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Isolation of the plasma-membrane of the halotolerant alga *Dunaliella salina* using sulforhodamine B as a probe

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Sulforhodamine B, a fluorescent substance, was found suitable for specific labeling of the plasma-membrane of *Dunaliella*. Fluorescence microscopy showed that sulforhodamine B labeled the cell surface but did not penetrate the cells. More than one-third of the fluorescence of the cells and about 90% of the fluorescence of the membrane fractions were covalently bound to proteins. Much of the sulforhodamine B was bound to proteins which appeared in the soluble fractions and are apparently peripheral membrane proteins, possibly belonging to the proposed 'cell coat'. Methods for labeling the cells and for quantitative measurement of sulforhodamine B fluorescence in the various fractions were developed. A method was developed for mildly breaking the cells and so minimizing rupture of intracellular organelles. It involves a 2-fold osmotic dilution at low ionic strength. Under these conditions, a substantial amount of plasma-membrane was released from the cells in the form of tiny vesicles. Due to the gentle breakage of the cells, chloroplasts – and apparently other large organelles – remained intact and were removed completely by differential centrifugation. Partial purification of the plasma membrane was achieved by a short centrifugation on a layer of Ficoll, followed by centrifugation on a 0–60% sucrose density gradient. The purified plasma membrane preparation possessed a magnesium-dependent and vanadate-sensitive ATPase activity and was essentially free of chlorophyll, and low in galactolipids.

Introduction

Dunaliella is a genus of cell-wall-less unicellular green algae which are found naturally in medium to extreme saline habitats. Some of these habitats, such as shallow ponds, undergo extreme fluctuations of salinity and temperature due to evaporation and flooding. *Dunaliella* rapidly adapts to such fluctuations through an efficient osmoregulatory mechanism. Since *Dunaliella* lacks a rigid cell-wall, a rare phenomenon in the plant kingdom, osmotic changes in the external medium result in rapid changes of cell volume. The high salt tolerance of *Dunaliella* and its osmoregulatory mechanism have been subjects of extensive studies [1]. It has been found that glycerol is the major intracellular osmotic component. At high osmolarity, glycerol content exceeds 50% of the total algal dry weight, and its average intracellular concentration is over 4 M.

In the absence of a cell wall, the plasma membrane acts as the only barrier between the intracellular content, with its high glycerol concentration, and the extracellular medium, with its high salt concentration. Some authors concluded that the plasma-membrane is very permeable to inorganic ions and even to relatively high-molecu-

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lar-weight compounds [2,3]. Intracellular salt concentrations were reported to be around half the extracellular values [4–6]. Other investigators measured much lower values [7–12] even when the cells were grown in high salt concentrations. This discrepancy seems to arise from the methodological difficulties involved in determining low amounts (intracellular salt content) in the presence of massive amounts (external salt content). Independent findings support the notion that the intracellular salt (NaCl) concentrations are much lower than those in the ambient medium. (a) A variety of biochemical activities in cell-free systems from *Dunaliella* have been found to be sensitive to salt concentrations in a manner resembling the respective systems from non-halotolerant species [7,13–16]. They are severely inhibited by sodium chloride in concentrations much lower than those in which the cells thrive [7–9,13–15, 17–20]. (b) The intracellular glycerol concentration accounts, in high-salt grown cells, for at least 80% of the required intracellular osmotic pressure [4,21,22]. The suggestion that the plasma-membrane but not the chloroplast membrane is freely permeable to salts [6] is inconsistent with the observation that microscopically the chloroplast and the cytoplasm respond similarly to osmotic changes (e.g., Ref. 23).

The plasma-membrane of *Dunaliella* must therefore withstand high concentration gradients of both salt and glycerol, in opposite directions, in order to protect the cell interior from penetration of deleterious amounts of salts and from extensive loss of glycerol. This implies low permeability to salts and to glycerol, and an energy-consuming mechanism to exclude excess salt which may penetrate. Indeed, Brown et al. [24], using NMR methods, found that glycerol permeability of intact cells was extremely low (half-time at 17°C of 400 h). Degani and Avron [25] found by similar techniques that the water permeability of such cells was similar to other cells or model membranes.

The very unusual properties which the plasma-membrane must possess in order to fulfill its function in *Dunaliella*, prompted us to undertake a more careful study of this cell component. Two previous attempts to isolate and characterize the plasma-membrane of *Dunaliella* have been reported. Jokela [14] obtained a heterogeneous mixture of membranes, but little evidence was provided for its origins. Kaaden and Gimmler [26] and Gilmour et al. [27] recently described a vanadate-sensitive, magnesium-dependent ATPase activity in a purified fraction which they concluded to be the plasma-membrane, but no clear evidence was provided for its identity or purity.

Materials and Methods

Growth conditions

Dunaliella salina was obtained from the culture collection of Dr. Thomas, La Jolla, CA. The cells were grown in batch culture, with periodic dilution to maintain them in the logarithmic growth phase.

The growth-medium contained 1 M NaCl, 50 mM $NaHCO_3$, 5 mM KNO_3, 5 mM $MgSO_4$, 0.3 mM $CaCl_2$, 0.2 mM KH_2PO_4, 0.185 mM H_3BO_3, 7 μM $MnCl_2$, 6 μM Na_2EDTA, 1.5 μM $FeCl_3$, 0.8 μM $ZnCl_2$, 20 nM $CoCl_2$ and 0.2 nM $CuCl_2$; initial pH was 8.0. The growth-medium was not sterilized, since, at this salt concentration, significant contamination by other algae did not occur. Cell suspensions in low-form culture flasks were shaken (80 rpm) in a New Brunswick controlled environment incubator shaker model G-27 at 26°C. Continuous illumination of 300–350 foot-candles was supplied from cool white fluorescent tubes (Sylvania). Under these conditions the doubling time was 6–7 h.

Cell concentration was determined by a model F Coulter Counter with a 100 μm orifice, after dilution in 1 M NaCl to $(1–4) \cdot 10^4$ cells $\cdot$ ml^{-1}.

For harvesting, about $7 \cdot 10^9$ cells (approx. $1 \cdot 10^6$ cells $\cdot$ ml^{-1}) were filtered through cotton wool and Miracloth followed by centrifugation in a swinging bucket rotor at $750 \times g$ at 4°C for 5 min. They were washed by centrifugation and resuspension in 50–100 ml growth medium three times before cell breakage.

Labeling of the cell surface with sulforhodamine B

Sulforhodamine B (Lissamine rhodamine B) was obtained from Polysciences Inc. The sulfonyl-chloride form of sulforhodamine B was prepared according to Chadwick et al. [28]. Appprox. 0.2 g sulforhodamine B was mixed for a few minutes in

a mortar with 0.4 g phosphorus pentachloride. The paste thus formed was dissolved in about 5 ml anhydrous acetone and filtered through filter paper. The solution, of 15–20 mM sulforhodamine B chloride, was stored anhydrous at 20°C and was sufficiently active for at least 2 months. All labeling steps were carried out at 0–4°C. Cells were harvested with one washing in growth-medium which had been preadjusted to pH 9.2. They were then resuspended in a small volume to obtain $(3–4) \cdot 10^8$ cells $\cdot$ ml^{-1}. The sulforhodamine B chloride solution was added while vortexing, to obtain a final concentration of 10–12 μM. After 10 min, the suspension was diluted to 100 ml with growth medium (pH 9.2) and the cells were spun down. One additional washing was made in this medium and then two washings in growth-medium.

Fluorescence microscopy and micrography

Zeiss Standard or Photomicroscope-III microscopes equipped with epi-illumination fluorescence accessories were used. The light source was a 50 W mercury lamp, the standard filter set contained 510-560 BP as exciter filter; 580 FT as dichromatic beam splitter; and 590 LP as barrier filter. The objective was Planapochromat 63/1.4. To eliminate chlorophyll fluorescence, a 620 SP filter was inserted as an additional barrier filter. For photomicrography, sulforhodamine B labeled cells in growth-medium were fixed in 0.03% glutaraldehyde. Kodak Tri-X film was exposed for 15–20 s and development-enhanced by Diafine 1600.

Fluorimetry

Samples were dissolved in 1–5% SDS, either directly or after precipitation in 96%(v/v) acetone. Sulforhodamine B fluorescence was measured in a model MPF-44A Perkin-Elmer spectrophotofluorimeter. Excitation light was 530 nm. Arbitrary fluorescence intensity units were determined using standard settings of the instrument.

Preparation of the plasma-membrane

All steps were at 0–4°C, except when otherwise stated. A pellet containing $(7–8) \cdot 10^9$ cells was resuspended in 50 ml 1.6 M glycerol/10 mM Mops(Na) (pH 7.5) (buffer A), and spun down immediately ($1200 \times g$ for 3 min). The pellet was resuspended in 60 ml 1.6 M glycerol/5 mM NaCl/10 mM Mops(Na) (pH 7.5) (buffer B). The suspension was slowly stirred with a magnetic rod, an equal volume of water was added and the mixture stirred for 25 min (10 min for the experiments reported here, see below) at 10–12°C. It was centrifuged in a Sorvall HB-4 swinging-bucket rotor at 5000 rpm ($3000 \times g$) for 5 min. The supernatant was centrifuged in SW27 rotor at 24000 rpm ($76000 \times g$) for 1 h, and the pellet was resuspended and homogenized in 6 ml 0.8 M glycerol/5 mM Mops(Na) (pH 7.5) (buffer C). It was then layered in four SW41 tubes each containing 9.5 ml of 8% (w/v) Ficoll-400 in buffer C. Following centrifugation at 30000 rpm ($110000 \times g$) for 15 min, a white band was formed at the interface. This band was loaded in two SW41 tubes on 10 ml of 0–60% (w/w) linear sucrose density gradient in buffer C and centrifuged at 35000 rpm ($150000 \times g$) for 150 min. Fractions of 0.4 ml were collected through a capillary starting at the bottom of tube. The five fractions rich in plasma-membrane (peaking at 35% sucrose: band II) were combined, diluted to 24 ml with buffer C and centrifuged at 35000 rpm ($150000 \times g$) for 1 h. The pellet was resuspended and homogenized in a small volume of 20% ethylene glycol in Buffer C. The plasma-membrane suspension was then stored at −70°C until used.

General methods

Protein precipitation. Acetone was added at 0°C to obtain 80%(v/v), and, after vortexing, more acetone added to obtain 96%(v/v). After 10 min, the suspension was centrifuged at $2000 \times g$ for 5 min and the precipitate dissolved in 1–5%(w/v) SDS.

Protein. Protein was determined according to Lowry et al. [29] as modified for lipid-rich samples by Markwell et al. [30], except that the absorbance was measured at 740 nm. Bovine serum albumin was used as a standard. Appropriate corrections were made for the small interferences by sucrose and glycerol.

Sucrose. Sucrose concentration in the sucrose gradient was determined by refractometry, using refraction index values [31] after correcting for the interference by 0.8 M glycerol.

Chlorophyll. Chlorophyll was determined in

80–96% (v/v) acetone extracts according to Arnon [32].

Results

Labeling of the plasma-membrane with sulforhodamine B

As can be seen in the fluorescence micrograph of Fig. 1, sulforhodamine B labeled the cell surface but did not penetrate intact cells. A relatively uniform fluorescence intensity is seen throughout the cell population and throughout the individual cell surfaces, including the two flagella. The cells had normal shape and – when not fixed by glutaraldehyde – were in rapid motion. When the sulforhodamine B chloride to cell ratio was $(1–8) \cdot 10^{-17}$ mol · cell^{-1}, the labeling intensity was linearly correlated to the sulforhodamine B chloride to cell ratio (Fig. 2); and the cells were sufficiently labeled as determined by fluorescence microscopy. Under these conditions, excess sulforhodamine B could be washed by three or four centrifugations. When the cells were added to a labeling solution which contained the non-reactive (sulfonate) form of sulforhodamine B, hardly any fluorescence was seen on the cell surface (not shown), indicating that the sulfonyl chloride group is essential for binding.

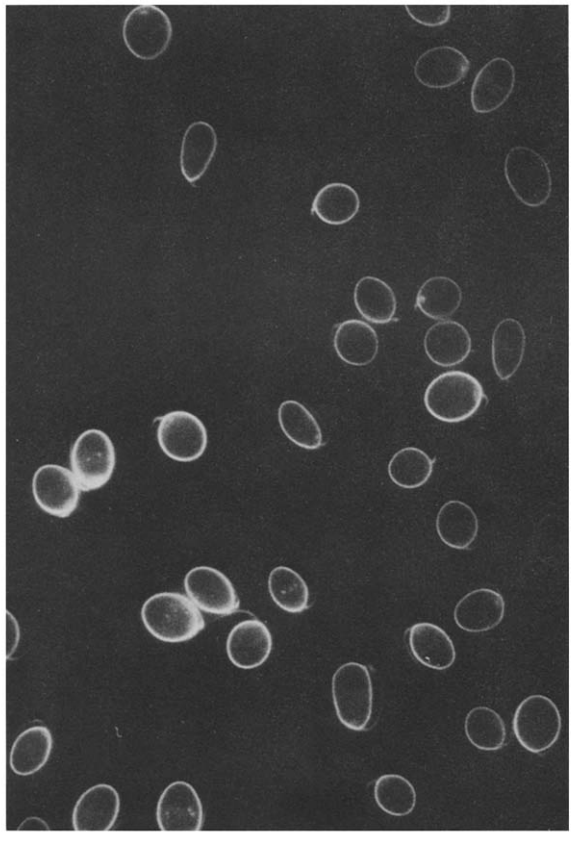


Fig. 1. A fluorescence micrograph of sulforhodamine B-labeled cells of *D. salina*. Cells were exposed for 20 min to sulforhodamine B chloride at $5.8 \cdot 10^{-17}$ mol·cell^{-1}, as described under Materials and Methods.

Not all the nonwashable sulforhodamine B was covalently bound. Most of it was found in the acetone-soluble fraction of the cells, indicating that it was not covalently bound to proteins (Fig. 3; Table I). Thin-layer chromatography of the acetone-soluble fraction on silica gel G showed two sulforhodamine B-fluorescent spots: one migrated like free sulforhodamine B and the other, with a higher R_F value, appeared to be of sulforhodamine B covalently bound to a neutral membrane lipid.

For quantitative measurements, samples were dissolved in 1–5% (w/v) SDS, which yielded a homogeneous suspension and largely eliminated artifacts due to light scattering. Sulforhodamine B fluorescence intensity of membrane fractions in

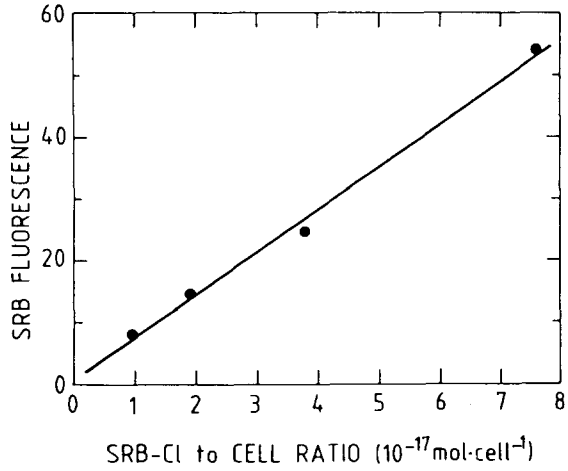


Fig. 2. Labeling intensity as a function of sulforhodamine B chloride to cell ratio. The sulforhodamine B chloride to cell ratio was varied by the volume of the labeling suspension, using a constant amount of cells and a constant sulforhodamine B chloride concentration of 12 μM. Sulforhodamine B fluorescence intensity was determined after washing the cells of the unbound sulforhodamine B and dissolving directly in 1% SDS.

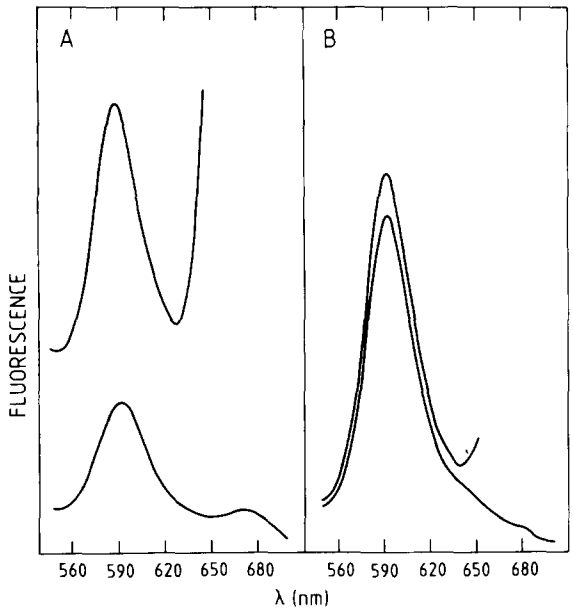


Fig. 3. Uncorrected fluorescence emission spectra of sulforhodamine B-labeled fractions. Whole cells (A); or Pellet 2 (B) were dissolved in SDS either directly (upper curves) or after acetone precipitation (lower curves). Excitation was at 530 nm.

SDS was linear with concentration and the recovery of fluorescence was complete throughout the purification steps.

TABLE I

PARTITIONING OF SULFORHODAMINE B FLUORESCENCE BETWEEN ACETONE-SOLUBLE AND INSOLUBLE FRACTIONS IN LABELED WHOLE CELLS AND IN PELLET 2

Samples were measured in 5% (w/v) SDS either directly or after extraction in 96% (v/v) acetone. The acetone-soluble fraction was measured in 96% (v/v) acetone. Excitation of all samples was at 530 nm.

Sample	Sulforhodamine B fluorescence emission	
	Approx. λ_{max}	relative intensity at λ_{max}
Whole cells		
direct	589	(1.00)
acetone-insoluble	593	0.36
acetone-soluble	573	0.68
Pellet 2		
direct	593	(1.00)
acetone-insoluble	593	0.88
acetone-soluble	575	0.13

Cell breakage and the release of the plasma-membrane

To obtain cell breakage under mild conditions, we utilized a previous observation that cells were much more fragile when suspended in isotonic glycerol. To ensure complete removal of the salts carried over from the growth-medium, the cells were rapidly washed and resuspended in buffered isotonic glycerol. The conditions were then varied to obtain an efficient cell breakage with maximal release of the sulforhodamine B-labeled plasma-membrane, while keeping rupture of intracellular organelles to a minimum. Cell breakage and the release of the plasma-membrane were followed by fluorescence microscopy, and quantitatively by estimation of plasma-membrane release by fluorimetry of Pellet 2. Early steps of purification are summarized in Table II.

Only a fraction of the cells broke when the final suspension was in isotonic solution, after mild homogenization. A 2-fold osmotic dilution with continuous stirring was found sufficient to ensure fast and complete cell breakage, without apparent harm to the chloroplasts. Homogenization at the end of the cell breakage caused aggregation of membranes with broken cells and resulted in a lower yield of free membranes. Somewhat higher yields of plasma membrane were obtained when stirring was continued for 20–30 min rather than the 10 min used in the experiments reported herein.

The release of plasma-membranes increased with temperature and, up to 15°C, resulted in a proportional increase of total protein in Pellet 2. Above 20°C, cell breakage and the release of plasma-membranes was faster and somewhat more extensive but was accompanied by breakage of the chloroplasts. Cell concentration during breakage was kept around $5 \cdot 10^7$ cells $\cdot$ ml^{-1}. Higher cell concentrations (above $1 \cdot 10^8$ cells $\cdot$ ml^{-1}) resulted in poorer yields, and more extensive breakage of chloroplasts.

As was ascertained by fluorimetry (Table II) and fluorescence microscopy, much plasma-membrane remained in Pellet 1 under all conditions. Resuspension of this pellet resulted in the rupture of many chloroplasts with little release of additional plasma-membrane.

TABLE II

SULFORHODAMINE B (SRB) PROTEIN AND CHLOROPHYLL RETENTION DURING THE EARLY PURIFICATION STEPS

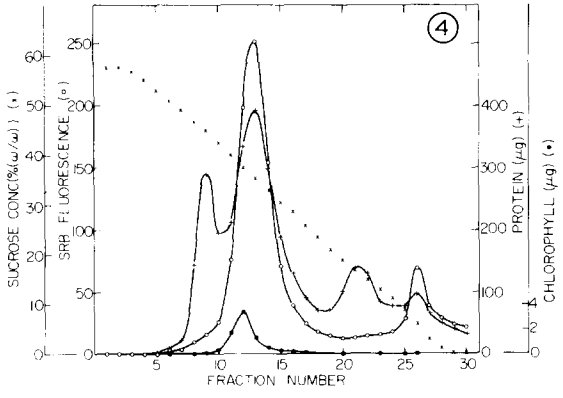
Supernatant 0 was centrifuged in SW41 tubes at 30000 rpm to separate particulate from soluble material. Sulforhodamine B and protein were determined after acetone precipitation, as in Materials and Methods. Values in parentheses are percent of whole cells, or – for sulforhodamine/protein – relative to whole cells.

Fraction	SRB	Protein ($pg \cdot cell^{-1}$)	SRB/protein	Chlorophyll ($pg \cdot cell^{-1}$)
Supernatant 0	106 (10.0)	0.28 (1.0)	375 (10.0)	0.008 (0.3)
soluble	52 (4.9)	0.19 (0.7)	270 (7.2)	0
particulate	38 (3.6)	0.07 (0.2)	555 (14.8)	0.008 (0.3)
Homogenate	958 (90.0)	28.1 (99.0)	34.1 (0.91)	2.340 (99.7)
Pellet 1	535 (50.3)	17.5 (61.6)	30.6 (0.82)	2.30 (98.0)
Supernatant 1	336 (31.6)	8.27 (29.1)	40.6 (1.08)	0.020 (0.9)
Pellet 2	138 (13.0)	1.35 (4.8)	102 (2.7)	0.020 (0.9)
Supernatant 2	203 (19.1)	6.56 (23.1)	31.0 (0.83)	0

Purification

Following centrifugation, most of the plasma-membrane is retarded at the interface when layered on a Ficoll cushion at low Ficoll concentrations, while the residual thylakoids are found in the pellet.

The purifying effect of Ficoll layering is demonstrated by comparing Figs. 4 and 5, which show sucrose density gradients of Pellet 2 as is or following Ficoll layering, respectively. Clearly, Ficoll layering has not only removed most of the remaining thylakoids, but has been also quite efficient in removing other non-relevant cell components. Four protein bands were separated on a sucrose density gradient of Pellet 2 (fig. 4). Band I, at 45% sucrose, is presumably mitochondria, and has high activity of alkaline ATPase. This band was removed to a large extent by Ficoll layering (Fig. 5),



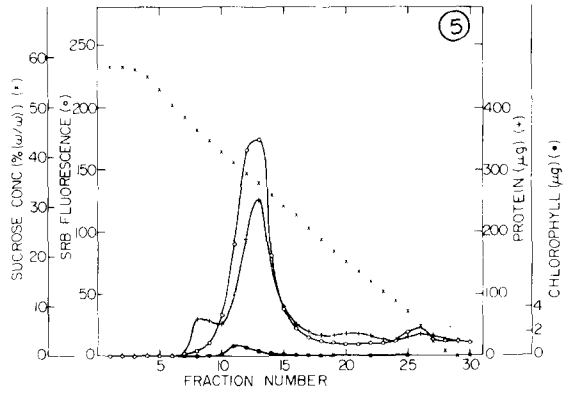


Fig. 4. Sucrose density gradient of Pellet 2. Pellet 2 was resuspended in 1.8 ml of buffer C and loaded on 11 ml of 0–60% (w/w) sucrose gradient as in Materials and Methods. Sulforhodamine B (○), protein (+), chlorophyll (●), and sucrose (×) were determined as in Materials and Methods.

Fig. 5. Sucrose density gradient of Pellet 2 after Ficoll layering. Pellet 2, from the same experiment as that of Fig. 4, was layered on Ficoll as in Materials and Methods. The band at interface was collected and loaded directly in 11 ml of 0–60% (w/v) sucrose gradient, as in Materials and Methods. Sulforhodamine B (○), protein (+), chlorophyll (●), and sucrose (×) were determined as in Materials and Methods.

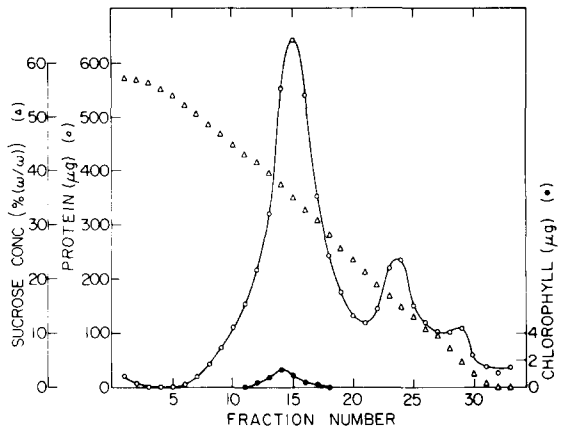


Fig. 6. Sucrose density gradient of modified Pellet 2. Supernatant 1 was centrifuged in a Sorvall HB-4 rotor at 8000 rpm for 15 min. The resulting supernatant was centrifuged as in Materials and Methods for Supernatant 1. The pellet was resuspended in 1.5 ml of buffer C, and loaded on 10.5 ml of 0–60% (w/w) sucrose gradient, as in Materials and Methods. Protein (○), chlorophyll (●), and sucrose (△) were determined as in Materials and Methods.

or by differential centrifugation of Supernatant 1 (Fig. 6). Band II, at 35% sucrose, is the largest protein band of the gradient, and corresponds to the major part of the sulforhodamine B-labeled plasma-membrane. Thylakoid membranes band at 37.5% sucrose, and overlap the lower part of band II. Most of the thylakoids were removed either by Ficoll layering (Fig. 5) or by differential centrifugation (Fig. 6). Thylakoid membranes contained about 1% of the total protein of the plasma-membrane preparation (see Discussion). Band III, at 16% sucrose, was largely removed by Ficoll layering. Its identity is unknown. Band IV, at 6% sucrose, although of low protein content, has high sulforhodamine B fluorescence and thus might be related to the plasma-membrane.

As shown in Table III, Ficoll layering followed by sucrose density gradient resulted in membranes which were 2.3-times purer than Pellet 2. The sucrose density gradient alone resulted in a somewhat smaller purity but a higher yield.

The plasma-membrane preparation contained two magnesium-dependent ATPase activities with maximal activities around pH 7.0 and pH 9.0, respectively, each with a specific activity of about 5 μmol P_i · mg protein per · h. The phosphohydrolase activities were lower (20–40%) for ADP and very low (1–5%) for AMP and *para*-nitrophenylphosphate. No evidence was found for a specific

TABLE III

SULFORHODAMINE B (SRB) PROTEIN AND CHLOROPHYLL CONTENT OF FRACTIONS DURING PURIFICATION

Sulforhodamine B and protein were determined either directly or after acetone precipitation, as indicated. Sucrose gradient refers to the combined five largest fractions of band II. Values in parentheses are percent of whole cells, or – for SRB/protein – relative to whole cells.

	SRB	Protein (pg·cell^{-1})	SRB/protein	Chlorophyll (pg·cell^{-1})
Cells				
direct	11930	35.9	–	–
acetone-precipitate	4290 (100)	32.2 (100)	133 (1)	–
acetone-extract	8080	–	–	2.155 (100)
Pellet 2				
direct	575 (13.4)	1.88 (5.8)	306 (2.30)	–
acetone-precipitate	502 (11.7)	1.79 (5.6)	280 (2.10)	–
acetone-extract	70	–	–	0.004 (0.17)
Ficoll layering				
direct	377 (8.8)	0.67 (2.1)	562 (4.22)	0.001 (0.05)
Sucrose gradient				
direct	383 (8.9)	0.73 (2.3)	527 (3.96)	0.003 (0.16)
Ficoll layering and sucrose gradient				
direct	259 (6.0)	0.36 (1.1)	717 (5.39)	0.001 (0.04)

enhancement of these activities by potassium or sodium. The neutral ATPase activity was partly inhibited by orthovanadate and calcium.

The lipid composition of the plasma-membrane preparation was studied in detail and is described in the accompanying article [33].

Discussion

Since *Dunaliella* lacks a rigid cell wall, the isolation of its plasma-membrane should, in principle, be easier than that of other plants. Nevertheless, there are very few reports dealing with cell fractionation of *Dunaliella*. Kombrink and Wober [34] used polycations for controlled cell disruption and obtained a fraction of intact chloroplasts after sucrose density centrifugation. Lynch and Thompson [35] disrupted the cells in a hypotonic solution of low ionic strength in a Parr Bomb, and fractionated the cells by differential centrifugation into three particulate fractions: chloroplast, intermediate (containing mitochondria, Golgi apparatus and flagella), and 'microsomal fraction'. Gimmler and co-workers [20,26,27] used a Yeda Press to facilitate cell disruption in a hypotonic solution. They fractionated the microsomal fraction by discontinuous sucrose gradient centrifugation and described three ATPases in the microsomal fraction, one of which was attributed to the plasma-membrane. In an attempt to isolate the plasma-membrane, Jokela [14] broke the cells by a 20-fold osmotic dilution. After discontinuous sucrose density centrifugation she obtained a preparation which was contaminated heavily by thylakoid membranes.

The plasma-membrane of *Dunaliella* has no known property that can be used a priori as a specific marker to be traced during isolation. However, plasma-membranes of intact cells can be labeled specifically with a detectable nonpermeant extrinsic probe that can be traced as if it were an inherent constituent of the plasma-membrane. Several radioactive and fluorescent labels have been introduced in the last 2 decades, but not all have proved satisfactory [36–39]. Sulforhodamine B (synonyms: Lissamine Rhodamine B; RB 200), a bright orange fluorescent substance, was introduced in 1958 by Chadwick et al. [28,40] as a protein tracer and has since been used in immunological studies. The low membrane permeability expected from its chemical nature has been confirmed in mammalian cells [41] and in this report in *Dunaliella salina*.

Sulforhodamine B was shown by fluorescence microscopy to label the cell surface without penetrating the cells and without affecting cell viability. The sulfonyl chloride group is essential for binding, since the nonreactive (sulfonate) form does not bind significantly to the cell surface. However, a large fraction of the label does not bind covalently and is extracted by acetone (or chloroform/methanol) as a free dye. The fast hydrolysis in water of sulforhodamine B chloride to the nonreactive form rules out the possibility that this free dye can bind covalently to other cell components after cell breakage. The method suffers from the inevitable disadvantages of nonspecific covalent labeling of an external cell surface: it results not only the labeling of the external surface of the plasma-membrane, but also in the labeling of extracellular constituents and extraneous particles. Nevertheless, sulforhodamine B labeling has proved to be an efficient and reliable method for general labeling of the plasma-membrane. The possibility that sulforhodamine B chloride does penetrate but that its fluorescence is quenched within the cells is unlikely for two major reasons: (a) its fluorescence intensity is not affected over a wide range of conditions, [42,43]; and (b) the very few dead cells present in each preparation were labeled intracellularly very strongly. Other advantages of sulforhodamine B are its being water-soluble, rapidly reacting and non-perturbant. We show that sulforhodamine B can be used for quantitative tracing, by solubilizing the samples in SDS and so eliminating light scattering artifacts. By acetone treatment one can separate protein-bound from soluble sulforhodamine B (comprised of free and lipid-bound sulforhodamine B).

The enrichment of plasma-membranes relative to other cell components was determined by the ratio of sulforhodamine B fluorescence to the total protein. This approach leads to an underestimation of the purification when whole cells, the Homogenate, and Pellet 1 were measured. A large quantity of peripheral proteins, many of which probably comprise the 'cell coat' [44–46], are ex-

posed to the external medium and hence are labeled even more extensively than the integral constituents of the plasma-membrane. These proteins are released into the soluble fraction upon cell breakage. The approach is, however, appropriate in particulate fractions which do not contain extraneous particles, i.e., Pellet 2 and more purified fractions of the plasma-membrane. Consequently, the enrichment of the plasma-membranes in Pellet 2 as compared to the whole cells is very much underestimated.

A 2-fold osmotic dilution at low ionic strength proved very efficient in ensuring fast and complete cell breakage. Practically no cells in Pellet 1 had intact plasma-membranes, as observed under the fluorescence microscope. Mechanical force, i.e., homogenization, did not significantly increase the release of the plasma-membrane.

The relatively simple composition of Pellet 2, as determined by the sucrose density gradient, indicates that the procedure for cell breakage and the release of the plasma-membrane was indeed mild and efficient resulting in a preferential release of the plasma-membrane. The plasma-membrane is the major fraction of the membranes in Pellet 2, based on the protein profile of the sucrose gradient. The damage to chloroplasts, as probably also to other organelles, is minimal. The mild breakage of the cells enabled the removal of the larger organelles, by short differential centrifugation. The good overlapping between sulforhodamine B fluorescence intensity and protein content in band II of the sucrose gradient suggests that the majority of the protein in this band is indeed plasma-membranes. The buoyant density agrees with the literature values for plasma-membranes of other plants [47–51].

Thylakoids are the most abundant membranes in the cells and are apt to be the major source of contamination, particularly because of the similarity of the buoyant densities of thylakoids and plasma membranes [51,52]. Calculation of the results in Table III and Fig. 6 show that the contamination from thylakoid membranes is very small. Assuming a protein-to-chlorophyll ratio in the thylakoids of 5 [14,53,54], thylakoid proteins comprise about 1% of the total protein in the plasma-membrane preparations. Digalactosyldiacylglycerol and monogalactosyldiacylglycerol, which together comprise about 6% of the total lipids in the plasma-membrane preparation [33], are the major lipids of chloroplasts [55]. Since galactolipids comprise about 60% of the total lipids in the chloroplast envelope [56], the content of chloroplast envelopes in the plasma-membrane preparation cannot exceed 8%. Finally, the high content of sterols in the preparation [33] also supports the conclusion that it is a relatively pure plasma-membrane preparation.

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