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Membranes of *Rhodopseudomonas spheroides*: Interactions of Chromatophores with the Cell Envelope

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Under carefully controlled ionic conditions, large-scale preparations of highly purified chromatophores and cell envelopes were obtained from phototrophically grown *Rhodopseudomonas spheroides* by zonal ultracentrifugation. The majority of the bacteriochlorophyll a was located in a single, discrete chromatophore band, whereas the envelopes were nearly devoid of photopigment. The envelope fraction contained substantial quantities of succinic dehydrogenase and cytochromes, confirming that phototrophically grown cells contain a photopigment-deficient cytoplasmic membrane. Magnesium at concentrations of 1.0 mM or higher caused chromatophores to reversibly aggregate with the cell envelope. Significant aggregation was also promoted by other divalent metals ($Co^{2+} > Mn^{2+} > Ca^{2+} > Mg^{2+}$), but aggregation was less extensive with monovalent cations. These results account for the distribution of photopigments in two bands reported by others and further suggest that the photosynthetic apparatus of *R. spheroides* is located on membranes largely distinct from the cell wall-cytoplasmic membrane complex.

Through density gradient ultracentrifugation, Gibson (9) and Niederman and Gibson (23) have isolated virtually all of the photosynthetic pigment from extracts of *Rhodopseudomonas spheroides* in a single, rather homogeneous chromatophore band whose sedimentation coefficient was 160*S*. In sucrose gradients, it was also possible to isolate a more rapidly sedimenting fraction that was identified as material of cell envelope origin nearly devoid of photopigment (23). In contrast, Worden and Sistrom (31) observed two pigmented bands in sucrose gradients of *R. spheroides* extracts; these bands were termed "light" and "heavy" chromatophores. From the characterization of these fractions, they concluded that the photosynthetic apparatus has two distinct forms within the cell (31). Since the gradients observed by Worden and Sistrom (31) contained 0.01 M magnesium, whereas magnesium was excluded from those observed by Niederman and Gibson (23), it seemed possible that the different results could be explained by magnesium-induced aggregation between light chromatophores and the cell envelope.

In this communication, the further characterization of isolated cell envelope and chromatophore fractions is reported. In addition, it is shown in resolution and reconstitution studies that the bulk of the photopigment found in heavy chromatophores results from the addition of high concentrations of ionic species during the isolation procedure.

MATERIALS AND METHODS

R. spheroides NCIB 8253 was phototrophically grown as described previously (9). Aerobic growth was carried out on a gyrotory shaker at 250 rpm for 24 h in 2.8-liter Fernbach flasks containing 500 ml of medium (5, 9). In some experiments, an alternative procedure (24) was employed for aerobic growth.

Cell-free extracts were prepared by passage through a French pressure cell and differential centrifugation (23). Subcellular particles were isolated from the 156,000 × *g* precipitate on sucrose density gradients in a Beckman SW27 rotor (23) or by zonal ultracentrifugation as described previously (24), except that the latter procedure was carried out in a Beckman Ti-14 rotor that was centrifuged at 40,000 rpm for 65 min. Gradients were collected by displacement, and the positions of the bands in the gradients were determined as described previously (23).

Dry weights were obtained as previously described (24). Protein was determined by the method of Lowry et al. (21), with crystalline bovine serum albumin as a standard. All samples were held at 40 C for 60 min in 0.5 N NaOH prior to the color reaction (28). Bacteriochlorophyll a (BCHL) was determined by acetone-methanol (7:2, vol/vol) extraction by using the method of Cohen-Bazire et al. (5) and the extinction coefficient of Clayton (2). Lipid was extracted by the method of Folch et al. (7), and the extracts were dried

and weighed as described previously (24). Total phosphorus was determined by the method of Chen et al. (1) after ashing of samples as described by LePage (20). Carbohydrate was determined by the phenol-sulfuric acid procedure of Dubois et al. (6). Total nucleic acid was determined as described previously (24).

Succinic dehydrogenase (succinate:phenazine methosulfate oxidoreductase, EC 1.3.99.1) activity was determined by the phenazine methosulfate-2,6-dichlorophenolindophenol-coupled assay of King (17). The initial velocity of 2,6-dichlorophenolindophenol reduction was followed at 600 nm in a Cary 14R spectrophotometer.

Cytochrome content was determined from reduced-minus-oxidized difference spectra obtained with a Cary 14R spectrophotometer using a 1-cm light path. Cuvettes containing cell envelopes (400 μg of protein) or chromatophores (29 μg of protein) were diluted in 0.01 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride buffer (pH 7.5) to a final volume of 1.0 ml; in each case the sample contained 2.5 μg of BCHL. Equal amounts of fraction were placed in the sample and reference beams, and the oxidation baseline was obtained. A few crystals of sodium hydrosulfite were added to the material in the sample beam, and the reduced difference spectrum was determined.

Heavy chromatophores were obtained from crude 156,000 × *g* precipitates by sucrose density gradient centrifugation in the presence of Tris buffer containing 0.01 M $MgCl_2$ (23, 31). To resolve them into light chromatophores and photopigment-depleted envelopes, they were dialyzed against 1.0 mM Tris buffer and placed on gradients (23) prepared in aqueous sucrose or in sucrose solutions containing 1.0 mM Tris.

Heavy chromatophores were also prepared by combining the isolated light chromatophore fraction (3 mg of protein) with cell envelope or cytoplasmic membrane-enriched material (1 mg of protein), followed by dialysis against Tris buffer containing the ionic species indicated in the text. The ratio of chromatophore-to-envelope protein in these experiments approaches that found in phototrophically grown cells harvested in late exponential growth. The dialyzed samples (0.5 ml) were placed on 13-ml sucrose gradients prepared in the homologous buffer and centrifuged at 40,000 rpm (200,000 × *g*) for 90 min in a Beckman SW40Ti rotor at 4 C in a Beckman Spinco L2-65B preparative ultracentrifuge.

RESULTS

We have reported previously a quantitative separation of chromatophores from the cell envelope in *R. spheroides* by carefully controlling the ionic strength in sucrose gradients used in their isolation (23). To obtain sufficient material for extensive characterization of the chromatophore and cell envelope fractions, an extract was prepared from a 2-liter culture of phototrophically grown cells harvested in late exponential growth. After differential centrifugation (23), the resulting subcellular particles were placed in a zonal rotor containing a linear 5 to 60% (wt/wt) sucrose gradient formed as described previously (24). The 5 to 28% (wt/wt) sucrose portion of the gradient was prepared in Tris buffer containing 0.1 mM $MgCl_2$; the remainder of the gradient was prepared with aqueous sucrose. The separation achieved is presented in Fig. 1. In the absorbance profile in the top panel, it is shown that a rather homogeneous chromatophore band (peak B) has been clearly resolved from ribosomes (peak A) in the portion of the gradient closest to the center of rotation. In the remainder of the gradient, a heterogeneous cell envelope band (peak C) appeared that is almost devoid of BCHL as shown by the low absorbance at 850 nm. The bottom panel shows the protein profile of the zonal gradient and the distribution of succinic dehydrogenase, an enzyme that serves as a marker for membranes (30). The protein profile follows closely the absorbance at 280 nm. Succinic dehydrogenase was present in both the chromatophore and cell envelope fractions; however, the specific activity in the leading edge of the cell envelope band is approximately twice that of chromatophores. This suggests that the cell envelope band contains fragments of succinic dehydrogenase-rich cytoplasmic membrane that are depleted in BCHL.

To characterize further the isolated chromatophores and cell envelopes, fractions 13 to 19 and 27 to 36 were separately pooled and washed in distilled water as described previously (23). The specific BCHL content of the chromatophores was 90.0 μg of BCHL/mg of protein, whereas that for the cell envelope material was 6.2 μg/mg. This suggests that the isolated envelopes were 93% free of chromatophores. The chemical composition of the isolated structures on a dry weight basis is given in Table 1. Although the chromatophores are somewhat enriched in protein content, the lipid, total phosphorus, and nucleic acid contents of each structure are very similar. The major differences in composition are in the greatly enriched BCHL content of the chromatophores and in the increased carbohydrate content of the envelope fraction. The carotenoid content of similar preparations has been reported previously (23). The low nucleic acid content of each of the structures is consistent with their apparent freedom from ribosome contamination shown in the absorbance profile of the zonal gradient (Fig. 1).

As a further test of the purity of chromatophores isolated by sucrose gradient centrifuga-

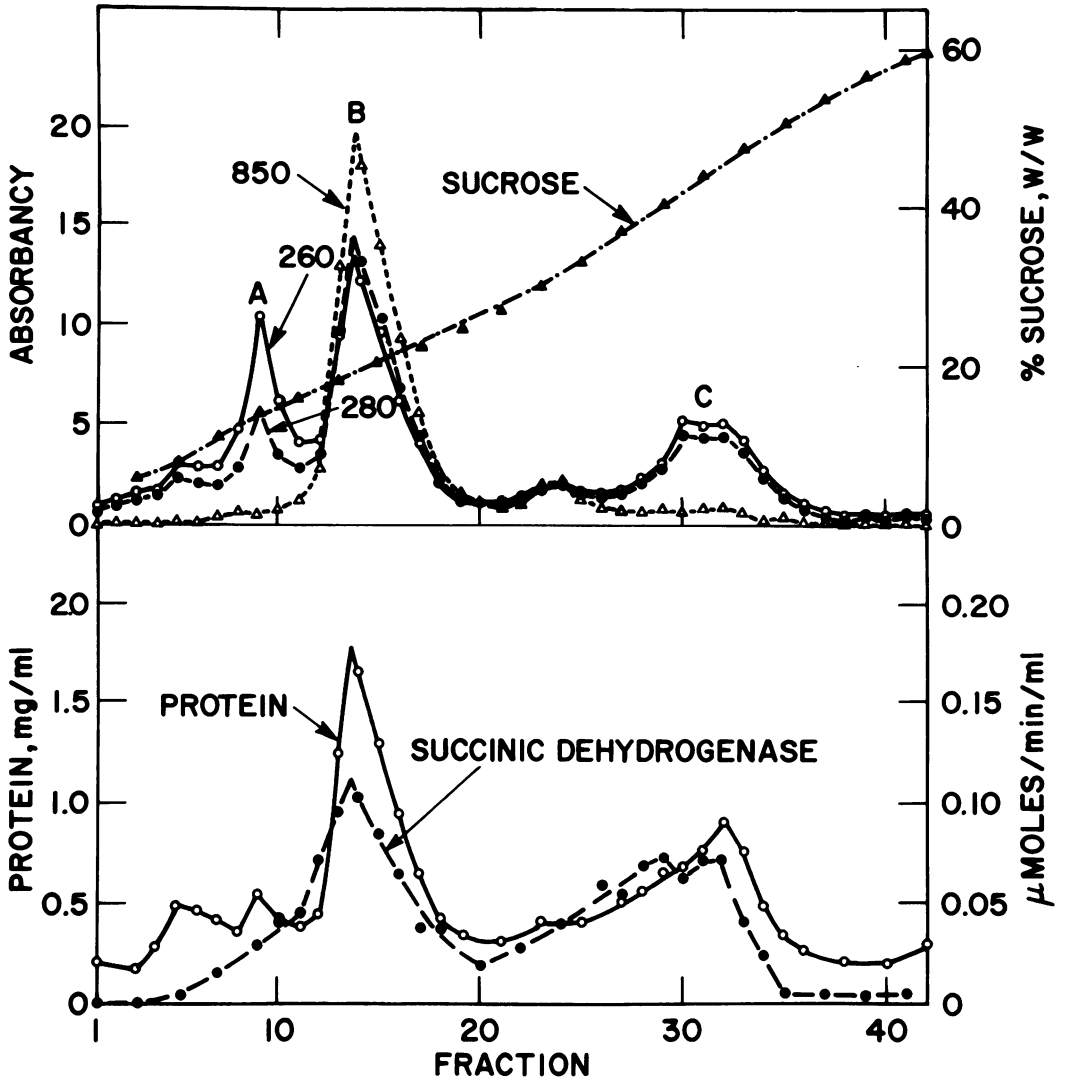

FIG. 1. *Separation of subcellular particles by zonal ultracentrifugation. Zonal ultracentrifugation was performed at 120,000 × g in a Beckman Ti-14 rotor as described previously (24). Sucrose concentrations were determined on a refractometer. Absorbancy of diluted fractions was determined at 260, 280, and 850 nm.*

TABLE 1. *Chemical composition of isolated chromatophores and cell envelopes*

Constituent	Chromatophore (% dry wt)	Cell envelope (% dry wt)
Protein	63	53
Lipid	30	27
Bchl	5.9	0.3
Total phosphorus	0.93	0.89
Carbohydrate[a]	2.3	4.5
Total nucleic acid	0.64	0.42

[a] Expressed as glucose equivalents.

tion, a reconstruction experiment similar to that reported by Fraker and Kaplan (8) was performed. In this procedure, aerobic cells devoid of chromatophores were grown in the presence of L-[4,5-^{3}H]leucine (New England Nuclear Corp., Boston, Mass.). They were added to unlabeled cells that were grown under phototrophic conditions, and the mixture was passed through a French pressure cell. Subcellular particles were prepared (23), and the 230,000 × *g* 60-min precipitate was placed on a 5 to 60% (wt/wt) sucrose gradient prepared in Tris buffer, and centrifuged in a Beckman

SW27 rotor. The results (Fig. 2) show that very little label is present in the discrete chromatophore band, but rather the vast majority is seen in the ribosome and cell envelope peaks. The specific radioactivity for the chromatophore band (fractions 10 to 14) was 4,270 counts per min per mg of protein, whereas that for the total 230,000 $\times$ g precipitate from the aerobic extract alone was 80,450 counts per min per mg of protein. This indicates that the isolated chromatophores were 95% free from contamination by particulate protein of non-chromatophore origin.

In Fig. 3, reduced-minus-oxidized difference spectra of isolated cell envelope and chromatophore fractions are shown. The amount of each fraction was adjusted to give equal quantities of BCHL (see Materials and Methods). The α bands in the 560- and 550-nm region and the Soret peak at approximately 428 nm suggest that each fraction contains b- and c-type cytochromes. The chromatophore spectrum essentially corroborates that published by Worden and Sistrom (31). When the absorbance of the 428-nm absorption maximum is subtracted from that of the troughs at approximately 410 nm, it can be seen that 60% of the cytochrome content of the envelopes can not be accounted for on the basis of contamination by chromatophores. This suggests further that the envelope fraction contains a BCHL-depleted cytoplasmic membrane.

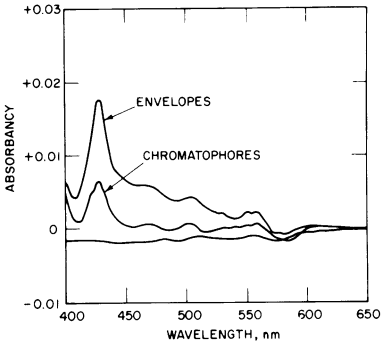

FIG. 3. *Reduced-minus-oxidized difference spectra of cell envelope and chromatophore fractions.*

In electron micrographs, negatively stained preparations of cell envelopes from phototrophically grown cells were similar in appearance to those from aerobic cells (24), except that a few chromatophores could be detected in the phototrophic preparations (B. J. Segen, R. A. Niederman, and K. D. Gibson, unpublished data).

In earlier attempts to isolate the photosyn-

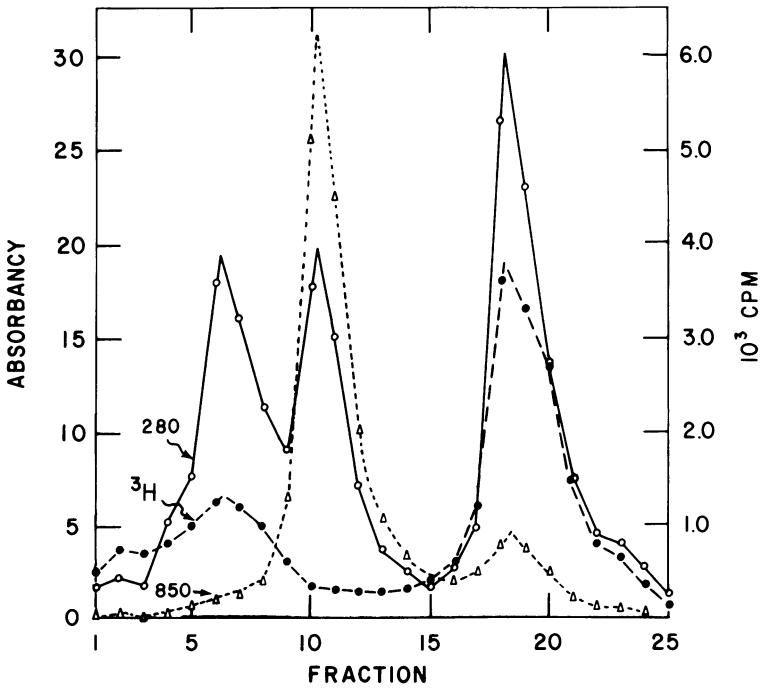

FIG. 2. *Reconstruction experiment assessing purity of isolated chromatophores. Radioactivity was determined by the method of Mans and Novelli (22) modified as previously described (13).*

thetic apparatus in *R. spheroides*, Worden and Sistrom (31) observed that photopigments were nearly equally distributed between two bands in sucrose gradients containing 0.01 M Mg^{2+}. The light band which had a sedimentation coefficient of 153*S* was identified as a purified chromatophore fraction, whereas the heavy chromatophore band was thought to be a complex of pigmented and nonpigmented material. Later, Gibson (9) reported that 85% or more of the photopigment in extracts of *R. spheroides* occurred as a largely monodisperse chromatophore fraction with a sedimentation coefficient of 160*S*. By moving boundary sedimentation, it was observed that some chromatophore preparations underwent aggregation (10). Aggregation was very sensitive to the ionic environment and was influenced by both ionic strength and the nature of the ions present. In particular, magnesium and other divalent cations were shown to cause extensive chromatophore aggregation at ionic strengths above 0.01 μ.

To test whether the heavy chromatophore fraction resulted from aggregation between light chromatophores and the cell envelope, the heavy fraction was prepared essentially as described by Worden and Sistrom (31) and dialyzed against Tris buffer. When this material was placed on a sucrose gradient prepared in the absence of ionic species (Fig. 4), an extensive light chromatophore band appeared in a position identical to the sole, pigmented band isolated from extracts in the absence of Mg^{2+} (23). In addition, a cell envelope band markedly depleted in photopigment was found in the original position of heavy chromatophores.

Further evidence that heavy chromatophores are magnesium-induced aggregates of the light

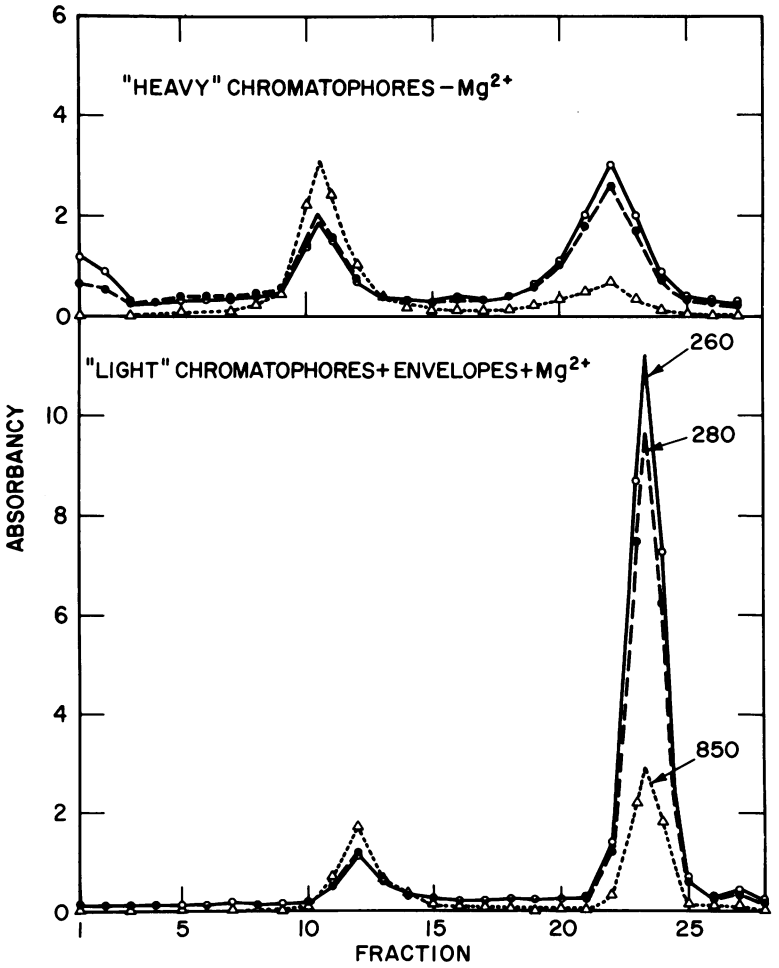

FIG. 4. *Resolution and reconstitution of heavy chromatophores. Fraction 1 is at the top of the gradient.*

chromatophore fraction and the cell envelope is provided in the bottom panel of Fig. 4. In this experiment, isolated light chromatophores and cell envelopes were combined, dialyzed in the presence of Tris buffer containing 0.01 M $MgCl_2$, and placed on a sucrose gradient prepared with the same buffer. It can be seen that the majority of the photopigment banded in the usual position of the light-scattering heavy chromatophore fraction. That the heavy fraction has not resulted from light chromatophore aggregates banding in a position coincident with the cell envelope fraction is suggested by a separate gradient layered with a sample of light chromatophores alone, that was dialyzed in the same manner. Only a single, somewhat more diffuse band appeared in the usual position of light chromatophores (not shown).

In Table 2, the specific BCHL content of the resolved and reconstituted heavy chromatophore fractions is presented. In the initial heavy chromatophores, about half of the protein could be accounted for on the basis of contamination with light chromatophores. The resolution of this fraction resulted in the release of a light chromatophore fraction with a specific BCHL content similar to that found in highly purified chromatophores, and an envelope fraction from which most of the photopigment has been removed. The reconstitution experiment resulted in the formation of heavy chromatophores whose specific BCHL approached that of the fraction isolated by the method of Worden and Sistrom (31). In addition, these reconstituted heavy chromatophores can be dissociated into light chromatophores and a photopigment-depleted cell envelope fraction by the above resolution procedures (M. A. Levine and R. A. Niederman, unpublished data).

TABLE 2. *Specific BCHL content of resolved and reconstituted heavy chromatophores*

Fraction	Specific BCHL (μg of BCHL/mg of protein)
1. Initial heavy chromatophore	45.0
1. After resolution	
Light chromatophore	80.1
Cell envelope	10.1
2. Initial light chromatophore	71.2
3. Initial cell envelope	6.0
2. + 3. + magnesium[a]	
Heavy chromatophore	41.4

[a] Combined and dialyzed against 0.01 M Tris buffer containing 0.01 M $MgCl_2$.

Since chromatophores of *R. spheroides* are found peripherally in cells grown at high light intensity (3, 12), it was previously suggested that divalent cation-induced aggregation of chromatophores with the cytoplasmic membrane may have physiological significance (10). This was tested by comparing the chromatophore-binding ability of chromatophore-free envelope and cytoplasmic membrane fractions isolated from aerobically grown cells as described previously (24). If the aggregation phenomenom has a function within the cell, it would be expected that the cytoplasmic membrane fraction would have a higher affinity for chromatophores. The results (Table 3) indicate that, to the contrary, the cell envelope binds chromatophores to a greater extent than does the cytoplasmic membrane fraction. Chromatophore binding by the latter fraction in the presence of magnesium was approximately the same as that obtained previously in its absence (24). Aggregation with aerobic envelopes was largely magnesium dependent, and the resulting heavy chromatophores had virtually the same specific BCHL as those formed with envelopes from phototrophically grown cells. This suggests that the phototrophic cell envelope has no specific chromatophore-binding properties. It is also shown in Table 3 that much of the chromatophores aggregated to the aerobic envelope were released by the resolution procedure. Since aerobic envelope preparations are initially free from chromatophores but bind them as well as their phototrophic counterparts,

TABLE 3. *Formation of heavy chromatophores using envelope fractions from aerobically-grown cells*

Fraction	Specific BCHL (μg of BCHL/mg of protein)
1. Initial light chromatophore	71.2
2. Initial aerobic cell envelope	0.5
3. Initial aerobic cytoplasmic membrane-enriched	1.8
1. + 2.[a]	14.7
1. + 2. + magnesium[b]	41.8
1. + 3. + magnesium[b]	26.9
4. Heavy chromatophore from 1. + 2. + magnesium after resolution	
Light chromatophores	68.1
Cell envelope	22.5

[a] Combined and dialyzed against 0.01 M Tris buffer.

[b] Combined and dialyzed against 0.01 M Tris buffer containing 0.01 M $MgCl_2$.

aerobic envelopes were employed in the remaining studies.

In Fig. 5, the effect of various magnesium chloride levels on heavy chromatophore formation is shown. In this experiment, samples were dialyzed against 1.0 mM Tris buffer containing the indicated magnesium concentrations, since at this Tris concentration magnesium-independent aggregation is greatly reduced. Although only limited aggregation was observed at 0.1 mM magnesium, it was extensive at 1.0 mM and reached a plateau at 5.0 to 10.0 mM magnesium. Thereafter, aggregation was somewhat reduced. Thus, in subsequent experiments, cations were tested at a concentration of 10.0 mM.

Since chromatophore aggregation is greatly influenced by the nature of the ions present (10), the effects of several monovalent and divalent cations on interactions between chromatophores and the cell envelope were tested (Fig. 6). Of the divalent cations examined, the lowest yield of heavy chromatophores (fractions 13 to 16) and the largest amount of unbound light chromatophores (fractions 6 to 9) were obtained with magnesium. Increasing chromatophore binding was obtained with calcium, manganese, and cobalt, respectively, and in the latter case no unbound chromatophores were detectable. In the case of spermine, extensive heavy chromatophore formation also occurred; however, major and minor bands of heavy chromatophores were observed. With lithium and sodium, only limited interaction between chromatophores and the cell envelope occurred.

In Table 4, the specific BCHL levels of the heavy chromatophores formed with the various cations is presented. The values follow closely the photopigment distributions in Fig. 6. Among the divalent metals, the highest value was obtained with cobalt and the lowest with magnesium. Although some heavy chromatophore formation occurred with each of the alkali metal ions, none gave values as high as those encountered with divalent cations. Aside from some specific differences, these results are in general aggrement with those obtained previously for the effects of cations on chromatophore aggregation (10); they also establish that the nature of the ions present markedly influences chromatophore-cell envelope interactions.

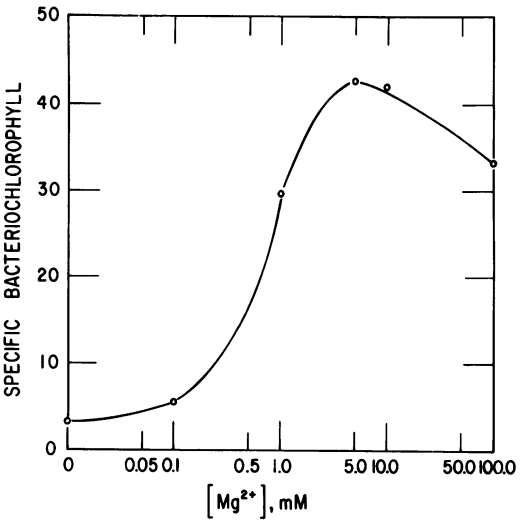

FIG. 5. *Effect of various magnesium concentrations on the formation of heavy chromatophores. Specific BCHL is micrograms of BCHL per milligram of protein.*

DISCUSSION

In this study, it is shown that zonal ultracentrifugation is a satisfactory method for large-scale preparation of highly purified chromatophore and cell envelope fractions from photosynthetically grown *R. spheroides*. Under carefully controlled ionic conditions, nearly all of the BCHL appeared in a single, discrete chromatophore band essentially free from material of nonchromatophore origin. This band was located in the same position as the light chromatophore band described by Worden and Sistrom (31). The envelope band had a very low photopigment content and appeared in the position of heavy chromatophores (31). Under our conditions of cell disruption, a portion of the peripheral cytoplasmic membrane is apparently released from the envelope in small fragments that are subsequently removed by differential centrifugation (24). Thus, the cell envelope preparations are somewhat depleted in cytoplasmic membrane. Nevertheless, the isolated envelopes contain substantial cytochromes and succinic dehydrogenase in addition to the increments expected from contaminating chromatophores. On this basis, it is proposed that the cell envelope of *R. spheroides* retains a BCHL-deficient cytoplasmic membrane during phototrophic growth. Evidence for a BCHL-deficient cytoplasmic membrane in photosynthetically grown *Rhodospirillum rubrum* has been presented by Oelze et al. (25) and Ketchum and Holt (16).

Based upon the isolation of two heavily pigmented bands from sucrose gradients, Cohen-Bazire and Kunisawa (4) and Worden and Sistrom (31) concluded that the bacterial photosynthetic apparatus has two distinct forms

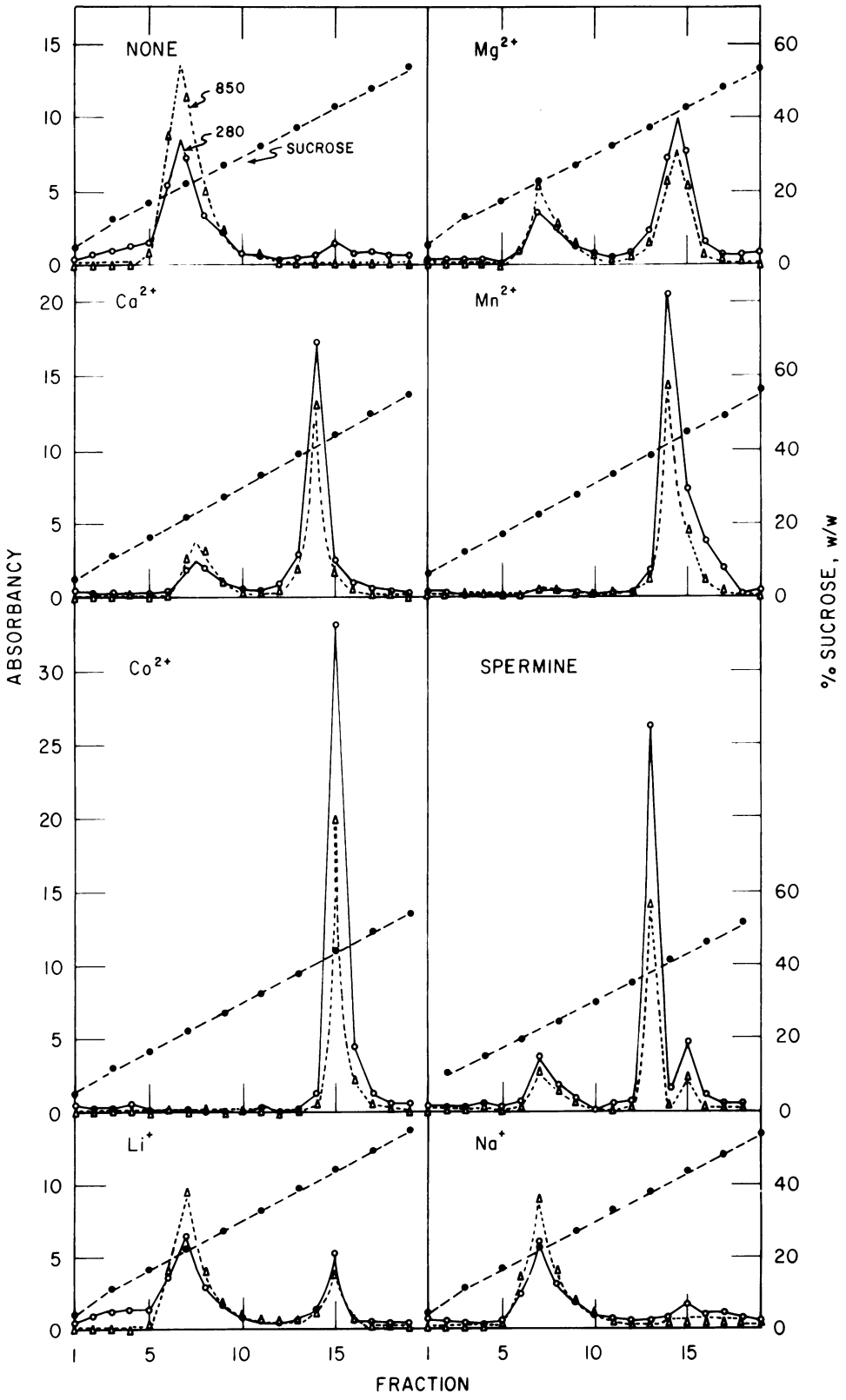

FIG. 6. *Effect of various cations on the formation of heavy chromatophores. Chloride salts of the various metal ions were used.*

TABLE 4. *Specific BCHL content of heavy chromatophores formed with various cations*

Fraction	Cation	Specific BCHL (μg BCHL/mg of protein)
1. Initial light chromatophores		80.4
2. Initial aerobic cell envelope		0.5
1. + 2.[a]	None	2.5
	Divalents[b]	
	Cobalt	70.4
	Manganese	60.2
	Calcium	52.5
	Magnesium	47.3
	Spermine[c]	
	Major band	55.3
	Minor band	30.5
	Monovalents[b]	
	Lithium	36.5
	Sodium	27.5
	Cesium	24.7
	Potassium	19.5

[a] Combined and dialyzed against 1 mM Tris Buffer containing the indicated ionic species at a concentration of 0.01 M.

[b] Chloride salts of the various metal ions were used.

[c] Spermine tetrahydrochloride (Sigma Chemical Co., St. Louis, Mo.).

within the cell. However, high magnesium concentrations were present throughout their isolation procedures. In the present study, it was shown that the majority of the photopigment in the heavy fraction results from the magnesium present during its preparation. The formation of heavy chromatophores from light chromatophores and cell envelopes in the presence of 0.01 M magnesium, together with their resolution into these components after dialysis in its absence, supports the previous conclusion that the photosynthetic apparatus in *R. spheroides* comprises a membrane system distinct from the peripheral cell wall-cytoplasmic membrane complex (23). Thus, the release of light chromatophore material from the heavy band by treatment with lipase (4) or further passage through the French pressure cell (16, 31) is obviated merely by dialysis and centrifugation with reagents of low ionic strength. The suggestion that heavy chromatophores represent a partially differentiated intermediate structure between pigmented internal membrane and the nonpigmented peripheral membrane (18) must be reevaluated in light of the present data.

With regard to a possible mechanism for heavy chromatophore formation, the divalent cations may increase the stability of chromatophore-cell envelope interactions by interchain cross-linking through lipid phosphate or protein carboxyl groups at the surface of the respective structures. This would be analagous to the mechanism proposed by Razin (26) for the role of divalent metals in the reaggregation of detergent-solubilized membranes such as the sodium dodecyl sulfate-resolved chromatophores of *Thiocapsa floridana* (29). This interpretation is supported by the more limited chromatophore-cell envelope interactions noted in the presence of monovalent cations. Reconstituted mycoplasma membranes formed in the presence of monovalent cations were also less stable than those produced with divalent metals (27). This has been attributed to the contribution of divalent, but not monovalent, cations to the stability of cross-linking between negatively charged groups in the membrane (27). Since much of the phospholipid is apparently at the surface of the chromatophore (11) and phosphatidylglycerol is a major phospholipid in *R. spheroides* (14, 19), these highly acidic molecules would be likely candidates for electrostatic interactions with divalent metal ions. This is consistent with the recent finding of Kahane et al. (15) that the phosphatidylglycerol-rich phospholipids provide the major binding sites for cations in mycoplasma membranes. At least 60 to 80% of the magnesium in these membranes was bound to lipid (15). Their results also suggested that the affinity of membranes was greater for calcium than for magnesium (15). This could explain why calcium caused more extensive heavy chromatophore formation than did magnesium.

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