Biochimica et Biophysica Acta, 507 (1978) 353—368

BBA 77952

STRUCTURAL AND FUNCTIONAL PROPERTIES OF CHROMATOPHORES AND MEMBRANE VESICLES FROM *RHODOPSEUDOMONAS SPHAEROIDES*

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(Received August 9th, 1977)

Summary

Membrane vesicles have been isolated by a modified procedure from *Rhodopseudomonas sphaeroides*, grown phototrophically under high light intensity. In addition, chromatophores have been isolated from this organism grown phototrophically with low light intensities.

Structural, chemical and functional properties of both preparations have been investigated and compared. The orientation of the membrane preparations has been studied by freeze-etch electron microscopy, the localization of cytochrome c_2, and light-driven active transport of amino acids and Ca^{2+}. The results demonstrate that the orientation of the vesicle membrane is the same as the cytoplasmic membrane of intact cells; the membranes in chromatophores, however, have an inverted orientation.

On a dry weight basis, the membrane vesicles contain less protein, carotenoids and bacteriochlorophyll and more lipids than do chromatophores. Qualitatively, however, the composition of both preparations is similar.

It is concluded that the intracytoplasmic structures from which the chromatophores are derived are structurally and functionally similar to (and most likely continuous with) the cytoplasmic membranes from which the vesicles are derived.

Introduction

Membrane-bound functions, such as electron transfer and enzyme activities, have been studied extensively in isolated cytoplasmic membrane preparations from bacteria. The diffusion barrier property of the membrane is essential for several functions, such as the generation of an energized membrane state or the

Abbreviations: SDS, sodium dodecyl sulphate; tricine, *N*-tris(hydroxymethyl)methylglycine.

utilization of this energized state for ATP synthesis or active solute transport, and these functions can only be studied in membrane preparations which have special properties. It is essential to use membrane vesicles. Most of these integrated membrane functions have vectorial properties and it is, therefore, equally important to perform the studies in membrane preparations which are homogeneous in their orientation. That means in membrane preparations, which are oriented as the cytoplasmic membranes of intact cells (right-side out) or in inverted membrane preparations (inside out). Maximal information about several membrane functions can be obtained from studies in both right-side out and inside out membrane preparations.

A number of isolation procedures has been described for the isolation of right-side out and inside out cytoplasmic membrane preparations from bacteria. Kaback [1,2] has introduced an isolation procedure of membrane vesicles by osmotic lysis of lysozyme-EDTA-treated cells. Such membrane vesicles have been prepared from many bacteria [3]. It has been shown that this isolation procedure results for *Escherichia coli* and *Bacillus subtilis* in right-side out membrane vesicles [4,5].

Several procedures have been used for the isolation of inside out membranes, especially from *E. coli*. However, none of these procedures produces a homogeneous population of inside out membranes. Membrane preparations obtained by French press disruption or by sonication of cells contain varying ratios of right-side out to inside out vesicles [6,7]. French Press treatment of spheroplasts from *E. coli* even results in right-side out membrane vesicles which perform active transport of amino acids [8].

For *Rhodopseudomonas sphaeroides*, isolation procedures have been described for membrane preparations which are supposed to be right-side out [9] or inside out [10]. *R. sphaeroides* is a phototrophic bacterium which can grow aerobically in the dark or anaerobically in the presence of light [10]. When grown under phototrophic conditions the cytoplasmic membrane forms invaginations (Fig. 1) which can be isolated as the so-called chromatophores [10]. These chromatophores are supposedly inside out. They have been used extensively in the study of light-dependent cyclic electron transfer processes in the photosynthetic apparatus [11,12], proton translocation across the membrane [11—13] and photophosphorylation [13,14]. Another preparation, the membrane vesicles, is isolated mainly from the cytoplasmic membrane (Fig. 1) and these vesicles are probably right-side out [9]. Cytoplasmic membrane vesicles from *R. sphaeroides* were first used in our studies on the mechanism of light-driven active transport in *R. sphaeroides* [9]. In the present paper a modification of that procedure, consistently giving membrane vesicles with high active transport activity, is described.

Chromatophores and membrane vesicles will be used in future studies on the generation of an electrochemical proton gradient upon illumination and the relation between this gradient (or its components, the membrane potential and the transmembrane pH gradient) and the translocation of solutes in both directions through the membrane. For these studies it is essential to know the orientation of the membrane preparation. The results presented in this paper demonstrate that the membrane vesicles are homogeneously right-side out and chromatophores homogeneously inside out. Membrane vesicles and chromatophores

are isolated by different procedures from *R. sphaeroides* grown under different conditions. Several structural and chemical aspects of both preparations were measured and compared.

Materials and Methods

Culture media and growth conditions. R. sphaeroides, strain 2.4.1., was grown anaerobically in the light, in the medium described by Sistrom [15], at 30°C, as described previously [9,16]. For the isolation of membrane vesicles cells were grown under high light intensity by illuminating the cultures with 8 × 150 W incandescent light 30 cm from the culture bath. Cells from which chromatophores were isolated were grown under lower light intensities supplied by 2 × 150 W incandescent light 30 cm from the culture.

Preparation of chromatophores. Cells were harvested in the middle of the exponential growth phase $A_{660\,nm}$ approx. 2.0) and washed once with 50 mM potassium phosphate, pH 7.0, containing 10 mM potassium EDTA and resuspended in 50 mM potassium phosphate, pH 7.0, containing 5 mM $MgSO_4$ at a final concentration of 1 g wet weight per 5 ml (Scheme 1). The cells were broken at 4°C by a 2-fold passage with a pressure of 20000 lb/inch^2 through a French pressure cell (RM 1, Sorvall Inc., Norwalk, Conn., U.S.A.) in the presence of 25 μg/ml deoxyribonuclease (DNAase, EC 3.1.4.5) and ribonuclease (RNAase, EC 3.1.4.22) (Miles Laboratories Ltd., Berkshire, England). Unbroken cells and large debris were removed by centrifugation at 50000 × *g* for 30 min. Chromatophores in the supernatant were sedimented by centrifugation at 140000 × *g* for 2 h and resuspended at a protein concentration of about 20 mg/ml in 50 mM potassium phosphate, pH 7.0, supplemented with 5 mM $MgSO_4$.

Preparation of membrane vesicles. The procedure for the isolation of membrane vesicles [9] has been modified (Scheme 1). Cells were harvested at the end of exponential growth ($A_{660\,nm}$ approx. 2.5) after the addition of chloramphenicol (50 μg/ml) and suspended in 120 mM potassium phosphate (pH 8.0) supplemented with 10 mM sodium EDTA, 10 mM potassium ascorbate and 20 μM dichloroindophenol at a concentration of 1 g wet weight per 20 ml. The cell suspension was incubated for 10 min at room temperature with 200 μg/ml lysozyme (E. Merck A.G., Darmstadt, Germany). The suspension was then diluted 1 : 1 with 10 mM potassium ascorbate (pH 6.0) in order to facilitate the penetration of lysozyme into the cell wall [17]. After 30 min incubation the spheroplasts were lysed by dilution with three volumes of 10 mM potassium ascorbate (pH 6.0) plus 20 μM dichloroindophenol. Deoxyribonuclease and ribonuclease were added to final concentrations of 10 μg/ml; after 15 min incubation $MgSO_4$ was added to a final concentration of 5 mM. The incubation was continued for another 15 min. Cells and unlysed spheroplasts were removed by centrifugation at 800 × *g* for 40 min. The supernatant was decanted carefully and centrifuged at 50000 × *g* for 30 min. The pellet was suspended to a final protein concentration of approx. 5 mg/ml in 50 mM potassium phosphate, pH 7.0, supplemented with 5 mM $MgSO_4$ and 5 mM potassium ascorbate.

All incubations were carried out anaerobically under nitrogen, in the dark at

room temperature. Membrane vesicles and chromatophores were stored anaerobically at 4°C in the dark. Under these conditions the preparations remained active for several days.

Determination of the internal volume of membrane vesicles and chromatophores. To 3 ml of chromatophores (38 mg of protein/ml) and 4 ml of membrane vesicles (5.7 mg/ml) 50 μl of [^{3}H]dextran (molecular weight 60000—90000, 250 μCi/ml) were added. After 10 min incubation at room temperature, the membrane preparations were centrifuged; chromatophores at 140000 × *g* for 2 h, membrane vesicles at 50000 × *g* for 30 min. The pellets were divided into two fractions. One fraction was used for the determination of the total water content by measuring the difference between wet and dry weight (dried 16 h at 110°C) of the fraction. The wet weight of the other fraction was measured, and total radioactivity determined as follows: the fraction was combusted in a Packard Sample Oxidizer 306 (Packard Instr. Comp., Downers Grove, Ill., U.S.A.). The oxidation products were collected in Monophase 40 (Packard) and their radioactivity determined in a liquid scintillation counter. The interstitial volume was calculated by comparing the radioactivity of the membranous pellet with the specific activity of the supernatant. The internal volume of the membrane particles could be determined by subtracting the interstitial volume from the total water content.

Transport assays. Uptake studies were carried out using the filtration technique described previously [2,16,18] at a temperature of 30°C, using cellulose-nitrate membrane filters (Schleicher and Schüll, Dassel, Germany) with a pore size of 0.45 μm for membrane vesicles or of 0.15 μm for chromatophores. Calcium transport was assayed as described by Tsuchiya and Rosen [7,19].

Enzyme assays. For the determination of enzyme activities chromatophores and membrane vesicles were prepared by the procedures given in Scheme 1, except that for the ATPase assay the phosphate buffers were replaced by tricine buffers of the same molarity and pH.

NADH dehydrogenase (EC 1.6.99.3) and succinate dehydrogenase (EC 1.3.99.1) activities were measured by following the reduction of 2,6-dichloroindophenol at 600 nm in a Perkin-Elmer 124 double beam spectrophotometer at 25°C. The reaction mixture contained 50 mM potassium phosphate, pH 7.0, for NADH dehydrogenase and pH 8.0 for succinate dehydrogenase, supplemented with 10 mM KCN, 67 μM dichloroindephenol and 0.5—1.0 mg membrane protein in a final volume of 3 ml. For measurement of succinate dehydrogenase activity, phenazine methosulphate was included (200 μM final concentration). The reactions were started by the addition of NADH or sodium succinate at final concentrations of 67 μM and 20 mM, respectively. The molar extinction coefficient of dichloroindophenol at 600 nm was taken to be 18800 $M^{-1} \cdot cm^{-1}$ [20].

NADH oxidase and succinate oxidase activities were determined by oxygen utilization measurements as described previously [16].

ATPase activity (EC 3.6.1.3) was determined as described by Cox and Downie [21].

Pigment content of the membrane preparations. Pigments were extracted from membranes by treatment with an acetone/methanol mixture (7 : 2, v/v) [22]. The bacteriochlorophyll content was determined from the absorbance at

770 nm, using the extinction coefficient of 82.4 $mg^{-1} \cdot cm^{-1} \cdot ml^{-1}$ [22]. The carotenoid content of the extract was determined from the absorbance at 483 nm, using an extinction coefficient of 220 $mg^{-1} \cdot cm^{-1} \cdot ml^{-1}$ [23].

Lipids. Lipids were extracted from chromatophores and membrane vesicles with chloroform/methanol (1 : 2, v/v) at 4°C. The solvents were removed by means of a rotary evaporator, and the lipid material stored in chloroform at −20°C. Lipids were separated by one-dimensional thin-layer chromatography on silica gel (E. Merck A.G., Darmstadt, Germany) with acetone/benzol/H_2O (91 : 30 : 8, v/v), as described by Pohl et al. [24], and identified under ultraviolet light after spraying the plate with an alkaline 0.003% Rhodamin-6-G solution.

Fatty acid composition of the membranes was determined by analysis of their methyl esters by gas-liquid chromatography on a Packard 421 gas chromatograph (Packard, Instr. Comp., Downers Grove, Ill., U.S.A.) equipped with an electronic integrator (Autolab 6300, Vidar Corp. MT. View, Calif., U.S.A.). The fatty acids were liberated by saponification and were converted to methyl esters by treatment with BCl_3 in methanol. The methyl esters were analyzed and quantified on a column of 5% Silar 10 C (Applied Science Laboratories Inc., State College, U.S.A.) on Gaschrom Q (100—120 mesh) at 160°C.

Protein. Protein was determined by th method of Lowry et al. [25].

Electron microscopy. Freeze etched replicas of cells, chromatophores and membrane vesicles were made and examined as described by Konings et al. [5]. This section electron microscopy was carried out as described previously [9].

Cytochrome spectra. Reduced versus oxidized difference spectra of cytochromes of chromatophores and membrane vesicles were measured with a Cary 14 R spectrophotometer (Applied Physics Corp., Monrovia, Calif., U.S.A.) at room temperature.

The reaction mixture (2.5 ml final volume) contained 1—1.25 mg membrane protein in 50 mM potassium phosphate, pH 7.0, supplemented with 5 mM $MgSO_4$. Cytochromes were reduced by addition of 30 µl of a saturated dithionite solution to the test cuvette, and were oxidized by the addition of $K_3Fe(CN)_6$ to a final concentration of 5 mM.

Polyacrylamide gel electrophoresis. Membranes were solubilized by treatment with a solution of 1% sodium dodecyl sulphate (SDS), 3% 2-mercaptoethanol and 10% glycerol in 0.05 M Tris buffer (pH 6.8) for 10 min at 100°C. Samples containing 100—140 µg solubilized protein were layered on 12.5% acrylamide gels, polymerized with 0.33% bisacrylamide. Disc electrophoresis was performed in 25 mM Tris buffer (pH 8.4), containing 200 mM glycine and 0.1% SDS, at a constant voltage of 18 V/cm.

Gels were stained overnight in 0.1% Coomassie Brilliant Blue R-250 in 45% methanol and 10% acetic acid. They were then destained by washing with the methanol/acetic acid solvent several times over a period of 24 h. An absorbance profile of photographic negatives of stained gels was made as described by Hoekstra et al. [44].

Isopycnic centrifugation. The buoyant density of the membrane was determined in linear 25—55% (w/w) sucrose gradients. Sucrose solutions were prepared in 50 mM potassium phosphate (pH 7.0). Samples of 0.25—0.40 ml, containing 4.6—8.4 mg of protein, were layered on the gradients, which were

centrifuged in a SW 27 rotor (Beckman Instr. Inc., Fullerton, Calif,., U.S.A.) at 25000 rev./min for 72 h at 4°C. Fractions of 15 drops were collected by upward flow displacement with 5.7 M potassium phosphate. Aliquots of each fraction were diluted 20—50 times with 50 mM potassium phosphate plus 25% sucrose and absorbance was measured at 260, 280 and 870 nm (the absorption maximum of *R. sphaeroides* bacteriochlorophyll).

Materials. [U-^{14}C]Alanine (164 Ci/mol), [^{3}H]dextran (10 mCi/g) and $^{45}CaCl_2$ (3.4 Ci/mmol) were obtained from the Radiochemical Centre (Amersham, Bucks., England). For transport studies $^{45}CaCl_2$ was diluted with non-radioactive 50 mM $CaCl_2$ to a specific activity of 38 Ci/mol. All other chemicals were reagent grade.

Results

Isolation of the membrane preparations

R. sphaeroides grown anaerobically under growth-limiting light intensities forms many invaginations of the cytoplasmic membrane [10]. These invaginations can be separated from the cytoplasmic membrane by mechanical breakage of the cells with a high pressure cell [10]. After removal of the larger cell fragments by centrifugation (50000 $\times g$ for 30 min), these invaginations, the so-called "chromatophores", can be sedimented by high speed centrifugation (140000 $\times g$ for 120 min) (Fig. 1, Scheme 1).

The isolation of cytoplasmic membrane vesicles is usually performed by lysis of osmotically sensitive forms of bacteria [2,9]. It has been reported previously

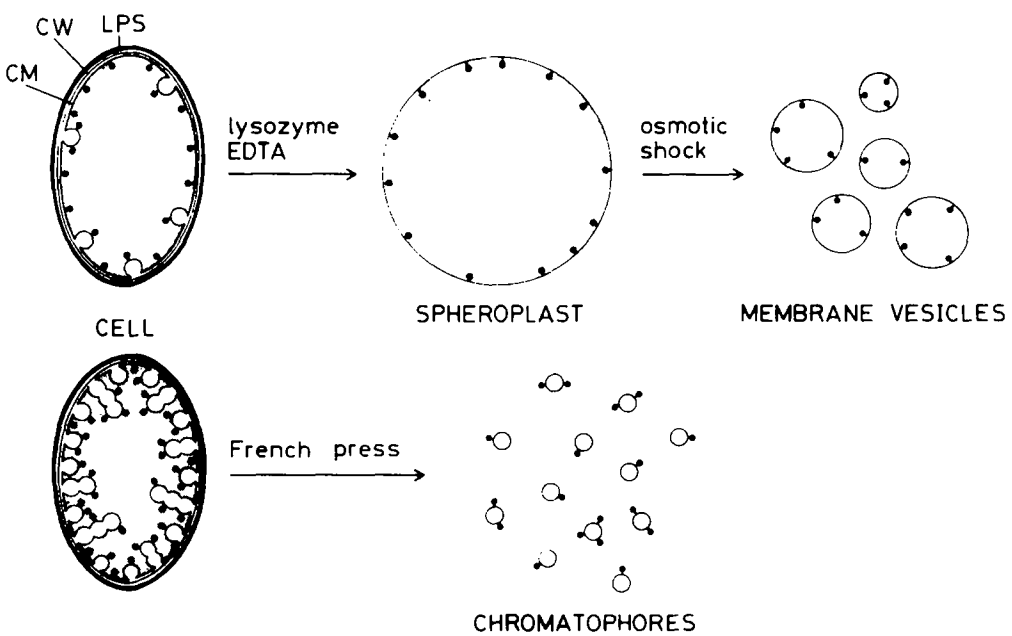

Fig. 1. Schematic representation of the isolation procedures for membranes from *R. sphaeroides*. The pin's heads represent ATPase molecules which are drawn in order to indicate the orientation of the membranes. LPS, outer membrane; CW, cell wall; CM, cytoplasmic membrane.

SCHEME I

SCHEME FOR THE ISOLATION OF CHROMATOPHORES AND MEMBRANE VESICLES FROM RHODOPSEUDOMONAS SPHAEROIDES

Chromatophores	Membrane vesicles
Cells, grown at low light intensity	Cells, grown at high light intensity
washed with 50 mM potassium phosphate (pH 7.0) + 10 mM potassium EDTA	resuspension 1 g wet wt./20 ml in 120 mM potassium phosphate (pH 8.0) + 10 mM EDTA + 10 mM potassium ascorbate + 20 μM DCIP + 200 μg/ml lysozyme
↓	↓
	10 min room temperature
	↓
8000 × *g*, 15 min	1 : 1 dilution with 10 mM potassium ascorbate, pH 6.0
↓	↓
	30 min room temperature
	↓
Pellet	+ DNAase 10 μg/ml + RNAase 10 μg/ml
	↓
resuspension 1 g wet. wt./5 ml in 50 mM potassium phosphate (pH 7.0) + 5 mM $MgSO_4$ + DNAase 25 μg/ml + RNAase 25 μg/ml	1 : 4 dilution with 10 mM potassium ascorbate (pH 6.0) + 20 μM DCIP
↓	↓
	15 min room temperature
	↓
French press treatment, twice at 20 000 lb/inch^2, 4°C	+ 5 mM $MgSO_4$
↓	↓
	15 min room temperature
	↓
50 000 × *g*, 30 min	800 × *g*, 40 min
↓	↓
Supernatant	Supernatant
↓	↓
140 000 × *g*, 120 min	50 000 × *g*, 30 min
↓	↓
Pellet (chromatophores)	Pellet (membrane vesicles)
resuspension 20 mg protein/ml in 50 mM potassium phosphate, pH 7.0, + 5 mM $MgSO_4$	resuspension 5 mg protein/ml in 50 mM potassium phosphate (pH 7.0) + 5 mM $MgSO_4$ and 5 mM potassium ascorbate

Scheme 1. Diagrammatic representation of the isolation procedures for chromatophores and membrane vesicles from *R. sphaeroides*. DCIP, dichloroindophenol.

[9] that such an osmotically sensitive form (i.e. spheroplasts for gram negative bacteria) cannot be obtained from cells grown with limiting light intensities. Under hypotonic conditions the internal osmotic pressure decreases rapidly due to the volume increase caused by unfolding of the invaginations. Growth of *R. sphaeroides* under high light intensities represses drastically the formation of invaginations, and osmotically sensitive spheroplasts are easily derived from these cells (Fig. 1). The modifications made to the procedure described in ref. 9 for the isolation of membrane vesicles consistently yields membrane vesicles with high transport activity (Scheme 1). The most important of these modifications are the following. During the spheroplast formation step osmotic stabilization with hypertonic solutions of sugars such as sucrose, sorbitol or mannitol was avoided because such treatment drastically reduces transport activity. For instance, washing cells with 0.25 M sucrose in 50 mM potassium phosphate (pH 7.0) reduced the accumulation of L-alanine by 60%, while washing with phosphate buffer alone hardly affected this activity.

The lysozyme treatment and lysis were performed in phosphate buffers of moderate ionic strength (0.15—0.5) in order to retain membrane-bound cytochrome c_2 (see below). For the same reason mechanical treatments such as centrifugation and resuspension were reduced as much as possible.

More effective formation of spheroplasts was obtained by introducing an osmotic shock step in the presence of lysozyme (Scheme 1). Such treatment facilitates the penetration of lysozyme through the outer membrane [17].

Physical properties of vesicles and chromatophores. Electron micrographs of thin sections of chromatophores and membrane vesicles show that both preparations consist essentially of trilaminar membrane structures in which no internal structures are detectable, indicating that the preparations are deprived of cytoplasmic components (see also ref. 9). Despite several attempts, we have not obtained thin section electron micrographs showing good contrasting membrane structures of chromatophores.

The chromatophores vary in size between 30 and 80 nm with a mean diameter of about 60 nm, while the diameter of the membrane vesicles varies between 100 and 500 nm with a mean diameter of 250 nm. Membrane vesicles are, on average, several-fold larger than chromatophores. The internal volumes of chromatophores (1.2 μl per mg protein) and membrane vesicles (11.9 μl per mg protein) are consistent with this observation.

The buoyant density of chromatophores and membrane vesicles was deter-

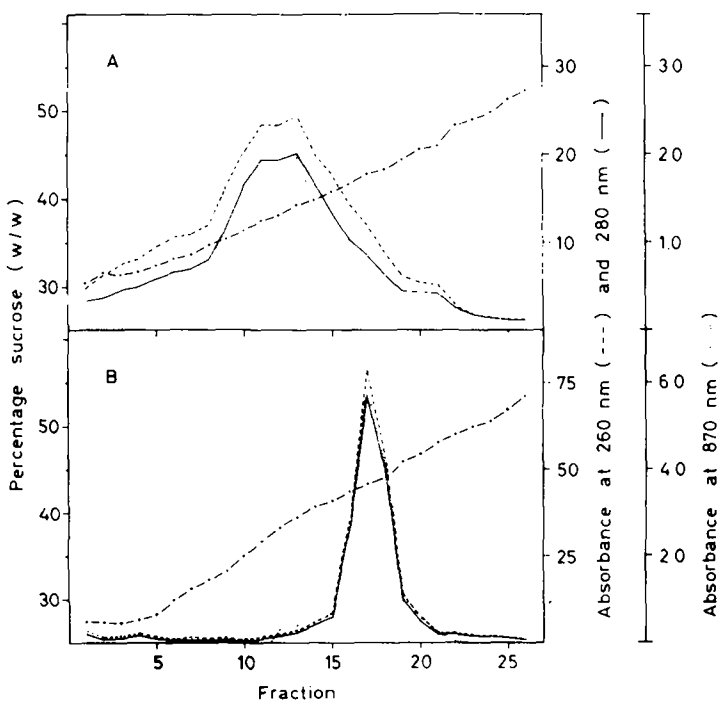

Fig. 2. Sucrose density gradient profile of chromatophores (A) and membrane vesicles (B) from *R. sphaeroides* after centrifugation for 72 h. Preparation of linear gradients, centrifugation and absorbance measurements of fractions were performed as described in Materials and Methods. o———o, sucrose concentration; - - - - - -, $A_{260\ nm}$; ———, $A_{280\ nm}$; · · · · · ·, $A_{870\ nm}$.

mined by isopycnic centrifugation on continuous sucrose gradients. In order to attain equilibrium, centrifugation for more than 16 h was necessary. The distribution patterns after 72 h centrifugation are shown in Fig. 2. The presence of bacteriochlorophyll, measured by the $A_{870\,nm}$, is used as an indication for the distribution of vesicles and chromatophores. This distribution follows that of protein in both preparations.

The buoyant density of the chromatophores varies between 1.12 and 1.22 g/ml, with a major band at 1.16 g/ml. The main buoyant density of the membrane vesicles is 1.19 g/ml, with a much smaller range of densities.

The slight increase of the $A_{260\,nm}/A_{280\,nm}$ at the top of the gradient of chromatophores indicates the presence of some ribosomal material in this preparations.

Freeze-etch electron microscopy

Several investigations have shown that the freeze-fracture faces of the cytoplasmic membrane of bacteria differ considerably in the particle density: the protoplasmic membrane half (P) is densely covered with particles, while the exoplasmic membrane half (E) has a low particle density [5,26]. In Fig. 3 the fracture faces of whole cells (Fig. 3A), membrane vesicles (Fig. 3B) and chromatophores (Fig. 3C) are shown. The convex half of the vesicle membrane contains many particles while the concave fracture face is very smooth, which agrees with the pattern observed in the P and E faces of the cytoplasmic membrane of whole cells. These observations therefore indicate that the orientation of the membrane in vesicles is the same as in whole cells.

The freeze-etch electron micrographs of chromatophores (Fig. 3C) are much less informative. Because of the small size of the chromatophores (mean diameter 60 nm) the number of particles on a fracture face is limited. However, it is possible to see more particles on the concave half of the chromatophores than on the convex half, which is indicative of an inverted orientation.

Cytochrome c_2

Dutton et al. [27] and Prince et al. [28] reported that cytochrome c_2, one of the electron carriers of the cyclic electron transport system, is loosely bound to the membrane, especially in the reduced state, and that it is easily detached at high ionic strength and by mechanical damage. Cytochrome c_2 appears to be located on the periplasmic side of the membrane [27,28]. Cytochrome c_2 should therefore be readily released from membrane vesicles but not from chromatophores. The data in Fig. 4 show that this is true. Washing of membrane vesicles with 50 mM potassium phosphate (pH 7.0) + 5 mM $MgSO_4$ reduces the cytochrome c_2 content by about 35% as estimated from the decrease of $A_{552\,nm} - A_{540\,nm}$ [27,28]. The light-driven active transport activity decreases concomitantly (data not shown). In contrast, washing of chromatophores with buffer did not reduce cytochrome c_2 content, and no cytochrome c_2 was detectable in the washings (Fig. 4).

Active transport activities

It has been shown previously [9] that membrane vesicles accumulate L-alanine actively upon illumination. Vesicles prepared according to the procedure

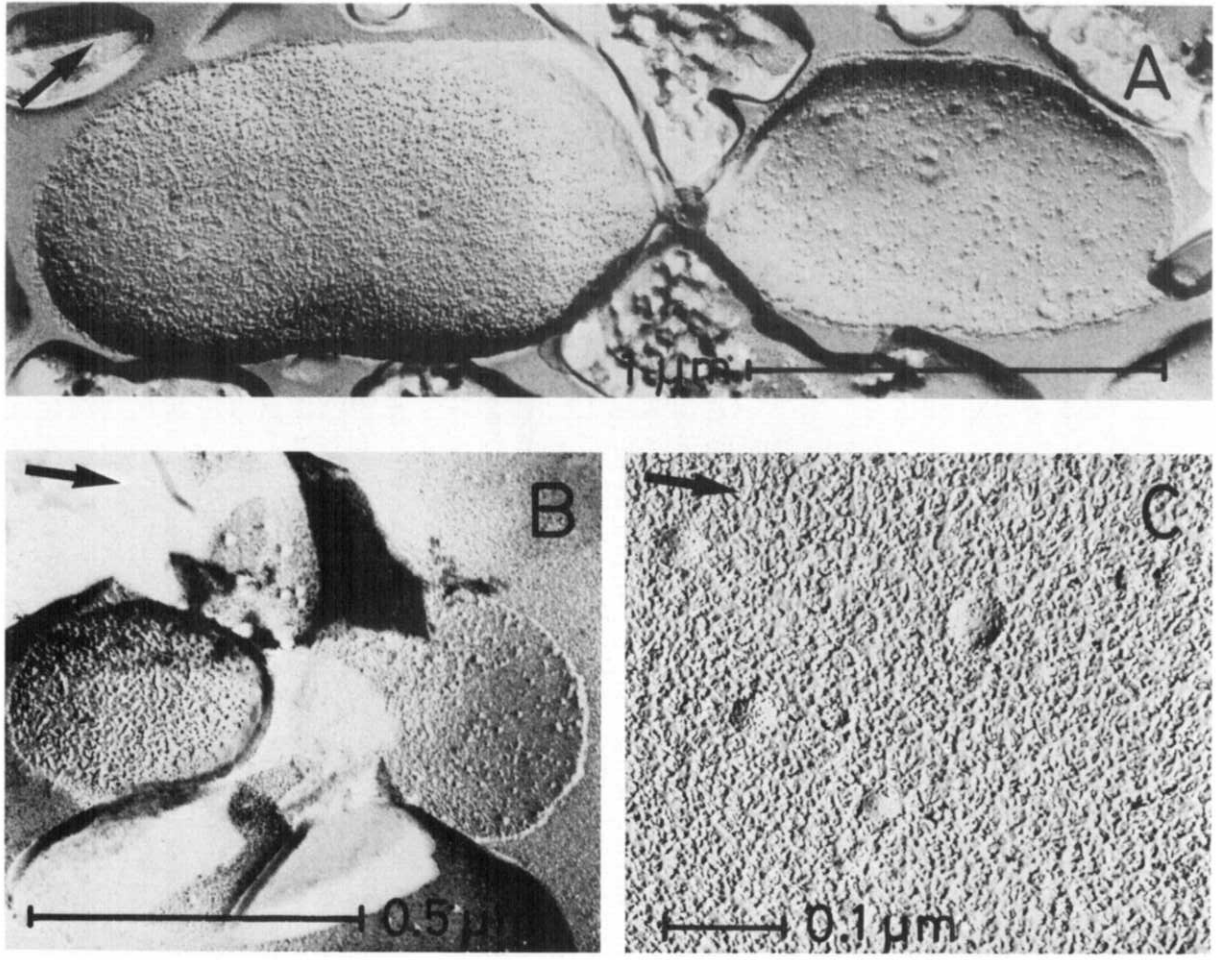

Fig. 3. (A) Replica of a freeze-etched cell of *R. sphaeroides*, showing two fracture faces of the cytoplasmic membrane: the convex protoplasmic half being densely covered with particles and the concave extracellular half-membrane with the low density of particles. The arrow in this and the next figures indicates the direction of the shadow. (B) Replica of a freeze-etched membrane vesicle showing a particle-rich convex membrane half and a smooth concave membrane half. (C) Replica of freeze-etched chromatophores, showing convex and concave membrane halves.

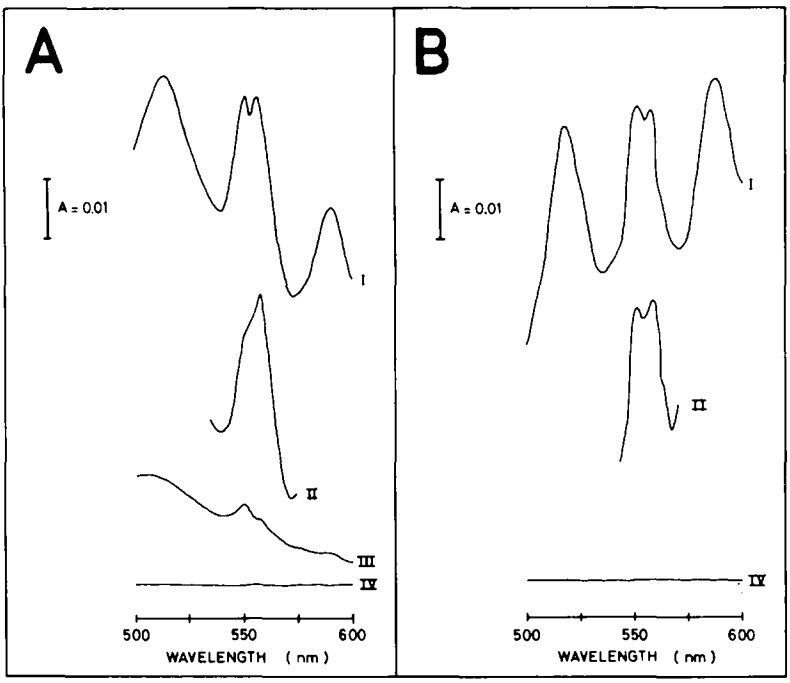

Fig. 4. The effects of washing on the cytochromes of membrane vesicles (A) and chromatophores (B) from *R. sphaeroides*. The difference spectra were recorded as described in Materials and Methods. Membrane vesicles were suspended at a concentration of 1.25 mg protein/ml, the chromatophores at a protein concentration of 1.1 mg/ml. I, Reduced minus oxidized before washing; II, reduced minus oxidized after washing; III, the difference spectrum of the supernatant after washing of the membrane vesicles; IV, baseline.

described above, have a 5-fold higher initial rate of light-driven active transport than membrane vesicles prepared by the original procedure and L-alanine is concentrated 25-fold after 10 min (Fig. 5B). Accumulation had not reached a steady state after 45 min illumination (data not shown).

Chromatophores, on the other hand, do not accumulate L-alanine in the presence of light energy. Neither was any uptake of L-alanine observed when a membrane potential of about 120 mV (inside negative) was imposed artifically by a 20-fold dilution of valinomycin-treated chromatophores (1 nmol valinomycin/mg membrane protein) into potassium-free buffer.

These chromatophores, however, accumulate Ca^{2+} upon illumination (Fig. 5A). An 8-fold concentration of calcium is obtained in chromatophores which contain potassium phosphate (100 mM; pH 7.0) and are suspended in Tris · HCl (10 mM; pH 8.0), KCl (150 mM), potassium phosphate (10 mM) and 1 mM Ca^{2+}. No light-driven Ca^{2+} accumulation is observed in chromatophores prepared in potassium phosphate (50 mM; pH 7.0) or in phosphate-free media such as glycylglycine (40 mM; pH 7.0) + 50 mM KCl. On the other hand, membrane vesicles prepared in either of these buffers do not actively accumulate Ca^{2+} upon illumination (Fig. 5A).

One of the advantages of bacterial membrane vesicles is that transport activity can be retained for several months by storage at low temperature, prefer-

TABLE I

THE COMPOSITION OF CHROMATOPHORES AND MEMBRANE VESICLES FROM *RHODOPSEUDOMONAS SPHAEROIDES*

Membrane component	Chromatophores	Membrane vesicles
Lipid/protein ratio (w/w)	0.28	0.90
Fatty acids (percentage of total fatty acids)		
16 : 0	2.3	2.6
16 : 1	3.2	3.4
18 : 0	4.3	2.9
18 : 1	89.3	88.4
20 : 1	0.9	2.7
Bacteriochlorophyll (μg/mg protein)	14	0.55
Carotenoid (μg/mg protein)	9.9	0.33
NADH dehydrogenase (nmol NADH · min^{-1} · mg^{-1} protein)	104	71.8
NADH oxidase (nmol O_2 · min^{-1} · mg^{-1} protein)	39.6	37.7
Succinate dehydrogenase (nmol succinate · min^{-1} · mg^{-1} protein)	115	223
Succinate oxidase (nmol O_2 · min^{-1} · mg^{-1} protein)	88.3	176
ATPase (nmol ATP · min^{-1} · mg^{-1} protein)	104	6

ably in liquid nitrogen. However, membrane vesicles and chromatophores of *R. sphaeroides* lose their light-dependent transport activities upon storage, and attempts to find storage conditions under which transport activity is retained have been unsuccessful.

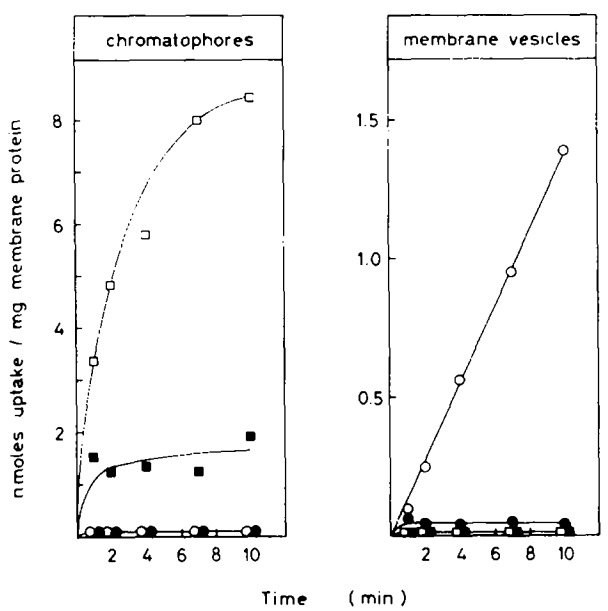

Fig. 5. Uptake of Ca^{2+} and L-alanine by chromatophores and membrane vesicles from *R. sphaeroides*. Transport studies were performed under anaerobic conditions as described in Materials and Methods. Alanine uptake was assayed with 6.0 mg vesicle protein per ml or 2.8 mg chromatophore protein per ml. L-Alanine concentration: 13.7 μM. For calcium transport measurements membranes were suspended in 10 mM Tris · HCl, 10 mM potassium phosphate and 150 mM KCl, adjusted to pH 8.0 to a protein concentration of 2.0 mg/ml for vesicles and of 7.2 mg/ml for chromatophores. Calcium concentration: 1 mM. ○, alanine uptake in the light; ●, alanine uptake in the dark; □, Ca^{2+} uptake in the presence of light; ■, Ca^{2+} uptake in the dark.

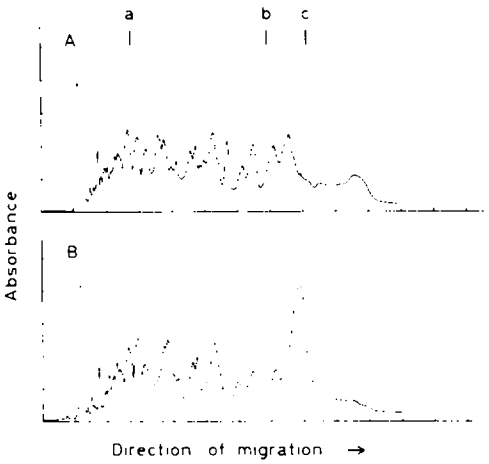

Fig. 6. Protein pattern of chromatophores (A) and membrane vesicles (B) from *R. sphaeroides* obtained by sodium dodecyl sulphate-polyacrylamide slab gel electrophoresis. Indications show position of reference proteins: a, bovine serum albumin (Sigma Chemical Co., Saint Louis, Mo., U.S.A.), M_r = 68 000; b, trypsin inhibitor, soybean (Sigma), M_r = 21 500; c, cytochrome *c*, horse heart (Boehringer, Mannheim GmbH, Mannheim), M_r = 13 400.

Chemical composition

Membrane vesicles contain about equal amounts of lipid and protein while chromatophores contain much less lipid than protein (Table I). In both preparations the lipids are predominantly phospholipids, and thin-layer chromatography showed phosphatidylethanolamine and phosphatidylglycerol to be the major components (data not shown). The fatty acid composition of the phospholipids is very similar in both preparations. The major fatty acid is a C_{18} monoenoic acid (18 : 1). A slightly higher content of a C_{18} saturated acid (18 : 1) is found in chromatophores, while vesicles contain slightly more C_{20} monoenoic acid (20 : 1).

Protein composition was studied by polyacrylamide electrophoresis of SDS-solubilized preparations. Fig. 6 shows that both preparations have a number of identical proteins.

Chromatophores contain 25—30-fold more bacteriochlorophyll and carotenoid than membrane vesicles (Table I).

The activity of several enzymes has been determined. Both membrane vesicles and chromatophores contain NADH dehydrogenase, NADH oxidase, succinate dehydrogenase and succinate oxidase. The activity of the NADH enzymes is slightly higher in chromatophores than in membrane vesicles while the activities of the succinate enzymes are about 2-fold higher in membrane vesicles than in chromatophores. The dehydrogenase activities were also determined in membrane preparations treated with toluene (1%, v/v). Such a treatment resulted in a 2-fold increase of the activity of succinate dehydrogenase in membrane vesicles, whereas the activity in chromatophores was not affected. No effect was observed on NADH dehydrogenase in membrane vesicles or chromatophores.

Discussion

For several reasons, the procedure described in this paper for the isolation of membrane vesicles from *R. sphaeroides* is much more complicated than those described for membrane vesicles of other bacteria [2,3]. First, spheroplasts which are osmotically sensitive can only be obtained from *R. sphaeroides* grown under light intensities which suppress the formation of invaginations of the cytoplasmic membrane. Secondly, light-driven cyclic electron transfer coupled to active solute transport is only retained when the redox potentials of the media are maintained between 0 and 100 mV during the isolation of membrane vesicles. Thirdly, in order to prevent loss of cytochrome c_2 loosely bound at the outer surface of the cytoplasmic membrane vesicles, gentle homogenization is required.

Previous attempts to isolate membranes from *Rhodospirillaceae* by osmotic lysis of spheroplasts resulted in ghost-like membranous material rather than vesicular structures [29,30]. Similar structures were observed upon lysozyme-EDTA treatment of *R. sphaeroides*, grown under limited light intensities [9]. The ghost-like structures result from the unfolding of the many invaginations of the cytoplasmic membrane due to internal hypertonic pressure. Hochman et al. [31] recently described a procedure that involves passage of cells through a Yeda pressure cell at mild pressures. In addition to the regular chromatophores a fraction of so-called heavy chromatophores is obtained, composed of broken cell envelopes containing closely packed vesicles enclosed in a cytoplasmic membrane.

A number of characteristics of membrane vesicles obtained by the lysozyme-EDTA procedure have been compared with chromatophores. Chromatophores have been isolated by mechanical breakage of cells which are grown under low light intensity and therefore produce many invaginations of the cytoplasmic membrane. These two types of membrane preparations from *R. sphaeroides* clearly have an opposite orientation: Chromatophores are inside out and membrane vesicles right-side out with respect to the orientation of the cytoplasmic membrane in intact cells. This is demonstrated by freeze-etch electron microscopy, and by studies on the localization of cytochrome c_2 and ATPase. Moreover, membrane vesicles perform just like whole cells [9] active transport of L-alanine but not of Ca^{2+}; chromatophores perform light-driven active transport of Ca^{2+} but not of L-alanine. Evidence has been presented that extrusion of Ca^{2+} is a general characteristic of bacterial cells [32] and that inverted membrane preparations of *Bacillus megatherium* [33], *E. coli* [7,19] and chromatophores of *R. capsulata* [32] perform energy-dependent accumulation of Ca^{2+}.

We have studied in detail the generation of a proton motive force upon illumination in membrane vesicles and chromatophores. These studies will be described in a subsequent paper [45] but several observations offer further support for a right-side out orientation of membrane vesicles and an inside out orientation of chromatophores. In short, illumination of chromatophores results in a transmembrane pH gradient, inside acid, and a membrane potential, inside positive; illumination of membrane vesicles on the other hand results in a transmembrane pH gradient, inside alkaline, and a membrane potential, inside negative.

Attempts have also been made to obtain information about the orientation of the membranes from enzyme activity measurements, but the results are difficult to interprete. Similar problems have been encountered in enzyme studies of membrane preparations from *E. coli* [34,35]. In order to explain the enzyme activities found in the membranes and the effects of toluene on these activities knowledge is required about the permeability of the membranes for these substrates and the exact site of interaction of the electron acceptor with the electron transfer system [34,35].

Chromatophores are isolated from cells which are grown under distinctly different conditions than the cells from which the vesicles are isolated. It is, therefore, not surprising that quantitative differences are found in the chemical composition of the membranes. The chromatophores have a higher protein lipid ratio and contain more photosynthetic pigments, i.e. bacteriochlorophyll and carotenoids, than the membrane vesicles. It is of interest that qualitatively the chemical compositions of the chromatophores and the membrane vesicles are very much alike. Disc gel electrophoresis of solubilized membranes demonstrate a number of corresponding protein bands in chromatophores and membrane vesicles. Furthermore, both preparations have a similar lipid composition; the lipids are nearly all phospholipids (especially phosphatidylethanolamine and phosphatidylglycerol). The fatty acid compositions in both preparations are almost identical and very similar to the fatty composition of intact cells [36,37].

Also in functional aspects both preparations differ only quantitatively. The photosynthetic apparatus is present in both preparations as is demonstrated by the content of phtosynthetic pigments and cytochrome c_2. This photosynthetic apparatus is functional in both preparations because light energy can be used to drive active transport processes. The photosynthetic pigment therefore has to be present in the cytoplasmic membrane and these observations argue strongly against the conclusion reached by Niederman et al. [38,39] that the photosynthetic apparatus is confined to intracytoplasmic membranes. This conclusion was based on the differences in pigment contents of different membrane fractions from *R. sphaeroides.*

These observations strongly suggest that the cytoplasmic membrane and the intracytoplasmic membranes are no distinct entities and that the intracytoplasmic membrane forms a continuous system with the cytoplasmic membrane. Based on other lines of evidence other investigators reached the same conclusion for *R. sphaeroides* [40—42], *Rhodospirillum rubrum* [40,43] and *R. capsulata* [42].

In view of this conclusion it is of interest that chromatophores do not perform active transport of L-alanine when an aritifical membrane potential (inside negative) is imposed. According to the chemiosmotic coupling theory transport carriers should be able to translocate solutes in both directions when the proper proton motive force is applied. Our observation seems to question this contention.

Acknowledgements

We are indebted to Mrs. M. Boekhout, Dr. P.J.C. Kuiper, Mr. J. van Randen and Mr. M. Veenhuis for their help in several technical aspects of this investigation.

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