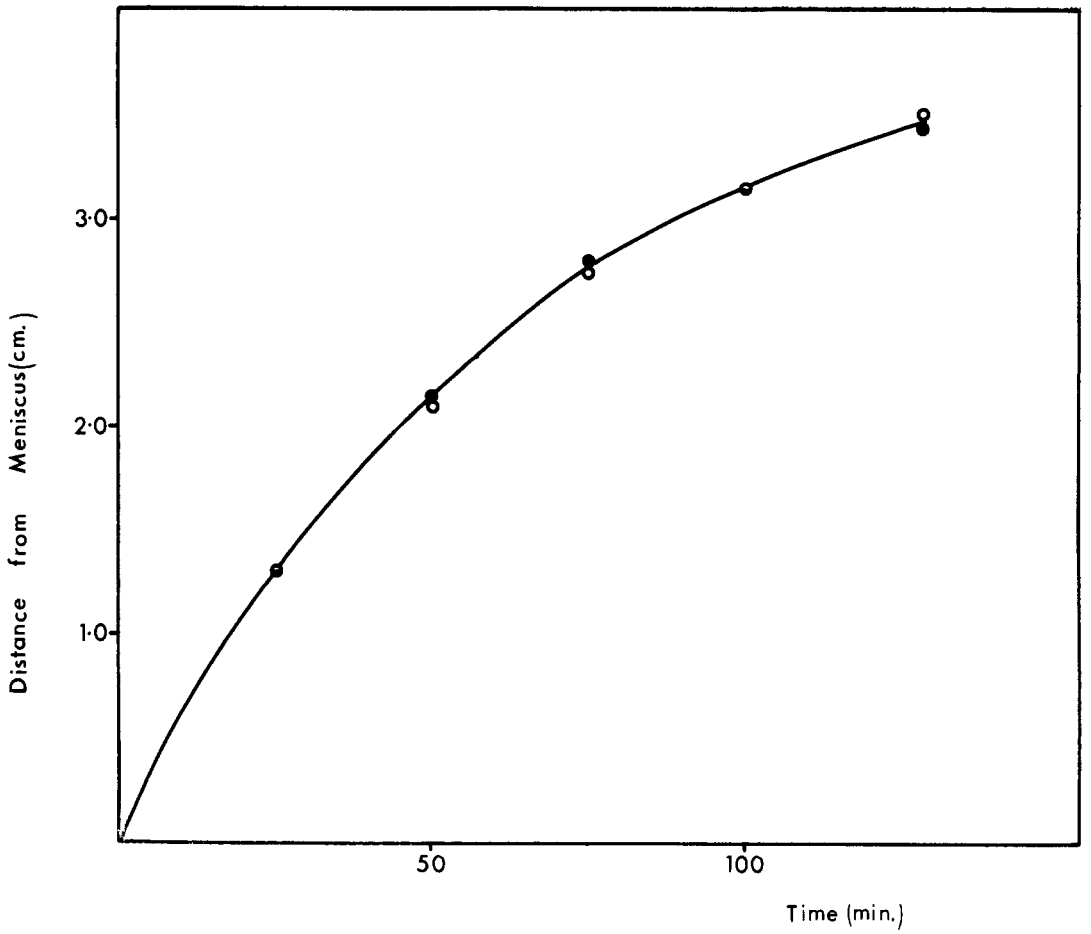

FIGURE 5: Movement of the pigmented band from whole extract (●) and "heavy" chromatophores (○) centrifuged through a linear CsCl gradient (0.60–1.66 M) at 39,000 rpm.

another CsCl gradient and both tubes were centrifuged together. Only a single pigmented band was observed in the tube containing the "heavy" fraction, and no pigment was found in the pellet. The rate at which the center of this band moved down the tube was the same as the rate at which the main pigmented band from the extract traveled (Figure 5), and the width of both bands remained exactly equal. Thus there was no significant difference in sedimentation behavior between the "heavy" fraction and the remainder of the chromatophores.

Photophosphorylation. In order to characterize further the purified chromatophores, their ability to catalyze cyclic photophosphorylation (Arnon, 1961) was investigated. It was consistently found that purified chromatophores had 15% or less of the activity present in the crude particles precipitated from the extract before they were centrifuged through a CsCl gradient (Table VI). The activities of crude and purified chromatophores increased to the same extent when *N*-methylphenazonium methosulfate was added, but crystalline horse heart cytochrome *c* did not stimulate either preparation. The loss of activity on purification was not due to the presence of CsCl, as crude particles kept at 5° in the presence of this salt for 24 hr had much the same activity at the end of this period as they did at the beginning, although when the same particles were purified by centrifugation through a CsCl gradient they became almost totally inactive (Table VII). Also when chromatophores were purified by centrifugation through a gradient of sucrose they had the same low activity as chromatophores prepared on a CsCl gradient (Table VIII). One further possibility was that omission of $MgCl_2$ during purification led to loss of activity; but when $MgCl_2$, $MnCl_2$, and $CaCl_2$ were added to a CsCl gradient in low concentrations, the activity was the same as it was when they were omitted (Table VIII). Finally, adding back the material in the pellet or any of the other particulate peaks in the gradient did not increase the amount of phosphate esterified by the chromatophores, nor was it possible to obtain consistent stimulation by the supernatant peak from the gradient.

In these experiments the activity even of fresh extracts was rather variable and much lower than has been observed with other species (Frenkel and Hickman, 1959; Fuller *et al.*, 1963). Unlike the chromatophores from *Rhodospirillum rubrum* (Nozaki *et al.*, 1963) the particles from *Rps. spheroides* were not stimulated at all

TABLE VI: Photophosphorylation by Purified Chromatophores.

Preparation	Addition	Phosphate Esterified[a] (μmole/hr/mg of bacteriochlorophyll)
Cell extract	...	2.7
Cell extract	10^{-3} M PMS[b]	3.7
Crude particles	...	4.7
Crude particles	10^{-3} M PMS[b]	6.5
Chromatophores	...	0.5
Chromatophores	10^{-3} M PMS[b]	0.7

[a] Assayed with ^{32}P; results based on a 15-min incubation. [b] *N*-Methylphenazonium methosulfate.

by the addition of $NADH_2$ or $NADPH_2$ even when a supernatant fraction of the cell was also added, or by the addition of ascorbate at concentrations up to 10^{-2} M, or by molecular hydrogen. This was true for purified and for crude preparations. However the activities of extracts and crude particles were increased by a factor of 10 when ascorbate at concentrations in the vicinity of 10^{-3} M was added to the cell suspension before crushing and maintained in all solutions used subsequently.

TABLE VII: Effect of CsCl on Photophosphorylation.

Preparation	Phosphate Esterified[a] (μmole/hr./mg of bacteriochlorophyll)
Crude particles	10.6
Crude particles + CsCl[b]	9.5
Chromatophores	1.3

[a] Assayed with ^{32}P, in the presence of 10^{-4} M *N*-methylphenazonium methosulfate; results based on a 30-min incubation. [b] Crude particles were mixed with 0.83 M CsCl and kept at 5° for 24 hr.

Extracts and crude particles prepared in this way had activities comparable to those observed in other species, but purified chromatophores prepared by centrifugation through a CsCl gradient were still very inactive, even though ascorbate was present at the same concentration in the gradient (Table IX). These chromatophores, like those prepared in the absence of ascorbate, were not activated by the addition of metal ions or by any other fraction from the CsCl gradient.

TABLE VIII: Photophosphorylation by Chromatophores Isolated under Different Conditions.

Preparation	Phosphate Esterified[a] (μmole/hr/mg of bacteriochlorophyll)
Crude particles	14.8
Chromatophores[b]	2.8
Chromatophores[c]	2.5
Crude particles	5.2
Chromatophores[b]	0.6
Chromatophores[d]	0.5

[a] Assayed with ^{32}P, in the presence of 10^{-4} M *N*-methylphenazonium methosulphate; results based on a 30-min incubation. [b] Chromatophores isolated on a gradient of CsCl. [c] Chromatophores isolated on a gradient of CsCl in the presence of 10^{-2} M $MgCl_2$, 10^{-3} M $CaCl_2$, and 10^{-4} M $MnCl_2$. [d] Chromatophores isolated by zone centrifugation through a linear gradient (0.15–0.75 M) of sucrose at 25,000 rpm for 1 hr at 5°.

TABLE IX: Photophosphorylation by Particles Prepared in the Presence of Ascorbate.

Concn of Ascorbate[a] (M)	Phosphate Esterified (μmole/hr/mg of bacteriochlorophyll) by [b] Extract	Crude Particles	Purified Chromatophores
4×10^{-4}	...[c]	125	5
5×10^{-3}	81	70	8

[a] Ascorbate was added to cells at this concentration before passage through French press and maintained at this concentration throughout. [b] Assayed with nonradioactive phosphate; results based on a 10-min incubation. [c] Not determined.

Discussion

One of the drawbacks that has prevented full acceptance of the view that chromatophores are particulate structures *in vivo* has been the difficulty of obtaining high yields of homogeneous preparations when mild procedures were used to rupture the cells. Although pigmented particulate fractions have been isolated in a number of studies there is a remarkable dearth of information about the over-all yields of photosynthetic pigment obtained in the isolated material. It appears to be possible to obtain highly purified preparations from extracts made by sonication in yields of 75% and more (Bull and Lascelles, 1963). However, the effect of sonica-

tion in disrupting lipid structures is well known and, hence, the isolation of particles by this technique is no guarantee that such particles ever existed *in vivo*. In the work reported here a homogeneous pigmented particulate fraction has been isolated in 80% yield from extracts made by rupture in a French press. While this technique must also cause disruption of structures, it is not nearly as suspect as sonic disintegration, and thus the isolation of this material lends some support to the view that the structure which contains the pigment *in vivo* is the same as the one in which it is found *in vitro*.

Sedimentation Coefficient of Chromatophores. The value of $s_{20,w}$ calculated from moving-boundary sedimentation in the preparative ultracentrifuge for the isolated chromatophores was 168 S. This agrees very well with the value of 160 S obtained for the larger of the pigmented particles that were observed directly in crude extracts of anaerobic cells and which accounted for more than 85% of the photosynthetic pigments (Gibson, 1965a). In this experiment the concentration of chromatophore protein was less than 0.05 mg/ml. A lower value for $s_{20,w}$ was obtained from the single boundary visible in the analytical ultracentrifuge; the concentration of chromatophore protein in this experiment was about 4 mg/ml. The two values suggest that there is a marked concentration dependence in the $s_{20,w}$ value of the particles. This also manifested itself as a strong self-sharpening effect which was seen in experiments in the analytical ultracentrifuge and which led to boundaries that suggest a higher degree of homogeneity than was found from other sedimentation studies. Worden and Sistrom (1964) also observed a considerable concentration dependence of $s_{20,w}$ for their purified chromatophores. In their experiments the value of s at a concentration of 2.5 mg/ml was about 140 S, whereas that obtained by extrapolation to infinite dilution was 153 S.

Aggregation of Chromatophores. Many of the contradictions which have been observed previously can be reconciled by taking account of the readiness with which chromatophores aggregate under conditions very similar to those that have been used in their isolation and study. Any technique involving freezing and thawing, even where this was carried out in whole cells before rupture, almost certainly leads to the irreversible formation of aggregated material. However the most significant observation in this respect is the aggregation caused by salts and especially by salts of divalent cations. It seems to be a common practice to include Mg^{2+} in the media for isolating chromatophores. Thus, Cohen-Bazire and Kunisawa (1960) using *R. rubrum* and Worden and Sistrom (1964) using *Rps. spheroides* both added Mg^{2+} to a concentration of 0.01 M before rupturing the cells in a French press, and maintained this concentration throughout their isolation procedure. This concentration of Mg^{2+} was quite sufficient to cause a large increase in turbidity in every preparation of chromatophores examined in the present work. Again, although they avoided the use of Mg^{2+} Newton and Newton (1957) disrupted *Chromatium* in the presence of 0.1 M Tris buffer and obtained a heavy fraction from their extracts. The chromatophores isolated in the present work became aggregated at that concentration of Tris. Although the experiments reported here make it clear that the aggregation of chromatophores is a somewhat complex process, it is probable that the high concentrations of salts used by all these investigators would promote aggregation; it is therefore quite possible that the heavy fractions reported previously are really aggregates of chromatophores which were formed after the cell was broken.

This view is supported by the experiment reported here in which a chromatophore fraction obtained by centrifugation at moderate speed, which ought to have consisted largely of "heavy" chromatophores, behaved in a manner indistinguishable from the main pigmented band in whole extracts when centrifuged in a gradient of CsCl. Perhaps high concentrations of CsCl disperse "heavy" chromatophores, but it is also possible that there was no such fraction and that the material which was collected by centrifugation at moderate speed was a random sample from all the chromatophores. Other attempts to detect a "heavy" fraction in fresh extracts, for instance, by centrifuging an extract through a 37–45% sucrose gradient as described by Worden and Sistrom (1964) (but omitting $MgSO_4$ from the solution), indicated the presence of only one particulate fraction containing pigment. It is significant that Worden and Sistrom themselves found that a fresh extract of cells gave rise to only one band of pigment when centrifuged in their sucrose gradient.

The observations reported here also explain the effectiveness of CsCl gradients in separating chromatophores as a single peak. Discontinuous sucrose gradients have been used to some effect with material obtained by sonication (Bull and Lascelles, 1963). However it seems likely that gradients of sucrose and similar materials will give low and variable yields of purified chromatophores unless great care is taken to exclude divalent cations; and indeed preliminary results with sucrose and sodium potassium tartrate were rather discouraging (Gibson, 1964b). On the other hand gradients constructed from cesium chloride have consistently given high yields of purified chromatophores, a fact which can clearly be traced to the ability of this salt to protect them against aggregation caused by other ionic species. Perhaps a similar effect was at work in the centrifugation step in the presence of rubidium chloride in the purification procedure used by Worden and Sistrom (1964).

Whether the aggregation phenomena reported here have any physiological significance is a matter for speculation. The salt concentrations in some of the solutions which caused chromatophores to aggregate were not too far removed from concentrations which might be found *in vivo*, and it is possible that for instance the aggregation due to divalent cations might have some function in the cell. Intracellular chromatophores tend to be found in clumps near the periphery of the cell, particularly when they are sparse as in organisms grown at high light intensities (Cohen-Bazire

and Kunisawa, 1963; Boatman, 1964; Gibson, 1965d). It has been suggested that this is because they originate at the periphery of the cell. However, it is also possible that the chromatophores become attached to the cytoplasmic membrane and to each other *in vivo* by the same forces as are responsible for their aggregation *in vitro*. In this connection it is of interest that there is a considerable similarity between the lipids in the cell walls and those in the chromatophores of *Rps. spheroides* (Gorchein, 1964), and that 70% of the lipid of the chromatophore lies in a thin layer on the surface of the particle (Gibson, 1965b).

Chromatophores and Nucleic Acid. Small amounts of nucleic acid have been found by other workers in preparations of chromatophores from *Rps. spheroides* (Bull and Lascelles, 1963; Worden and Sistrom, 1964). The result in Table I suggests that these were contaminants, since rigorous purification removed all but the merest trace of them. What remained was mostly DNA; if this were equally distributed among all the chromatophores, its molecular weight could not exceed 15,000, since the amount of protein per chromatophore was about $0.6 \times (3 \times 10^7)$, or 1.8×10^7, units of molecular weight (Bull and Lascelles, 1963; Gibson, 1965a) and their DNA content was about 0.7 μg/mg of protein. On the basis of present day information this is probably too small to be genuine. The trace of nucleic acid found in the chromatophores was therefore almost certainly a contaminant.

This observation has an important bearing on the problem of the origin of subcellular particulate organelles such as mitochondria and chloroplasts. Recently it has been found that mitochondria from yeasts (Schatz *et al.*, 1964; Luck and Reich, 1964) and chick embryos (Nass and Nass, 1963a,b) and chloroplasts from *Eugiena* (Edelman *et al.*, 1964) and some higher plants (Chun *et al.*, 1963) contain their own specific DNA with densities differing from the densities of the chromosomal DNA. It has been suggested that a specific cytoplasmic DNA is involved in the formation of every organelle either by acting as a template or by directing the formation of a template to which other constituents become attached (Danielli, 1962). Clearly it is of some importance to discover whether such a mechanism could be universal.

The chromatophores of *Rps. spheroides* are completely analogous to the particulate organelles of higher organisms in the following respects: their gross composition is very similar (Bull and Lascelles, 1963), their shape is uniform, their sizes are rather narrowly distributed about a mean (Gibson, 1965d), and they are specialized to perform a particular biochemical function (Geller, 1961). This suggests that they resemble other organelles at the level of their molecular structure, and that although they are much smaller and simpler their mode of origin should be similar. The experimental results show that the biosynthesis of chromatophores cannot involve DNA contained in the particles, and thus probably occurs in a nonautonomous manner. The implication for larger organelles is that their DNA does not play an essential part in the formation of the macromolecular structures from which these organelles are built (such as the electron-transport particles of mitochondria (Green and Fleischer, 1962; Fernandez-Moran *et al.*, 1964)), but must be involved at a higher level of organization.

Photophosphorylation in Rps. spheroides. Vernon (1963) found that chromatophores from *Rps. spheroides* could not photoreduce nicotine–adenine dinucleotide (NAD) or fumarate under conditions which allow a rapid reduction with *R. rubrum* chromatophores. On the other hand the particles from *Rps. spheroides* can photoreduce their own bound coenzyme Q (Clayton, 1962) and cytochromes (Smith *et al.*, 1960), and the light-induced absorption changes in these chromatophores provide ample evidence for changes in the physical or chemical state of other internal components (Smith and Ramirez, 1960; Worden and Sistrom, 1964). These observations suggest that in *Rps. spheroides* the chromatophores contain only the part of the electron-transport chain that is concerned directly with cyclic photophosphorylation, the remainder being located elsewhere in the cell. The part of the chain that is missing could comprise components that are involved specifically in the transfer of electrons from succinate, $NADH_2$, ascorbate, or molecular hydrogen.

This view can explain the fact that crude particles from *Rps. spheroides* had low and variable photophosphonylative activities unless they were isolated in the presence of sufficient ascorbate to prevent oxidation, and that the activity could not be restored by the addition of various naturally occurring hydrogen donors, in contrast to the chromatophores from *R. rubrum*, which are much more stable to oxidation and which can be readily reactivated by these hydrogen donors (Nozaki *et al.*, 1963). The different observations become compatible if the chromatophores of *Rps. spheroides* lack the electron carriers which couple these donors to the photophosphorylative system, while those of *R. rubrum* contain them.

However, this does not explain the large drop in activity when chromatophores were purified by zone centrifugation. The investigations that have been carried out show that this drop is not due to the use of CsCl or to the omission of divalent cations or to oxidation of the particles, but is a consequence of the separation of the particles from some unidentified factor. This factor is probably not a metal ion and it is not contained in any other particulate fraction of the cell. Possibly, it is a soluble coupling factor such as phosphodoxin (Black *et al.*, 1963) or one of the coupling factors involved in oxidative phosphorylation (Green *et al.*, 1963); however, the question clearly requires further investigation.

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