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ISOLATION AND CHARACTERIZATION OF THE MEMBRANES FROM *RHODOSPIRILLUM RUBRUM*

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SUMMARY

1. The specific bacteriochlorophyll *a* content of *Rhodospirillum rubrum* was inversely related to the specific growth rate of these cells. However, the lipid-phosphorus content of isolated and purified chromatophores was directly related to the specific growth rates of the cells from which they were isolated. The specific bacteriochlorophyll *a* content of these chromatophores was found to be constant and independent of the specific growth rate. This constancy in the specific bacteriochlorophyll *a* content was not observed in chromatophores isolated from cells in the stationary phase where increases in the specific bacteriochlorophyll *a* content of 100% were demonstrated.

2. Isolated chromatophores responded as osmometers to density gradient solutes of different osmotic properties. The density of chromatophores in Ficoll gradients increased when the non-permeable solute sucrose was added to the gradient, thus indicating that isolated chromatophores are vesicular structures which can be plasmolyzed in hypertonic solutions.

3. Soluble protein was released from purified chromatophores by subjecting them to osmotic shock and sonication. No bacteriochlorophyll *a*, and only 30% of the total membrane protein was released during 40 min of sonication. Comparison of the number of proteins released (acrylamide gels) and the release of succinate dehydrogenase by sonication and osmotic shock suggests that succinate dehydrogenase is tightly bound to the membrane whereas there is a group of unidentified proteins which are loosely bound or trapped inside the vesicular membrane.

4. In the absence of magnesium a heavy band was isolated which had a low bacteriochlorophyll *a*, phospholipid and succinate dehydrogenase content and high hexosamine content. Chromatophore-like material was isolated from this fraction by French pressure cell disruption followed by sucrose density centrifugation. Electron micrographs, and the ratio of bacteriochlorophyll *a* to phospholipid of the resulting fractions indicate that photosynthetically grown *R. rubrum* contains a bacteriochlorophyll *a*-deficient cytoplasmic membrane.

INTRODUCTION

The bacteriochlorophyll *a* content of photosynthetically grown *Rhodospirillum rubrum* and *Rhodopseudomonas spheroides* is an inverse function of light intensity

and specific growth rate[1]. Since all of the bacteriochlorophyll *a* in *R. rubrum* and *Rps. spheroides* is bound to membrane[2–6] there are two possible mechanisms for the relationship between specific growth and bacteriochlorophyll *a* content[1]: The quantity of "chromatophores"* of constant bacteriochlorophyll *a* composition may decrease with increasing specific growth rate, or the cell may contain a constant amount of "chromatophores" whose specific bacteriochlorophyll *a* content varies with specific growth rate, or both mechanisms may operate. Chemical and cytological investigations of *R. rubrum* have been reported which support both alternatives[2,7–10].

The bacteriochlorophyll *a* containing structures of *R. rubrum* can be separated into two distinct pigmented fractions. These fractions have been designated light and heavy according to their sedimentation characteristics in sucrose gradients[2]. The "light band" of *R. rubrum* has been extensively investigated[2,3] whereas the "heavy band" from *R. rubrum* has only recently been investigated by OELZE *et al.*[11] who characterized the large membrane material in Ficoll gradients and postulated that these components contained bacteriochlorophyll *a*-deficient cytoplasmic membrane. Similarly, experiments with the heavy band from *Rps. spheroides* suggest that it contains cell wall material[4] and a bacteriochlorophyll *a*-deficient cytoplasmic membrane[6,12].

The light and the heavy band from *R. rubrum* were isolated, purified and then characterized by physical and chemical methods. The bacteriochlorophyll *a* and total phosphorus content of the purified chromatophores were correlated with specific growth rates of *R. rubrum*. Both mechanisms postulated[1] for the inverse relationship between bacteriochlorophyll *a* content of whole cells and specific growth rate are shown to be operative in *R. rubrum*. Chemical characterization and electron microscopy of the isolated heavy band indicate that it is composed of cell wall and cytoplasmic membrane material.

The equilibrium densities of chromatophores, isolated from *R. rubrum* in density gradients made of solutes of different membrane permeability characteristics and different osmolalities were measured to determine the vesicular nature of *R. rubrum* chromatophores. Purified chromatophores were subjected to osmotic shock and sonication to investigate the effect of physical disruption on the structural integrity of chromatophores.

MATERIALS AND METHODS

R. rubrum (Strain S_1) was grown in 1-l Roux bottles in a water bath at 30° as previously described[3]. Illumination was provided by Sylvania superfloods maintained at 75 V (a.c.) with a variable voltage transformer. A Gossen Trilux light meter calibrated in ft candles was used to measure the light intensity incident on the culture flask. Light intensity was adjusted by altering the distance between the culture flask and the light source, or by interposing wire mesh screens between the light source and the water bath. When light intensities above 5000 ft candles were used, the voltage to the lamps was increased to 85 V(a.c.).

* Chromatophore is defined in this paper as the bacteriochlorophyll *a*-containing light band material isolated from cell-free extracts of *R. rubrum* and *Rps. spheroides*. Quotation marks around the term chromatophore indicate the photosynthetic membrane structure which exists in whole cells.

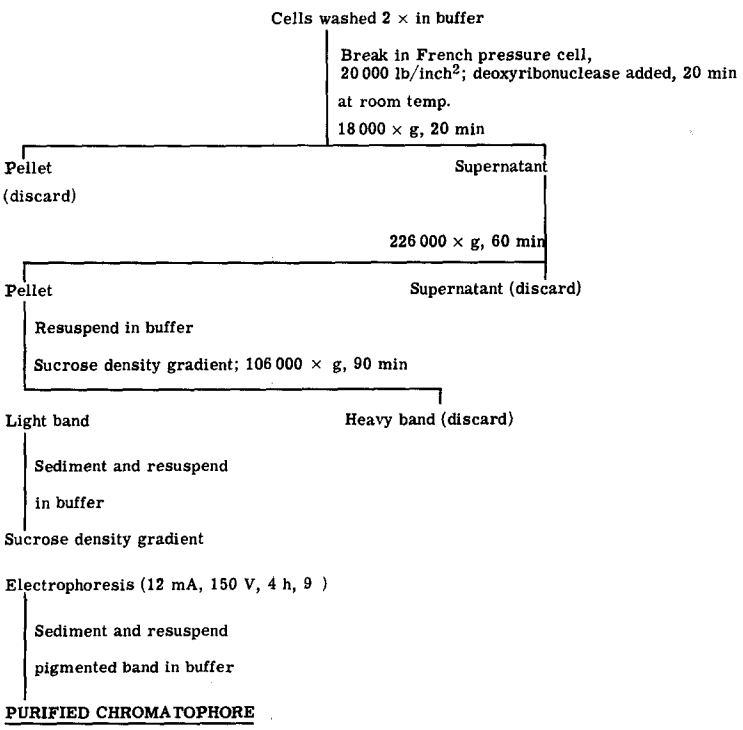
The increase in cellular dry mass of *R. rubrum* was measured at 680 nm and was a linear function of the absorbance between 0.000 and 0.450. All cultures were harvested during exponential growth (Table I) in order to insure that the cultures were in steady-state conditions. As evidence of a steady state, the differential rate of chlorophyll synthesis was determined to be constant prior to harvesting.

TABLE I

RELATIONSHIP BETWEEN LIGHT INTENSITY, SPECIFIC GROWTH RATE AND BACTERIOCHLOROPHYLL CONTENT OF *R. rubrum*

Light intensity (ft candles)	*Specific growth rate (h^{-1})*	*μg bacteriochlorophyll per mg dry wt.**	*Maximum mg dry wt. per ml culture** during balanced growth*
100	0.038	18.1	0.058
160	0.077	13.1	—
180	0.087	12.2	0.079
380	0.145	9.2	0.153
880	0.149	7.2	—
3 100	0.185	4.68	0.242
10 800	0.176	6.37	0.300

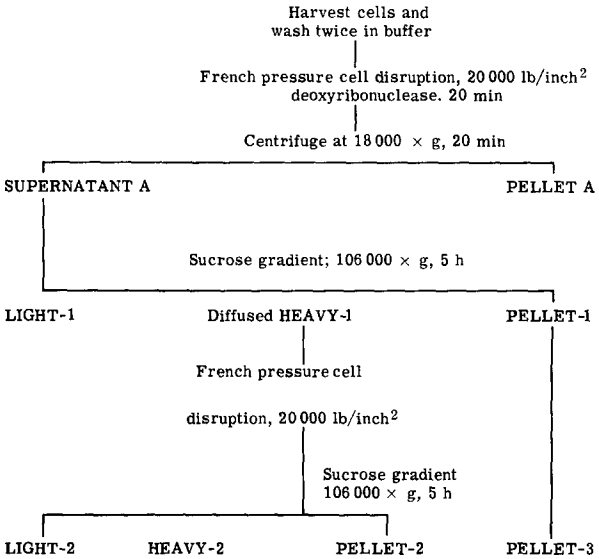
* Average of three determinations.
** Maximum cell density before self-shadowing (0.1 mg dry wt. per ml = 0.170, $A_{660\,nm}$).



Scheme 1. The isolation and purification of chromatophores from *R. rubrum*. Buffer: 0.02 M Tris·HCl (pH 8.1), 0.01 M $MgSO_4$. Sucrose density gradient: 0.8–1.7 M buffered sucrose; sample protein concn. < 1 mg/ml. Electrophoresis: protein concn. = 0.5 mg/ml of gradient.

Isolation and purification of chromatophores

Chromatophores were isolated and purified according to Scheme 1. Buffer containing 0.02 M tris(hydroxymethyl)aminomethane·HCl (pH 8.1) and 0.01 M $MgSO_4$ was used throughout. The electrophoretic apparatus described by HOLT AND MARR[13] was used for electrophoretic separations. All procedures were performed at 4°.



Scheme 2. The isolation of the diffused heavy band from *R. rubrum*. Buffer: 0.02 M Tris·HCl (pH 8.1). Sucrose gradient: 0.8–1.7 M buffered sucrose; < 1 mg protein per ml of gradient.

Isolation of the cell envelope "heavy band" fraction

The cell envelope (cell wall and cytoplasmic membrane) was isolated and purified according to the scheme presented in Scheme 2. Linear sucrose density gradients (55 ml) were centrifuged for 5 h in a Beckman SW 25.2 rotor in a Beckman Model L-2 ultracentrifuge equipped with a rotor stabilizer. Bands were collected by inserting a hypodermic needle through the side of the centrifuge tube or by collecting drops through a hole punctured just above the pellet in the bottom of the tube. The fractions were washed by slowly adding an equal volume of Tris buffer (0.02 M Tris–HCl (pH 8.1)) to the magnetically stirred solution. The material was then sedimented for 1 h at 226 000 × *g*. The sedimented material was resuspended in Tris buffer using a small magnetic stirrer. All procedures were performed at 4°. The distribution of protein and bacteriochlorophyll *a* during the isolation procedures is shown in Table II.

Electron microscopy

For negative staining, samples were placed directly on carbon-coated 300-mesh copper grids and stained with 1% (w/v) phosphotungstic acid (pH 6.8) for approx. 30 sec. All excess fluid was withdrawn with filter paper and the grid was air-dried for at least 30 min before examination in the electron microscope. All samples were examined in a Philips EM-200 electron microscope operating at 60 kV, equipped with

TABLE II

DISTRIBUTION OF PROTEIN AND BACTERIOCHLOROPHYLL DURING ISOLATION AND PURIFICATION

	Protein (mg)	*Bacterio-chlorophyll (μg)*	*μg bacterio-chlorophyll per mg protein*
(A) Purification of chromatophores			
Cell suspension*	—	880	—
18 000 × *g* supernatant	46.6	515	11.0
18 000 × *g* pellet	35.0	296	8.0
226 000 × *g* supernatant	23.2	—	—
226 000 × *g* pellet	21.5	445	20.6
Sucrose density gradient			
Light band	2.7	68.6	25.6
Heavy band	8.31	220.0	25.2
Electrophoretic band	1.93	44.2	23.0
(B) Isolation of the cell envelope fraction			
Cell suspension**	—	—	—
Supernatant A	1160	9583	8.25
Pellet A	79	367	4.64
Light-1	434	9100	21.3
Diffused heavy-1	53	640	9.7
Pellet-1	31	109	3.74

* 200 mg dry wt. suspended in 10 ml Tris–$MgSO_4$ buffer.
** 1.6 g dry wt. suspended in 30 ml Tris buffer.

a 10-objective aperture. Insertion of a liquid N_2 cold finger at the level of the specimen kept contamination at a minimum.

Sonication of chromatophores

Purified chromatophores were sonicated in a Raytheon 10-kcycles sonic oscillator at maximum output. The total volume in the cup was between 15 and 25 ml. The cup was gassed with nitrogen for at least 5 min prior to sonication in order to reduce the formation of free radicals[14].

Osmotic shock of chromatophores

Chromatophores were suspended in 3 M glycerol and stirred for 15–60 min at 4°. Glycerol was used because of its permeability characteristics[15]. The suspension was then rapidly expelled into 15 times its volume of rapidly stirring buffer.

Acrylamide gel electrophoresis

A Canalco model 6 or 66 disc electrophoresis apparatus with 5 mm × 50 mm gel columns was used for polyacrylamide gel electrophoresis. The gels were prepared in 35% acetic acid containing 4 M urea according to the procedure of TAKAYAMA *et al.*[16]. Samples containing between 120 and 170 μg protein in 20–40 μl were prepared in 8 M urea and layered on top of the gel column. Electrophoresis was run at a constant current of 6 mA per gel for 3 h at 4° with 10% acetic acid in both the upper and lower reservoir. The gels were removed from the glass tubes and stained with Amido Black for 1 h at room temperature as described by DAVIS[17]. Destaining was performed

electrophoretically in a Canalco disc apparatus at 12–15 mA per gel using 7% acetic acid as electrolyte.

Chemical assays

Bacteriochlorophyll a. Samples were extracted with acetone–methanol (7:2, v/v) and the absorption at 775 nm was measured in a Beckman DU-2 spectrophotometer[18]. Quantitative estimation of bacteriochlorophyll *a* was calculated using the extinction coefficient (75 $mM^{-1} \cdot cm^{-1}$) reported by CLAYTON[19]. The bacteriochlorophyll *a* content of whole cells was calculated using the extinction coefficient[20] (140 $mM^{-1} \cdot cm^{-1}$) after correcting the absorbance at 880 nm for light scattering[9].

Protein. Protein concentration was estimated using the Folin–Lowry method[21]. Samples containing glycerol were dialyzed before assay and samples containing Tricine (*N*-tris(hydroxymethyl)methylglycine (Calbiochem)) were run against a reagent blank containing an identical amount of Tricine due to the positive interference of these substances with the assay.

Total phosphorus. Samples were wet ashed in 5 M H_2SO_4 at 160° for 2 h. The samples were then oxidized with H_2O_2 for 90 min at 160° and assayed for inorganic phosphorus[23].

Phospholipid. All samples were washed by centrifugation to remove any soluble inorganic phosphorus. Samples containing between 0.9 and 4.3 μg bacteriochlorophyll *a* were extracted with 1 ml of chloroform–methanol (1:2, v/v), followed by centrifugation at 18000 × *g* for 20 min. The supernatant solution was removed and the pellet again extracted with chloroform: methanol (1:1, v/v). The supernatant fluids containing the extracted phospholipids were pooled and their inorganic phosphorus content was determined as described above. One mg of phosphorus was equated to 25 mg phospholipid[23].

Succinate dehydrogenase. Samples containing between 100 and 160 μg protein in a total volume of 1.0 ml were assayed in a Gilford spectrophotometer according to the procedure of MASSEY[24]. Specific activity is reported as the change in $A_{550\ nm}$ per h per mg protein.

Hexosamine. Samples containing between 3 and 4 mg protein were hydrolyzed in 2 M HCl at 100° for 15 h. The hexosamine content of the hydrolysate was determined by the method of BOAS[25], using glucosamine · HCl as the standard.

RESULTS

A distinct pigmented light and a heavy band were separated by sucrose density gradient centrifugation of cell-free extracts of *R. rubrum* which had been prepared by French pressure cell disruption in the presence of 0.01 M Mg^{2+}. The separation of these two fractions depended upon their sedimentation rates, and not entirely on their densities (Fig. 1). The heavy band reached an equilibrium density of 1.18 g/cm^3 in 2 h, while the light band did not reach its equilibrium density of 1.17 g/cm^3 until approx. 30 h.

Chemical characterization of chromatophores

The purified chromatophores were monitored by electron microscopy and shown to be relatively homogeneous in size and free of cellular debris such as flagella,

cell-wall components and ribosomes (Fig. 2). Purification, determined by comparing the specific bacteriochlorophyll *a* content of the chromatophores to that of the crude extract, varied from 2- to 6-fold depending on the specific growth rate at which the cells were grown (Table III).

The bacteriochlorophyll *a* and protein content of chromatophores isolated and purified from *R. rubrum* grown under balanced growth conditions were determined on at least three separately purified samples. With increasing specific growth rate (0.185–0.380 h^{-1}) the bacteriochlorophyll *a*/protein ratios of the purified chromato-

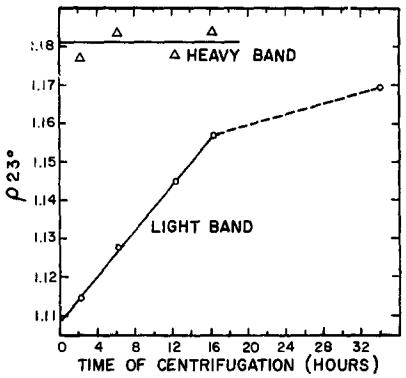


Fig. 1. Sedimentation characteristics of the pigmented components in cell-free extracts of *R. rubrum*. A 3-ml sample of crude extract was layered on a linear sucrose gradient (0.8–1.7 M sucrose in Tris–$MgSO_4$ buffer) and centrifuged for the time indicated at 75 000 × *g*. The density of the sample was determined in a tared 1-ml specific gravity bottle at 25° after the pigmented bands were removed with a syringe.

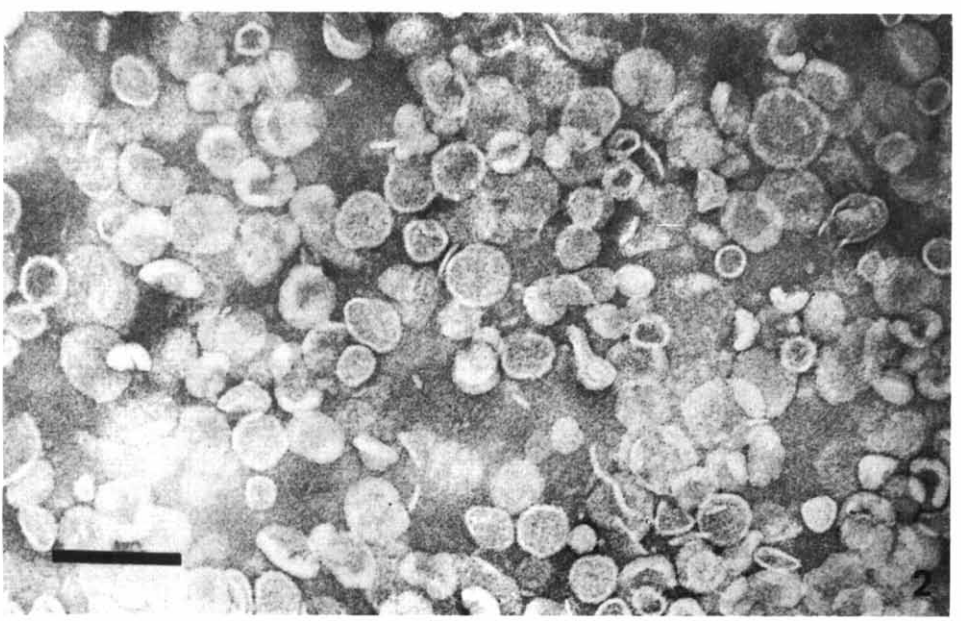


Fig. 2. Electron micrograph of purified chromatophores negatively stained with 1% phosphotungstic acid. The bar represents 200 nm.

TABLE III

EFFECT OF LIGHT INTENSITY AND SPECIFIC GROWTH RATE ON THE BACTERIOCHLOROPHYLL CONTENT OF CRUDE EXTRACTS AND PURIFIED CHROMATOPHORES

Specific growth rate (h^{-1})	*Light intensity* (*ft candles*)		*Expt. No.* 1	2	3	*Average*
0.038	100	Crude extract*	11.7	10.3	12.3	11.4
		Chromatophores*	23.4	24.8	23.1	23.6
0.087	180	Crude extract	8.5	10.5	10.4	9.8
		Chromatophores	21.4	25.7	28.6	25.2
0.146	380	Crude extract	9.5	8.0	6.7	8.1
		Chromatophores	23.1	22.7	22.0	22.9
0.180	3 100	Crude extract	4.1	4.5	6.4	5.0
		Chromatophores	20.9	21.1	21.4	21.1
0.176	10 000	Crude extract	3.9	3.7	4.1	3.9
		Chromatophores	22.3	26.4	25.6	24.7

* μg bacteriochlorophyll per mg protein.

phores remained essentially constant at 23 μg bacteriochlorophyll *a* per mg protein, while the bacteriochlorophyll *a*/protein ratio of the cell-free extract decreased approx. 3-fold from 11.4 to 3.89 μg bacteriochlorophyll *a* per mg protein (Table III) supporting the hypothesis that the inverse relationship between the specific growth rate and the bacteriochlorophyll *a* content of whole cells (Fig. 3) results from a change in the amount of "chromatophore" membrane of constant bacteriochlorophyll *a* composition.

When the growth rate of a culture growing at 400 ft candles decreases as the culture enters the stationary phase, the bacteriochlorophyll *a* content of the chromatophores increases by as much as 100% (Table IV). This was demonstrated by transferring cultures at 0.3 mg dry wt. per ml and allowing them to grow for 24 and 48 h. The bacteriochlorophyll *a* content of the chromatophores isolated from these cells increased to 36 μg bacteriochlorophyll *a* per mg protein at 24 h and to a maximum

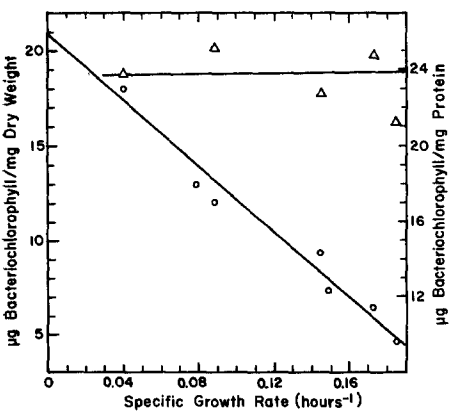


Fig. 3. The effect of specific growth rate of *R. rubrum* on the bacteriochlorophyll *a* content of whole cells and purified chromatophores. O—O, μg bacteriochlorophyll *a* per mg cellular dry wt.; △—△, μg bacteriochlorophyll *a* per mg chromatophore protein.

TABLE IV

EFFECT OF GROWTH PHASE ON THE RATIO OF BACTERIOCHLOROPHYLL/PROTEIN OF PURIFIED CHROMATOPHORES FROM *R. rubrum*

Growth conditions	*μg bacteriochlorophyll per mg protein*	*% Increase over balanced growth value*
Balanced growth	23.0*	—
Stationary (24 h)	36.0	57
	36.5	59
Stationary (48 h)	42.0	82
	47.0	102

* Average of six determinations.

of 47 μg bacteriochlorophyll *a* per mg protein at 48 h. This demonstrates that the cell has the capability of incorporating varying amounts of bacteriochlorophyll *a* into the chromatophore membrane, as much as twice the amount of bacteriochlorophyll *a* incorporated into the membrane when the cells are under balanced growth conditions.

The yield of purified chromatophore that is obtained from cells grown under balanced growth is extremely low; therefore, total phosphorus was used as an indicator of membrane phospholipid. Since the purified chromatophores are free of ribosomes (Fig. 2), and by using the RNA values reported by WORDEN AND SISTROM[4], the contaminating RNA should comprise less than 5% of the lowest phosphorus value reported here. Therefore, measuring the total phosphorus content of purified chromatophores is probably a good approximation of lipid-phosphorus content of purified samples.

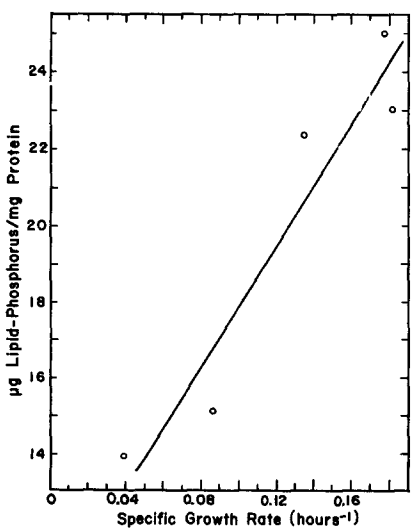


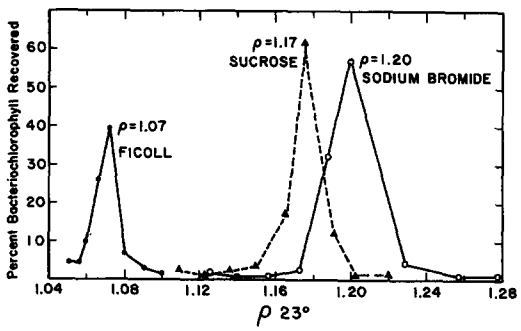
Fig. 4. The relationship between "lipid-phosphorus" content of purified chromatophores and the specific growth rate of *R. rubrum*. The values for lipid-phosphorus are an estimate which employs the assumption that 1 μg total phosphorus is equal to 25 μg lipid phosphorus.

TABLE V

EFFECT OF LIGHT INTENSITY AND SPECIFIC RATE ON THE LIPID-PHOSPHORUS* CONTENT OF PURIFIED CHROMATOPHORES

Specific growth rate (h^{-1})	*Light intensity* (*ft candles*)		*Expt. No.* 1	2	3	*Average*
0.038	100	Lipid-P/protein**	10.9	16.8	—	13.8
		Lipid-P/bacteriochlorophyll***	0.464	0.729	—	0.587
0.087	180	Lipid-P/protein	15.6	16.6	13.1	15.1
		Lipid-P/bacteriochlorophyll	0.609	0.912	0.456	0.659
0.146	380	Lipid-P/protein	18.5	20.1	27.7	22.3
		Lipid-P/bacteriochlorophyll	0.797	0.912	1.30	0.999
0.180	3 100	Lipid-P/protein	25.7	16.4	26.9	23.0
		Lipid-P/bacteriochlorophyll	1.23	0.771	1.27	1.09
0.176	10 000	Lipid-P/protein	32.9	22.9	19.2	25.0
		Lipid-P/bacteriochlorophyll	1.03	1.25	0.745	1.01

* Lipid-P = total P content of chromatophores corrected to lipid-P. See Fig. 4.
** μg/mg.
*** μg/μg.



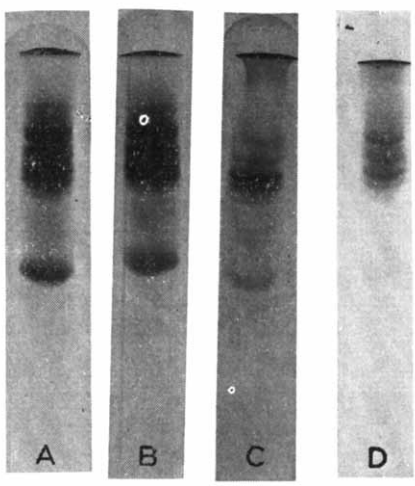


Fig. 5. Equilibrium density of chromatophores in NaBr, sucrose and Ficoll gradients. The light band chromatophore fraction was washed three times in Tris–$MgSO_4$ buffer and then a portion was suspended in 6.5 ml of 0.8 M sucrose and 6.5 ml of 1.7 M sucrose in Tris–$MgSO_4$ buffer. The density gradients were then formed with a Buchler gradient maker. Ficoll gradients were made as above from 10% and 30% purified Ficoll solutions in Tris–$MgSO_4$ buffer. Isopycnic gradients of NaBr were prepared by adding 24% NaBr (w/v) to a chromatophore suspension in Tris–$MgSO_4$ buffer. Each gradient contained approx. 2 μg bacteriochlorophyll *a*. The gradients were centrifuged at 40 000 rev./min in a Beckman SW 41 rotor for 36 h at 4°. Fractions were collected by puncturing the bottom of the centrifuge tube with a hypodermic needle and collecting 1.3-ml fractions. The bacteriochlorophyll *a* concentration was determined by measuring the absorption at 880 nm and the density of at least three fractions (top, pigmented band and bottom) were determined using a tared 1-ml specific gravity bottle.

Fig. 6. Acrylamide gels of the soluble components released from purified chromatophores by sonication and osmotic shock. A. 5 min of sonication, 148 μg protein. B. 10 min of sonication, 172 μg protein. C. 15 min of sonication, 143 μg protein. D. Osmotic shock, 174 μg protein.

The total phosphorus content of purified chromatophores is a linear function of the specific growth rate (Fig. 4). From the data in Table V the correlation coefficient of 0.66 was calculated ($P \leqq 2\%$) indicating that the lipid-phosphorus content of the chromatophore membranes is a direct function of the specific growth rate of *R. rubrum*.

Physical characterization of chromatophores

The equilibrium densities of chromatophores in sucrose, Ficoll and NaBr are shown in Fig. 5. As predicted by the theory governing the effect of gradient solutes on the density of vesicular structures[26], chromatophores exhibit their lowest density in the high molecular weight, non-permeable solute Ficoll, their highest density in the permeable solute NaBr and an intermediate density in the low molecular weight, non-permeable solute, sucrose. Thus, the equilibrium density of the chromatophore membrane is approx. 1.20 g/cm^3 (the chromatophore's density in NaBr) since NaBr is permeable to the membrane and its hydration effects are minimal. In sucrose, the chromatophores appear to be plasmolyzed by the high osmolality of the solute and band at an intermediate equilibrium density of 1.17 g/cm^3. This density is similar to the equilibrium densities observed in sucrose for chromatophores isolated from Chromatium[27] and for membranes isolated from Mycoplasma[28]. Since osmotic pressure is a colligative property of solutions, the osmotic pressure exerted on a vesicular structure by a solution of Ficoll of mol. wt. 400 000 is approx. 1000 times less than the osmotic pressure exerted by a solution containing the same concentration of sucrose. The interior volume of the chromatophore appears to be maintained in Ficoll solutions, presumably because of the low osmotic pressure, and the observed equilibrium density is correspondingly low, 1.08 g/cm^3.

To test the hypothesis that plasmolysis occurs when the osmolality of the gradient is increased, sucrose was added to Ficoll density gradients (Table VI). Ficoll gradients were prepared as described, however, sucrose was added in equal concentrations to the 10% and 30% Ficoll–chromatophore solutions. The equilibrium density of chromatophores in Ficoll gradients increased with increasing sucrose concentration (Table VI). This effect is interpreted to be the result of an increased plasmolysis of the chromatophore vesicle with increasing concentrations of sucrose, which results in a corresponding increase in equilibrium density.

TABLE VI

EQUILIBRIUM DENSITIES OF CHROMATOPHORES IN FICOLL GRADIENTS IN THE PRESENCE OF SUCROSE

Ficoll gradients were made as described for Fig. 5, with the exception that sucrose was added to the 10 and 30% Ficoll–chromatophore suspensions.

Sucrose (%)	*Density* (*g/cm*^{3})		
	Expt. 1	*Expt. 2*	*Average*
0	1.082	1.084	1.083
5	1.104	1.103	1.103
15	1.120	1.130	1.125
25	1.157	1.146	1.151

Effect of sonication and osmotic shock on chromatophores

The release of protein from the purified chromatophores during a drastic method of rupture, sonication, and a mild method, osmotic shock were compared in order to determine the structural stability of chromatophore protein. Soluble protein is defined as that protein not sedimented by centrifugation at 226 000 × *g* for 1 h. Succinate dehydrogenase activity was determined as an indicator of membrane bound protein[29] and acrylamide gel electrophoresis was used to estimate the number of proteins released.

The results of sonication of purified chromatophores are reported in Table VII. The amount of protein and succinate dehydrogenase released increased rapidly during the first 10 min and then leveled off, while the total succinate dehydrogenase activity remained constant until 20 min of sonication, after which there was a marked decrease in this activity in both the soluble and sedimentable samples. The release of soluble protein indicates that there is a finite amount of membrane protein, approx.

TABLE VII

THE RELEASE OF SOLUBLE PROTEIN AND SUCCINATE DEHYDROGENASE FROM PURIFIED CHROMATOPHORES DURING SONICATION

Time (min)	*Soluble protein** (%)	*Total succinate dehydrogenase activity in supernatant* (%)	*Specific activity succinate dehydrogenase in supernatant*	*Specific activity succinate dehydrogenase in pellet*
0	7.9	3.83	9.9	19.5
2	10.6	3.73	7.05	19.2
4	13.2	9.30	12.3	18.2
6	13.5	8.53	11.8	20.0
8	17.5	16.20	18.3	20.4
10	24.0	23.60	18.1	18.3
15	24.1	26.10	20.1	18.7
20	25.0	27.60	21.7	19.0
25	25.6	26.00	13.0	12.8
30	27.5	27.70	13.0	12.9

* Initial concn.: 20 mg chromatophore protein per 20 ml. Assay performed on 1-ml samples.

TABLE VIII

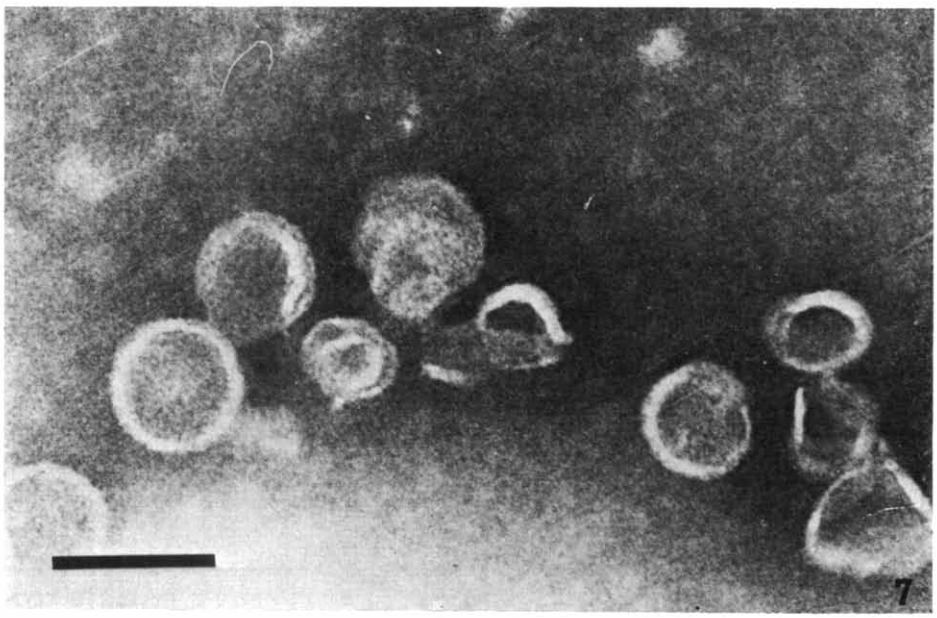
THE RELEASE OF SOLUBLE PROTEIN AND SUCCINATE DEHYDROGENASE FROM PURIFIED CHROMATOPHORES BY OSMOTIC SHOCK

The initial protein concentration was 2.0 mg protein in each experiment. 0.02 M Tricine buffer (pH 8.1) was used throughout.

Experiment	*Protein released corrected for control** (%)	*Specific activity succinate dehydrogenase*	
		Supernate	*Pellet*
(I) Chromatophores into Tricine buffer[3]	12.0	1.2	19.2
(II) Chromatophores diluted with Tricine buffer[3]	13.9	0.8	33.6

* The amount of protein solubilized without the osmotic shock treatment was 2.3% of the total protein.

30%, which can be released from the chromatophore by sonication under these conditions. The remaining 70% of the membrane protein appears to be bound to the sedimentable lipid portion of the membrane, which sediments at 226 000 × *g*. No



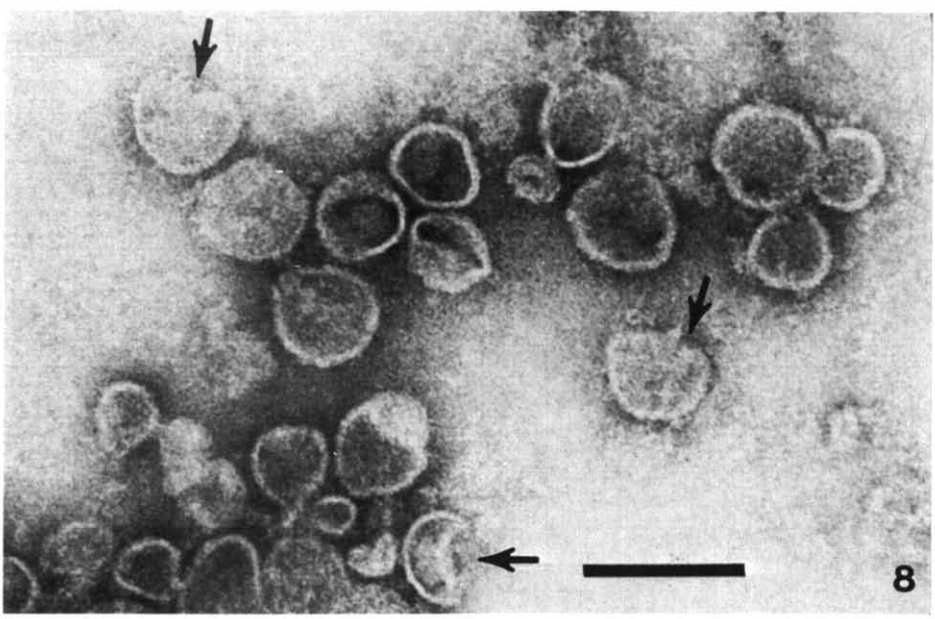


Fig. 7. Electron micrograph of purified chromatophores which were sonicated for 60 min and then negatively stained with 3% phosphotungstic acid. The bar represents 100 nm.

Fig. 8. Electron micrograph of osmotically shocked chromatophores (arrows) negatively stained with 1% phosphotungstic acid. The bar represents 100 nm.

bacteriochlorophyll *a* was released during 30 min of sonication and the initial concentration of bacteriochlorophyll *a* was equal to the bacteriochlorophyll *a* concentration in the pellet after 30 min of sonication.

The release of protein from chromatophores by osmotic shock (Table VIII) demonstrates that chromatophores are osmotically sensitive. Approximately the same amount of protein was released from the chromatophore–glycerol suspension when it was rapidly expelled into buffer or when the buffer was added to the stirring chromatophore–glycerol suspension.

The major differences between osmotic shock and sonication are that approximately twice as much protein and considerably more succinate dehydrogenase were released during 30 min of sonication than after osmotic shock, and acrylamide gel electrophoresis of the resulting supernatants revealed more protein bands in the sonicated samples than in the osmotic shock samples. However, even after sonication or osmotic shock treatment, the chromatophores maintained their basic morphological characteristics (Figs. 7, 8).

TABLE IX

CHEMICAL CHARACTERIZATION OF THE DIFFUSED HEAVY BAND ISOLATED FROM *R. rubrum*

Fraction	*μg bacteriochlorophyll per mg protein*	*Succinate dehydrogenase specific activity*	*μg phospholipid per μg bacteriochlorophyll*	*μg hexosamine per mg protein*
Supernatant A	8.25	1.12	—	—
Pellet-A	4.64	2.89	15.6	20.8
Light-1	21.3	8.13	7.76	trace
Heavy-1	9.75	2.69	9.9	31.2
Pellet-1	3.74	2.00	15.7	32.8
Light-2	22.6	6.64	6.5	8.65
Heavy-2	3.92	2.17	13.3	36.0
Pellet-2	0.80	1.08	47.8	40.0
Pellet-3	1.66	1.75	31.9	55.0

TABLE X

EFFECT OF MAGNESIUM ON THE BACTERIOCHLOROPHYLL CONTENT OF THE HEAVY BAND

Isolation buffer	*μg bacteriochlorophyll per mg protein*			*Average*
Expt. No.:	*1*	*2*	*3*	
0.02 M Tris–0.01 M $MgSO_4$	26.3	23.6	31.6	27.2
0.02 M Tris	7.4	5.3	4.4	5.7

Isolation and characterization of the "heavy band"

Omission of Mg^{2+} from the isolation and purification procedure (Scheme 2) markedly changed the sedimentation characteristics of the bacteriochlorophyll *a* containing components in cell-free extracts. The light band chromatophore material appeared to be unchanged in its density (1.129 g/cm^3), bacteriochlorophyll *a* content (Table X) and morphology from the light band material isolated in the presence of

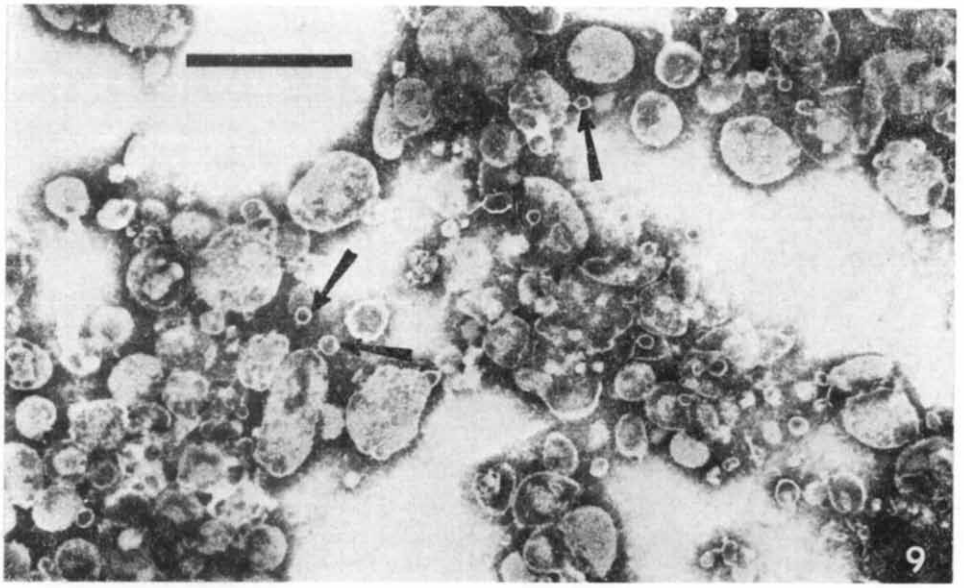


Fig. 9. Electron micrograph of the diffused heavy-1 fraction negatively stained with 1% phosphotungstic acid. The bar represents 500 nm. At arrows: chromatophores.

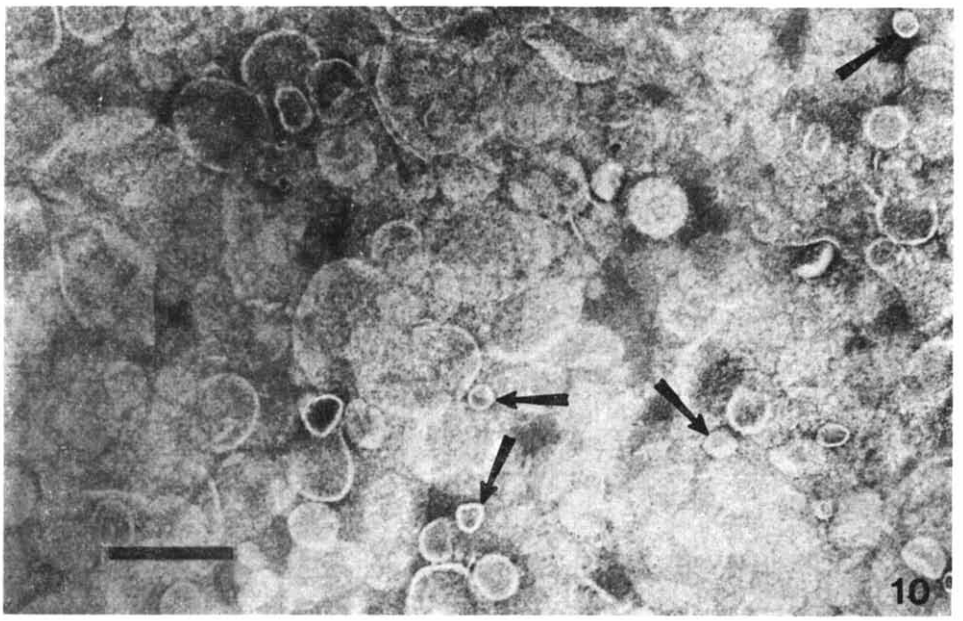


Fig. 10. Electron micrograph of the diffused heavy-2 fraction negatively stained with 1% phosphotungstic acid. The bar represents 240 nm. At arrows: chromatophores.

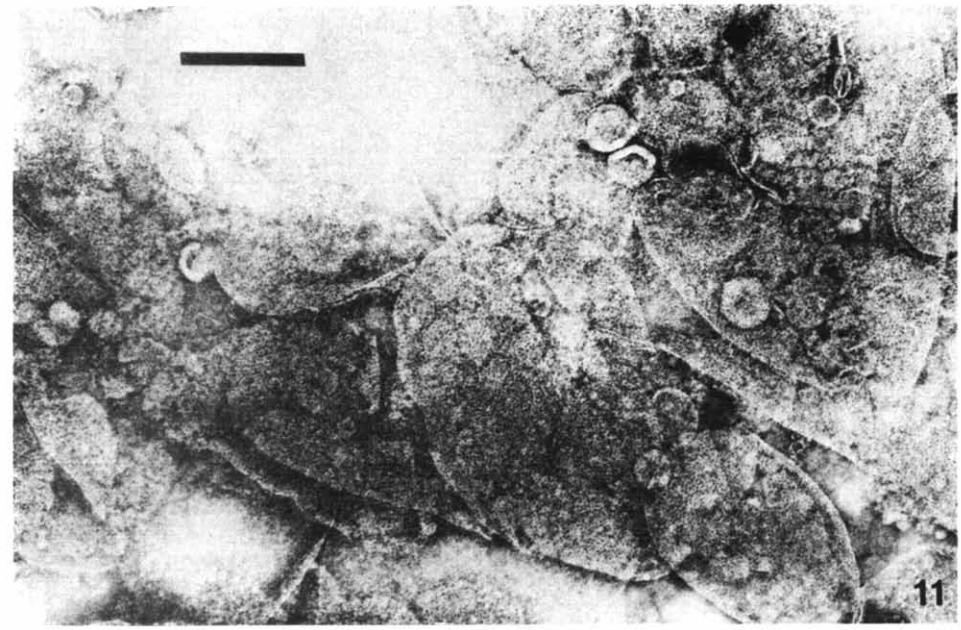


Fig. 11. Electron micrograph of the pellet-1 fraction negatively stained with 1% phosphotungstic acid. Fragments of cell wall and cytoplasmic membrane are present. Bar represents 25 nm.

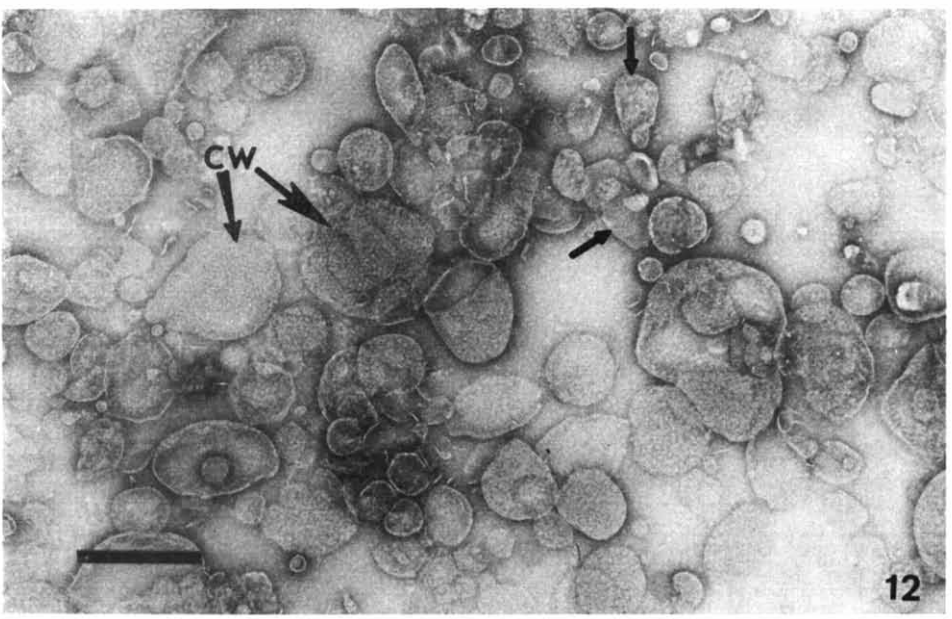


Fig. 12. Electron micrograph of the pellet-2 fraction negatively stained with 1% phosphotungstic acid. The fine structure on the cell wall (CW) fragments and the cytoplasmic membrane (small arrows) fragments can be seen. The bar represents 250 nm.

Mg^{2+}. However, the heavy band was markedly changed when Mg^{2+} was omitted. This material (diffused heavy band) had a decreased bacteriochlorophyll *a* content (Table X), was non-light-scattering and was distributed from the bottom of the gradient to just below the light band.

This diffused heavy band material was isolated and physically characterized by French pressure cell disruption and sucrose gradient centrifugation according to the scheme presented in Scheme 2. The distribution of protein, hexosamine, succinate dehydrogenase, bacteriochlorophyll *a*, and phospholipid is presented in Table XI. The high bacteriochlorophyll *a* content and the low hexosamine content in light-2 indicate that some chromatophore-like material is released from heavy-1 by French pressure cell disruption. The high hexosamine content in the diffused heavy-2 fraction indicates that this fraction contains most of the cell-wall material and some membrane material as indicated by the 28% phospholipid content. The pellet-2 fraction appears to be composed mainly of cell-wall material as indicated by its relatively low content of all components except hexosamine and protein.

TABLE XI

CONVERSION OF THE DIFFUSED HEAVY FRACTION INTO A LIGHT AND A PELLET FRACTION

Fraction	*Hexosamine* (%)	*Succinate dehydrogenase* (%)	*Bacterio-chlorophyll* (%)	*Phospholipid* (%)	*Protein* (%)
Diffused heavy-1*	100	66.5	100	100	100
Light-2	8.3	67.7	67.3	66.0	23.0
Diffused heavy-2	67.0	27.5	21.1	28.3	57.0
Pellet-2	24.0	2.9	1.4	6.1	19.0

* Samples containing 20.0 mg protein and 190 μg bacteriochlorophyll were separated on 55-ml sucrose density gradients.

By expressing the bacteriochlorophyll *a* content of the different fractions as the ratio of bacteriochlorophyll/protein (Table IX), it is apparent that the light-1 and light-2 fractions are greatly enriched in bacteriochlorophyll *a* over supernatant-A. Some of the increase in the bacteriochlorophyll *a*/protein ratio of light-1 and heavy-1 can be attributed to the removal of soluble protein in the supernatant-A fraction by density gradient centrifugation. Both the heavy-2 and the pellet-2 fractions show a decrease in bacteriochlorophyll *a* content after disruption of the heavy-1 fraction. This datum indicates that a bacteriochlorophyll-rich component, probably chromatophores, is being released from the diffused heavy-1 fraction. Similarly, the high ratio of hexosamine/protein in the heavy-1, heavy-2, pellet-1, pellet-2 and pellet-3 fractions indicates that these fractions contain cell-wall material. The high bacteriochlorophyll *a* content and succinate dehydrogenase activity and the low hexosamine content of the light fractions imply that these fractions are composed mainly of chromatophores. Also, the morphology of the membranes in the light fractions was almost identical to that shown for purified chromatophores (Fig. 2).

Since phospholipid and bacteriochlorophyll *a* have been localized only in cell membranes, the ratio of these two components in the fractions isolated from the diffused heavy band was measured to distinguish membranes of different bacteriochlorophyll *a* compositions. The ratio of phospholipid/bacteriochlorophyll *a* in the

chromatophores (light-1) is 7.76, compared to 15.7 and 47.8 in pellet-1 and pellet-2, respectively and 9.9 and 13.3 in the heavy-1 and heavy-2, respectively, supporting the concept that both the heavy and the pellet fractions contain a bacteriochlorophyll *a*-deficient membrane component as compared to the light-1 fraction.

Electron micrographs of the heavy-1 and heavy-2 fractions show large membrane-like structures measuring approx. 400 nm which are contaminated with chromatophore structures (Figs. 9, 10). The pellet fractions (Figs. 11, 12) contained two distinct membrane-like structures, a smooth surfaced membrane-type structure and a rough surfaced cell-wall-like structure. Chromatophore structures were noticeably absent in the pellet fractions.

DISCUSSION

The distinct "heavy" band isolated in the presence of Mg^{2+} from cell-free extracts of *R. rubrum* is probably the result of aggregation of chromatophore membranes and cell envelope materials. This hypothesis is supported by the observations that the distinct "heavy" band sediments at a faster rate than the "light" band, is absent when Mg^{2+} is omitted and is readily dissociated by 30 sec of sonication into a slower-moving component. Furthermore, the bacteriochlorophyll *a* content of the distinct "heavy" band is higher than the diffused heavy band isolated in the absence of Mg^{2+}. Similar aggregation phenomena have been attributed to Mg^{2+} on both smooth and rough microsomes[30]. GIBSON[31] has also demonstrated that divalent cations cause aggregation of *Rps. spheroides* chromatophores. The addition of Mg^{2+} to the isolation buffer may be a valid procedure in order to obtain the distinct separation of contaminating materials from the "light" band and to stabilize ribosomes. However, the distribution of bacteriochlorophyll *a* in density gradients and the resulting densities of the components involved must be interpreted with great care when Mg^{2+} is present. The observation was made that the bacteriochlorophyll *a*/protein values obtained for the distinct heavy band isolated in the presence of Mg^{2+} were consistently higher than those obtained by an identical assay procedure for the purified light band material. This inconsistency may be due to the inability of the protein assay to completely hydrolyze the protein in the aggregate found in the distinct heavy band.

During balanced growth conditions, the specific bacteriochlorophyll *a* content of *R. rubrum* cells is an inverse linear function of the specific growth rate[1]. However, the specific bacteriochlorophyll *a* content of the purified chromatophores is constant at 23 μg bacteriochlorophyll *a* per mg protein over the range of specific growth rates studied (0.038–0.180 h^{-1}) and therefore independent of the specific growth rate when chromatophores are isolated from cells grown under balanced growth conditions. This constancy in the bacteriochlorophyll *a* content of isolated chromatophores varies when chromatophores are isolated from cells in the stationary phase of growth. Under the latter conditions, the bacteriochlorophyll *a* content of chromatophores increases to 42–47 μg bacteriochlorophyll *a*/mg protein. The bacteriochlorophyll *a* contents of the purified chromatophores reported here are within the range reported by previous investigators (Table XII) when these values are corrected using the extinction coefficient for bacteriochlorophyll *a* reported by CLAYTON[19].

This datum indicates that under balanced growth conditions the bacteriochlorophyll *a* content of the whole cells is dependent on the regulation of the amount of

TABLE XII

COMPARISON OF THE BACTERIOCHLOROPHYLL* CONTENT OF CHROMATOPHORES FROM *R. rubrum*

Chromatophores prepared by	*Low light (100 ft candles)*	*High light (2000–3000 ft candles)*	*Extreme light (6000–10000 ft candles)*	*Shadowed (48 h)*
COHEN-BAZIRE AND KUNISAWA[2]	30.9	12.5	—	—
HOLT AND MARR[9]	46.7	37.9	23.9	—
KETCHUM AND HOLT	23.6	21.4	24.7	45.0

* Results are given as μg bacteriochlorophyll, using the extinction coefficient of CLAYTON[19] per mg protein.

chromatophore material of constant bacteriochlorophyll content, a conclusion which is supported by electron microscopic observation of whole cells[7–9] and by the chemical data presented by HOLT AND MARR[9]. A possible explanation for the increase in the bacteriochlorophyll *a* content of chromatophores isolated from cells in the stationary phase of growth is that bacteriochlorophyll *a* and chromatophore membranes are synthesized at equal rates (balanced growth) determined by some regulatory compound (see SISTROM[32]) whose level in the cell is a function of the specific growth rate. When the cells are in the stationary phase of growth, limitation of precursors for membrane synthesis (proteins, lipids, carbohydrates) may affect the synthesis of membranes before the synthesis of bacteriochlorophyll *a*. This explanation may account for both the constant bacteriochlorophyll *a* content of chromatophores under balanced growth conditions and the increased bacteriochlorophyll *a* content of chromatophores when the cells reach the stationary phase of growth. It does not rule out the correlation between protein synthesis and photopigment synthesis[33,34] since protein turnover or synthesis probably takes place in stationary phase cultures.

Our approximation of the lipid-phosphorus content of purified chromatophores (total inorganic phosphorus) indicates that the lipid-phosphorus content of chromatophore membrane is a direct linear function of the specific growth rate. This is in direct contrast to the relationship between bacteriochlorophyll *a* content and specific growth rate. Recently STEINER *et al.*[35] reported a similar phenomenon by showing that the total phospholipid content of whole cells of Chromatium increased with increasing light intensity. One may conclude that there is no single mechanism which controls the composition of chromatophores, but instead that chromatophores are dynamic structures whose synthesis and chemical composition depend on the environment in which the cells are grown.

The vesicular nature of chromatophores isolated from *R. rubrum* has been indicated by observations that chromatophores can form pH gradients across their membranes[36–38] and that they can preferentially bind dyes on the inside of their membranes[39]. The response of isolated chromatophores to density gradient solutes of differing osmotic and permeability characteristics also indicates that chromatophores are vesicular structures. Since the chromatophore membrane is theoretically permeable to NaBr (ref. 26), the density of chromatophore membranes in NaBr (1.20 g/cm^3) is probably the best approximation of the membrane's true density. The behavior of chromatophores in sucrose, Ficoll and sucrose–Ficoll mixtures conforms to the theo-

retical behavior of vesicular structures in these solutes[26]. It is interesting to note that the permeability characteristics of chromatophore membranes with respect to sucrose are similar to those of the grana membranes of chloroplasts. The grana membranes are permeable to sucrose, whereas the outer membrane is impermeable to sucrose[40].

Our sonication experiments of chromatophores revealed three interesting observations: (a) the amount of solubilized protein appears to approach 30% of the total protein, (b) the photopigments are not normally solubilized during sonication, and (c) the morphology of the sonicated membrane is not drastically altered. These results are not inconsistent with those of FRENKEL[41], who demonstrated the release of phosphorylating particles from chromatophores during sonication, since particles the size of those obtained by FRENKEL would sediment under the conditions of centrifugation employed in these experiments (224000 × *g*, 1 h). A comparison of acrylamide gels of the acid-soluble (run in 10% acetic acid) proteins released by osmotic shock and sonication from purified chromatophores showed that fewer proteins are released by osmotic shock than by sonication. These results are supported by the observation that succinate dehydrogenase was not released, or released in very small quantities by osmotic shock, whereas 27% of the initial succinate dehydrogenase content of the chromatophores was released by sonication. Since chromatophores are formed by invagination of the cytoplasmic membrane[3,8] and since the periplasmic region of certain bacteria has been shown to contain soluble proteins (see review by HEPPEL[42]) it is possible that the soluble proteins of the periplasmic region are incorporated into the interior of the chromatophore vesicle at the time of formation. Theoretically, these soluble proteins may be released preferentially by osmotic shock, whereas membrane-bound proteins (succinate dehydrogenase), as well as the soluble matrix proteins, may be released by sonication.

The heavy band from *Rps. spheroides* isolated in the absence of Mg^{2+} was postulated to contain cytoplasmic membrane which was shown to have a low bacteriochlorophyll *a* content[6,12]. The heavy band isolated from *R. rubrum* in the absence of Mg^{2+} also appears to contain a bacteriochlorophyll *a*-deficient cytoplasmic membrane as well as cell-wall components. Distinct separation between chromatophores, cell-wall material and the cytoplasmic membrane was not attained, however, experiments with heterotrophically grown *R. rubrum*[43,44] have shown that the cytoplasmic membrane sediments in a position corresponding to the heavy band from photosynthetically grown cells, suggesting that both photosynthetically grown *Rps. spheroides* and *R. rubrum* contain a bacteriochlorophyll *a*-deficient membrane. Recently, OELZE *et al.*[11], using Ficoll gradients, isolated a fraction from both photosynthetically and heterotrophically grown *R. rubrum* which they called cytoplasmic membrane. The size of the membranes in this fraction is smaller than the size of chromatophores, an observation which does not conform to our results concerning the size of the membranes found in the diffused heavy band. However, one would expect that large vesicular membrane fragments would be found at a lower density than chromatophores in Ficoll gradients.

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