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FUSION OF CHROMATOPHORES DERIVED FROM *RHODOPSEUDOMONAS SPHAEROIDES*

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Fusion of chromatophores, the photosynthetic membrane vesicles isolated from the intracytoplasmic membranes of *Rhodopseudomonas sphaeroides*, was achieved by the use of poly(ethylene glycol) 6000 as fusogen. Ultracentrifugation, electron microscopy, intrinsic density and isotope labeling were used to demonstrate chromatophore fusion. Although studies of the flash-induced shift in the carotenoid absorbance spectrum indicated that the membrane was rendered leaky to ions by either the fusion procedure or the increased size of the fused products, the orientation and integrity of fused chromatophores were otherwise demonstrated to be identical to control chromatophores by freeze-fracture electron microscopy, proteolytic enzyme digestion, enzymatic radioiodination, and transfer of chromatophore phospholipids mediated by phospholipid exchange protein extracted from *Rps. sphaeroides*.

Introduction

Rhodopseudomonas sphaeroides, a facultative photosynthetic bacterium, develops an intracytoplasmic membrane when grown under anaerobic conditions. The amount of intracytoplasmic membrane can be altered as a function of light intensity [1,2]. This membrane exhibits a well defined function, i.e., the entrapment of light quanta and the esterification of inorganic phosphate plus ADP to ATP via a photosynthetic electron transport chain [3]. Upon cell breakage, this membrane is disrupted and reseals as small, closed vesicles (60–70 nm in diameter) so called chromatophores, which are easily purified and chemically well defined [4–6]. Recent studies in our laboratory using synchronous cultures demonstrate a non-coordinate incorporation of protein and phospholipid into the intracytoplasmic membrane during the cell growth cycle [7–11]. The continuous insertion of newly synthesized polypeptides coupled with the discontinuous accumulation of phospholipids during the cell cycle results in a cyclic change in the protein/phospholipid ratio of the intracytoplasmic membrane. These changes in membrane composition are also accompanied by changes in membrane lipid structure [12].

Our interest in studying structure-function relationships employing chromatophores has prompted investigations of chromatophore fusion. In particular, we attempted to correlate membrane microviscosity, protein/phospholipid ratio (which varies from 2.2 to 4.6 during the cell growth cycle) and intramembrane particle density and size (as revealed by freeze-fracture electron microscopy) for the first time in a well defined biological membrane. However, due to the small size of isolated chromatophores and their high radius of curva-

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RCL, RCM and RCH, reaction-center polypeptides, L, M and H, respectively; LH, light-harvesting polypeptides; PMSF, phenylmethylsulfonyl fluoride.

ture, satisfactory freeze-fractured membrane faces with exposed areas large enough to allow statistical analysis of intramembrane particle density were not readily attainable. In order to offset this difficulty we considered the possibility of producing larger vesicles with a less restrictive radius of curvature. A number of investigations have been reported which deal with the fusion of both artificial and biological membranes [13–16] as well as the successful use of poly(ethylene glycol) to promote fusion in various native membrane systems [17–22]. Therefore, poly(ethylene glycol) 6000 was employed in our studies for the fusion of chromatophores. We report here the fusion of a native membrane, the chromatophores of *Rps. sphaeroides*, whose composition can be precisely controlled. Ultracentrifugation, electron microscopy, isotope and density labeling were all used to demonstrate fusion between chromatophores.

Studies on light-dependent proton gradient formation, ATPase localization, and the asymmetric distribution of intramembrane particles in the photosynthetic bacteria indicate that the orientation of isolated chromatophores is opposite to that of the intracytoplasmic membrane of intact cells [23–27]. In other words, the outer surface of chromatophores represents the cytoplasmic surface of the intracytoplasmic membrane of whole cells. The orientation and integrity of fused chromatophores were assessed by freeze-fracture electron microscopy, proteolytic enzyme digestion, enzymatic radioiodination and transfer of chromatophore phospholipids mediated by phospholipid exchange protein extracted from *Rps. sphaeroides*.

Materials and Methods

Organism, medium and growth condition

Rps. sphaeroides strain 2.4.1 was grown photoheterotrophically on succinic acid minimal medium supplemented with 0.2% casamino acids [7] as described by Cohen and Kaplan [5]. Cells in the logarithmic phase of growth ($1.3 \cdot 10^9$ cells/ml) were harvested by centrifugation in a Sharples Super Centrifuge and stored as a cell paste at $-20°C$.

Radioactive and density labeling of cells

L-[^{3}H]Leucine-labeled chromatophores were obtained by growing cells in succinic acid minimal medium containing 1 μCi/ml L-[^{3}H]leucine and 10 μg/ml unlabeled L-leucine. L-[^{14}C]Leucine-labeled, deuterated chromatophores were obtained from cells grown in 70% ^2H_2O-succinic acid minimal medium [7] supplemented with 0.2 μCi/ml L-[^{14}C]leucine and 10 μg/ml unlabeled L-leucine. ^{32}P-labeled chromatophores were obtained from cells grown on modified, low-phosphate, succinic acid minimal medium containing 10 μCi/ml [^{32}P]orthophosphate [11]. All inocula were previously adapted in unlabeled media and cultures were grown photoheterotrophically [7] for at least four mass doublings in labeled media. Culture growth was followed turbidimetrically using a Klett-Summerson colorimeter equipped with a No. 66 filter (1 Klett unit $= 1.0 \cdot 10^7$ cells/ml).

Chromatophore isolation

Highly purified chromatophores were prepared as described by Cohen and Kaplan [5] with the following modifications. 0.1 M sodium phosphate buffer, pH 7.6, containing 5 mM EDTA and 5 mM β-mercaptoethanol was used throughout the isolation procedure. Freshly prepared phenylmethylsulfonyl fluoride (PMSF) in dimethyl sulfoxide was added to cells at a final concentration of 1 mM immediately before cell breakage in a French pressure cell. Similar additions of PMSF were also made to the cell lysate after unbroken cells and large membrane debris were removed by low speed centrifugation, and to crude chromatophores before these were loaded onto a Sepharose 2B column. The highly purified chromatophores, banding at the 20/40 interface following discontinuous sucrose gradient centrifugation, were pooled, diluted with at least four times the volume of 10 mM sodium phosphate buffer, pH 7.0, and pelleted by centrifugation at $184000 \times g$ for 1 h. The pellets were resuspended in the same buffer and the washing procedure repeated once. Isolation of radioactively labeled or deuterium-labeled chromatophores were the same as described above.

Fusion of chromatophores

Chromatophore fusion mixture containing highly purified chromatophores (approx. 12 mg protein/ml), 55% poly(ethylene glycol) (M_r 6000),

and 0.5 mM $CaCl_2 \cdot 2H_2O$ in 10 mM sodium phosphate buffer, pH 7.0, all at final concentrations were incubated at 33°C for 1 h. The mixture was vortexed for 30 s every 10 min during incubation to enhance fusion. The mixture was then diluted with 10-times the volume of the same buffer and loaded onto a 60% sucrose (w/v) cushion and centrifuged at $83000 \times g$ for 2 h. Chromatophores banding on top of the cushion were pooled and diluted in the same buffer. This procedure which removes free poly(ethylene glycol) was repeated once. Residual poly(ethylene glycol) and sucrose were further removed by ultrafiltration using an Amicon XM 100 membrane filter. The total population of fused chromatophores consisted of vesicles of various sizes. Fused chromatophores were resolved into two major but still heterogeneous peaks by rate zonal centrifugation in a discontinuous sucrose gradient (10/20/35/50/60% sucrose, w/v) at 25000 rpm in either a Beckman Ti 15 zonal rotor for 2 h or in a Beckman SW41 rotor for 2 h depending upon the quantity of material. The gradient was fractionated and recovered chromatophores were diluted with the same buffer as above and pelleted by centrifugation at $43000 \times g$ for 18 h to remove sucrose. Pelleted chromatophores were washed as above one additional time. Chromatophores were then resuspended in the same buffer for further studies. Fused chromatophores could be stored for months at liquid nitrogen temperatures in the above buffer containing 10% glycerol, final concentration.

Fragmentation of chromatophores

Fragmentation of chromatophores was achieved by storing fused chromatophores at 4°C for 3–6 weeks, then sonicating a 1 ml sample on ice in a Falcon disposable 12×75 mm culture tube for 1 min with a Branson W-350 Sonifier Cell Disruptor (continuous mode, maximum energy output).

Isopycnic density gradient centrifugation

For density determinations, chromatophores (0.3–0.5 mg protein) in 0.3 ml were mixed with 6.0 ml CsCl solution (density = 1.1963) at pH 7.4 containing 0.10 M KH_2PO_4 and 10 mM EDTA, and centrifuged in a Beckman 50.3 rotor at 32000 rpm for 36 h. Gradient fractionations and density determinations were performed as described by Leuking et al. [7]. Radioactivity in gradient fractions was determined by counting aliquots in an aqueous scintillation system [5].

Proteolytic enzyme digestions of chromatophores

Chromatophores in 10 mM sodium phosphate buffer, pH 7.0, were diluted in 50 mM NH_4HCO_3 buffer, pH 8.2, pelleted by centrifugation at $184000 \times g$ for 1 h and resuspended in the latter buffer. A protease stock solution of 5 mg/ml in water was heated at 50°C for 20 min allowing limited self-digestion of any contaminating enzymes that might be present. The digestion mixture containing chromatophores at 20 μg protein/μl and 0.25 μg protease/μl (1.25% w/w or 0.05 units/mg protein) was incubated at 33°C for 30 min. The sample was then diluted with four times the volume of buffer and pelleted by centrifugation in a Beckman Airfuge at 26 lb/inch^2 (90000 rpm) for 10 min. The pellet was washed two additional times as described above. The pellet was resuspended in the same buffer and solubilized for sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis. Trypsin and α-chymotrypsin digestions were conducted in the same buffer and at the same chromatophore concentration (20 μg protein/μl) as the protease digestion. A final concentration of 0.6 μg trypsin/μl sample (3% w/w or 7.5 units/mg protein) or 3.0 μg α-chymotrypsin/μl sample (15% w/w or 7.5 units/mg protein) was added to the sample at 37°C to initiate digestion. 0.3 μg trypsin or 1.5 μg chymotrypsin/μl sample was added to the digestion mixture at 4 and 11 h after digestion. Aliquots were removed at 2, 8, and 22 h after digestion.

Protease bound to agarose beads (75–150 μm in diameter) was used as an alternative source of protease. 1.5 ml of the enzyme-agarose suspension was centrifuged at $8700 \times g$ for 4.5 min in a Beckman Microfuge B. The pellet was resuspended in 1.0 ml 50 mM NH_4HCO_3 buffer, pH 8.2, pelleted as above then resuspended in the same buffer. Chromatophores (at 15 mg protein/ml) were digested with agarose-bound protease (2.25 units of enzyme/mg chromatophore protein) in 50 mM NH_4HCO_3 buffer, pH 8.2, containing 50 μg/ml streptomycin and 25 μg/ml cycloheximide, in a Gyrotory Model G76 water bath shaker at 37°C with gentle shaking for 42 h.

Radioiodination of chromatophores

Carrier-free $Na^{125}I$ (100 μCi/mg protein) and lactoperoxidase (2.5% w/w or 4.2 units/mg protein) were added to chromatophores at 2 mg protein/ml in 10 mM sodium phosphate buffer, pH 7.0. H_2O_2 (100 ng/mg protein) was added last to initiate the iodination reaction which was conducted at room temperature. The reaction was stopped by addition, after 7 min, of 0.1 mg tyrosine/mg protein substrate. The chromatophores were washed three times by centrifugation at $82000 \times g$ for 2 h to remove free $Na^{125}I$.

SDS polyacrylamide gel electrophoresis

10 to 14% gradient polyacrylamide SDS slab gels ($120 \times 160 \times 0.8$ mm) were prepared and run according to the method of Laemmli and Favre [28]. Samples were solubilized before electrophoresis by heating at 100°C for 90 s in 2% SDS, 62.5 mM Tris-HCl (pH 8.0), 5 mM dithiothreitol, 10% glycerol, and 0.004% Bromophenol blue. Gels were subjected to electrophoresis at room temperature with a voltage of 120 V for 4 h. After electrophoresis the gels were stained and destained as described previously [29]. Vacuum drying and radioautography of gels were conducted as described [29].

Phospholipid transfer mediated by phospholipid exchange protein

Both ^{32}P-labeled control chromatophores and ^{32}P-labeled, fused chromatophores were used as phospholipid donor membrane substrates. Unilamellar liposomes (containing 70% phosphatidylcholine (PC), 30% phosphatidylethanolamine (PE) and trace of [^{14}C]triolein) were used as the acceptor membrane substrate. Phospholipid exchange protein from *Rps. sphaeroides* strain 2.4.1 and chromatophore-specific antibodies were all generous gifts from S.P. Tai and were prepared essentially as described [30]. Incubation of ^{32}P-labeled chromatophores (containing 12 μg phospholipids), ^{14}C-labeled unilamellar liposomes (160 μg phospholipids) and phospholipid exchange protein (280 μg protein) in a final volume of 0.25 ml 10 mM potassium phosphate buffer, pH 7.5, containing 1 mM EDTA, was conducted at 30°C for 3 h. At the end of the incubation period, 0.12 ml (0.5 mg protein) chromatophore-specific antibody was added to the mixture and after 10 min of incubation at 30°C the chromatophores were pelleted by centrifugation at $8700 \times g$ for 10 min in a Beckman Microfuge B. The supernatant was recovered and total phospholipids were extracted as described below.

Phospholipid extraction and radioactivity determination

Phospholipid extraction of ^{32}P-labeled chromatophores were conducted by the method of Bligh and Dyer as described by Ames [31]. Carrier cells (20 μg of phospholipid per ml of $CHCl_3$) were included and the extracts were washed with 1% NaCl. For bulk [^{32}P]phospholipid determinations, the extracted phospholipids were dried under a stream of nitrogen gas. Radioactivity was measured in a toluene-based scintillant [30]. Individual phospholipid species were resolved by two-dimensional thin-layer chromatography in chloroform/methanol/ammonium hydroxide/water (70:30:3:2, v/v) followed by development in chloroform/methanol/water (65:35:5, v/v) on plates of Silica gel G impregnated with 0.4 M boric acid [32]. Lipid areas were localized by iodine staining. The appropriate areas of the plate were scraped directly into scintillation vials and counted as described above.

Freeze-fracture and negative staining of chromatophores and electron microscopy

Glycerol was added dropwise to chromatophores in 10 mM sodium phosphate buffer, pH 7.0, to a final concentration of 20% (v/v). The sample was then incubated for 1 h at room temperature and centrifuged in a Beckman Airfuge at 26 lb/inch^2 (90000 rpm) for 10 min. Chromatophores were resuspended in a minimal volume of 20% glycerol in the same buffer to a final concentration of approx. 80 mg protein/ml. A drop of the sample (volume not exceeding 1 mm^3) was placed on a 3 mm diameter gold disc, immediately frozen in liquid Freon 12 or 22 for 5 s then transferred into liquid nitrogen. The frozen sample was fractured at −105°C and rotary shadowed with platinum and carbon in a Balzers 301 freeze-etch unit equipped with a rotary cold stage. The replica was picked up on a clean, uncoated 300-mesh copper grid, and allowed to dry before examina-

tion in the electron microscope. Negative staining of chromatophores and measurement of vesicle sizes were conducted as described [12]. 1-ml samples at a concentration of 0.3 mg protein/ml in 10 mM phosphate buffer, pH 7.0, were sonicated briefly (10 s) with a Branson W-350 Sonifier Cell Disruptor (continuous mode, low energy output) to disperse aggregated chromatophores immediately before negative staining. Freeze-fractured replicas were examined in a Siemens Elmiskop 102 electron microscope. Negatively stained samples were examined in a Jeol 100C electron microscope.

Carotenoid bandshift

Light-induced spectral changes were measured in a home-built kinetic spectrophotometer of conventional design. Chromatophores and fused vesicles were suspended in 100 mM KCl, 20 mM morpholinepropanesulfonic acid (Mops), pH 7.2, to an absorbance of 0.7 at 850 nm. Excitation was provided by saturating, single-turnover flashes of about 8 μs pulse duration. The flash-induced redshift in the carotenoid spectrum was measured point by point from 400 nm to 560 nm. Antimycin A (1 μg/ml) was routinely added to block electron flow through the cytochrome *b/c* oxidoreductase, thus simplifying the kinetics of the carotenoid bandshift [33]. Addition of 10 μM gramicidin plus 10 μM valinomycin completely abolished the transmembrane potential response of the carotenoids, leaving only the small amplitude signal of the reaction center carotenoid. Additional optical measurements were made of 550-minus-540 nm and at 602-minus-540 nm to monitor cytochrome *c* and reaction center oxidation-reduction kinetics. Reduced-minus-oxidized difference spectra of the cytochromes were run on an Aminco DW2 spectrophotometer in the wavelength region 540–580 nm. Dithionite (20% in 1 M Tris, pH 9.0) and ferricyanide (0.2 M) were added in 5-μl aliquots to the sample and reference cuvettes until a maximum difference spectrum was obtained.

Analytical method and chemicals

Protein concentrations were determined by a modified biuret reaction described by Munkres and Richards [34]. PMSF, protease Type VI, dithiothreitol and EDTA were purchased from Sigma Chemical Co. 99.8% ^2H_2O was purchased from BioRad. TRTPCK trypsin, CDS α-chymotrypsin (both are of the highest obtainable purity) and lactoperoxidase were obtained from Worthington. Agarose-bound protease was purchased from Miles. Acrylamide was purchased from Eastman Kodak Co. and was purified on a BioRad AG 501-X8 (D) column. L-[^{3}H]Leucine with a specific activity of 58.5 Ci/mmol, L-[^{14}C]leucine with a specific activity of 338 mCi/mmol, carrier free [^{32}P]orthophosphate and carrier free Na^{125}I with a specific activity of 2550 Ci/mmol were purchased from New England Nuclear Corp. All other chemicals were of reagent grade.

Results

Chromatophore fusion

Chromatophores were purified and fused in the presence of 55% poly(ethylene glycol) as described.

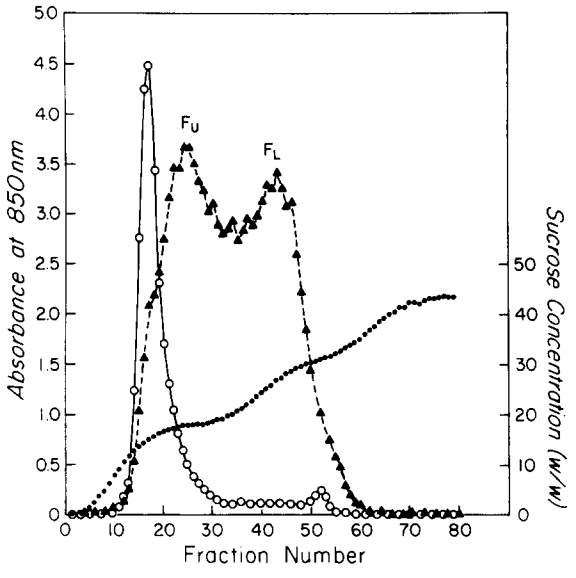

Fig. 1. Total fused chromatophores were centrifuged in a discontinuous sucrose gradient (10/20/35/50/60% sucrose, w/v or 10/19/31/42/49% sucrose, w/w) at 25000 rpm for 2 h in a Beckman Ti-15 zonal rotor. The gradient was fractionated into 10 ml-fractions. Absorbances at 850 nm and sucrose concentrations (w/w) of fractions were measured. Control chromatophores were obtained by processing purified chromatophores by the method as described for Fusion of chromatophores, except that poly(ethylene glycol) 6000 was eliminated. A chromatophore sample at a concentration of 0.28 mg protein/ml gives one absorbance unit at 850 nm. Open circles, absorbance profile of control chromatophores; triangles, absorbance profile of fused chromatophores; closed circles, sucrose concentration.

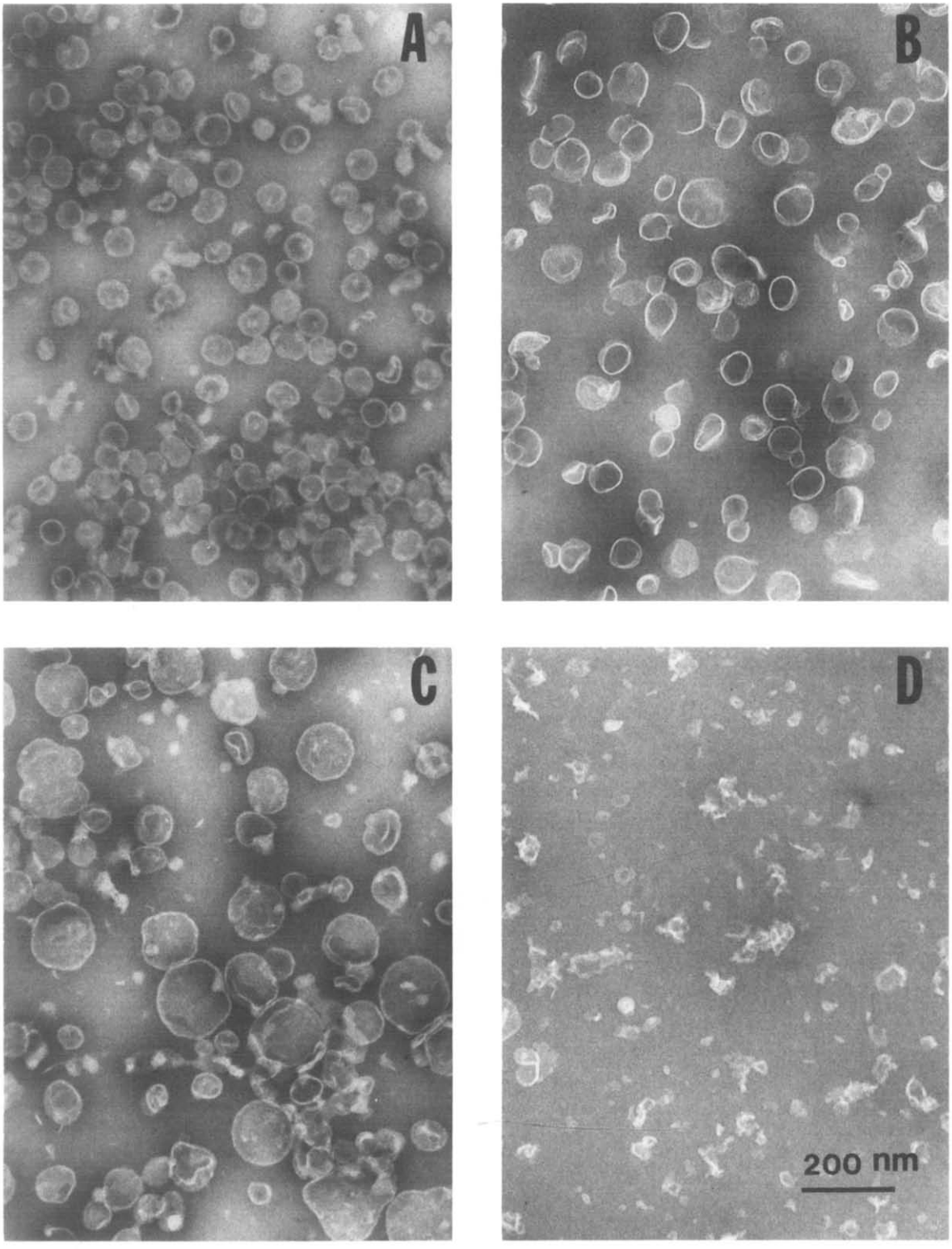

Fig. 2. (A–D) Electron microscopy of control, fused and fragmented chromatophores. Chromatophores were negatively stained with 1% ammonium molybdate as described before [12]. All pictures are of the same magnification (×75 000). Bar represents 200 nm. Fig. 2A, control chromatophores. Fig. 2B, fused chromatophores from fused-upper peak. Fig. 2C, fused chromatophores from fused-lower peak. Fig. 2D, fragmented chromatophores.

Total chromatophores after fusion were resolved into two major peaks (F_U and F_L, representing fused-upper peak and fused-lower peak respectively) by rate zonal centrifugation in a discontinuous sucrose gradient (Fig. 1). Purified chromatophores were processed as described under Fusion of chromatophores in the absence of poly(ethylene glycol), and were designated as control chromatophores. The position of peaks in the sucrose gradient suggests that the size of the chromatophores increased after being treated with poly(ethylene glycol). This was confirmed from electron micrographs of negatively stained samples (Fig. 2A–C).

The control chromatophores had an average diameter of 66.5 nm whereas chromatophores in the F_U peak and in the F_L peak had an average diameter of 86.9 nm and 104.4 nm, respectively. Histograms of control and fused chromatophores clearly indicated that the frequency of smaller chromatophores (40–79.9 nm in diameter) decreased and the frequency of larger chromatophores (80–219.9 nm in diameter) increased after fusion (Fig. 3). Although the negative-staining technique introduces considerable variability in chromatophore diameter due to the variable manner in which the highly hydrated chromatophores spread as they undergo dehydration, this process should be random as to which, larger or smaller, vesicles are affected. Therefore, the data shown in Fig. 3 should be viewed as demonstrating semiquantitative trends within either the 'upper' or 'lower' fused chromatophore populations. A Wilcoxon two-sample test with correction for ties was applied to samples F_U and F_L and yielded a normal deviate of 4.85, indicating a probability of less than one-millionth that the differences between sample F_U and F_L could be attributed to chance [35]. The net decrease in frequency of smaller chromatophores and the net increase in frequency of larger chromatophores indicated that a minimum of 60% of the starting chromatophores underwent fusion. There were 8.8%, 19.9% and 22.3% chromatophores designated as open (by electron microscopy) and present in control, F_U and F_L fractions with average diameters of 77.4, 106.7 and 140.6 nm respectively. The data from these open vesicles were not included in the histograms. This factor further contributes to an underestimation of degree of fusion since there were more open vesicles in fused than in control chromatophores and they were larger in size. The estimation of open vesicles must be considered as a maximum value since any vesicle appearing aberrant with respect to its shape or continuity was designated as open. Chromatophores from peak F_L were used as fused chromatophores for subsequent studies unless otherwise mentioned. It is worth pointing out that from Figs. 2B and 2C there is no aggregation of small chromatophore vesicles.

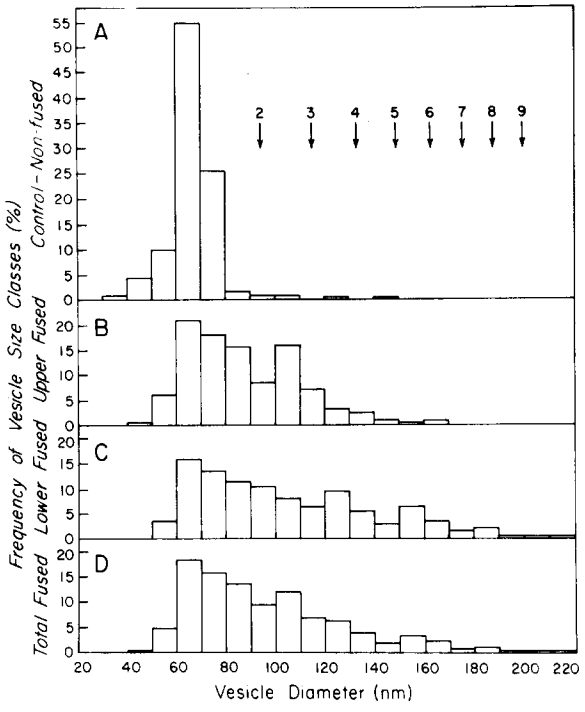

Fig. 3. Histograms of vesicle size classes before and after chromatophore fusion. The average diameters of control chromatophores (panel A), chromatophores from the F_U (upper fused) peak (panel B) and chromatophores from the F_L (lower fused) peak (panel C) were 66.5, 86.9 and 104.4 nm, respectively. At least 200 vesicles were traced for size measurement in panels A, B and C each. Panel D, derived from the sum of data in panel B and C, represents the total fused chromatophores. Arrows in panel A indicate the predicted diameters of fused chromatophore dimers, trimers, tetramers, etc. The predicted diameter of fused chromatophores originating from N control chromatophores is calculated as $\sqrt{N} \times$ (average diameter of control chromatophore) (i.e. $\sqrt{N} \times 66.5$ nm).

Fragmentation of chromatophores

When fused chromatophores were subjected to

sonication after 3–6 weeks of storage at 4°C, the closed membrane vesicles became fragmented therefore exposing both sides of the membrane (Fig. 2D). Control chromatophores stored at 4°C for the same length of time, however, were not fragmented upon sonication.

Fusion between normal and deuterated chromatophores

Fusion of chromatophores was further demonstrated by fusion between L-[^{3}H]leucine-labeled chromatophores (from cells grown in H_2O-based medium) and L-[^{14}C]leucine-labeled, deuterated chromatophores (from cells grown in ^2H_2O-based medium). Fusion of chromatophores was conducted employing L-[^{3}H]leucine-labeled chromatophores and L-[^{14}C]leucine-labeled deuterated chromatophores at an input substrate ratio of 3:1, 1:1 or 1:3 respectively. Poly(ethylene glycol) was removed and fused chromatophores resolved into two major peaks in a discontinuous sucrose gradient as described under Materials and Methods. Fractions from the F_L region were pooled as fused chromatophores for density determinations. The densities of normal, deuterated, and fused chromatophores were determined by isopycnic density centrifugation as described. When L-[^{3}H]leucine-labeled chromatophores and L-[^{14}C]leucine-labeled, deuterated chromatophores were mixed but not fused and then centrifuged in the same CsCl gradient, they separated cleanly from each other. The ^{3}H-radioactivity profile followed the absorbance profile of the normal chromatophore peak and the ^{14}C-radioactivity profile followed the absorbance profile of the deuterated chromatophore peak (data not presented). When fused chromatophores were centrifuged in a CsCl gradient, only one major broad (polydisperse) peak was observed and both ^{3}H- and ^{14}C-radioactivity profiles coincided with the absorbance profile (data not presented). Table I shows the densities of normal and deuterated chromatophores as well as the observed and predicted densities of fused chromatophore samples. Consistent with true fusion, the observed densities of fused chromatophores representing different input ratios of normal and deuterated chromatophores agreed very well with the predicted densities for the fused chromatophores. However, the increase in polydispersity of the fused preparation was consistent with the discrete nature of the fusion products, see below and Fig. 3.

Freeze-fracture

Freeze-fracture studies on fused chromatophores showed concave fracture faces with densely distributed intramembrane particles and convex fracture faces with a sparse distribution of particles (Fig. 4). The same asymmetrical distribution of intramembrane particles has been observed for unfused chromatophores (Fig. 4). Similar observations for native chromatophores of *Rps. sphae-*

TABLE I

DENSITIES OF NORMAL, DEUTERATED AND FUSED CHROMATOPHORES

Median densities of chromatophores were determined as described under Materials and Methods. The predicted median densities of fused chromatophores were calculated from the observed median densities of normal and deuterated chromatophores and from the input ratio of normal and deuterated chromatophores for each fusion preparation. For CsCl density gradient centrifugation of chromatophores see Refs. 7 and 36. Light refers to chromatophores derived from cells grown on normal medium and heavy refers to chromatophores derived from cells grown on ^2H_2O-supplemented medium.

Chromatophores	Substrate input ratio for fusion	Density	
		Observed	Predicted
Control chromatophores, normal	–	1.176	–
Control chromatophores, deuterated	–	1.210	–
Fused chromatophores	3 light: 1 heavy	1.187	1.185
Fused chromatophores	1 light: 1 heavy	1.195	1.193
Fused chromatophores	1 light: 3 heavy	1.200	1.202

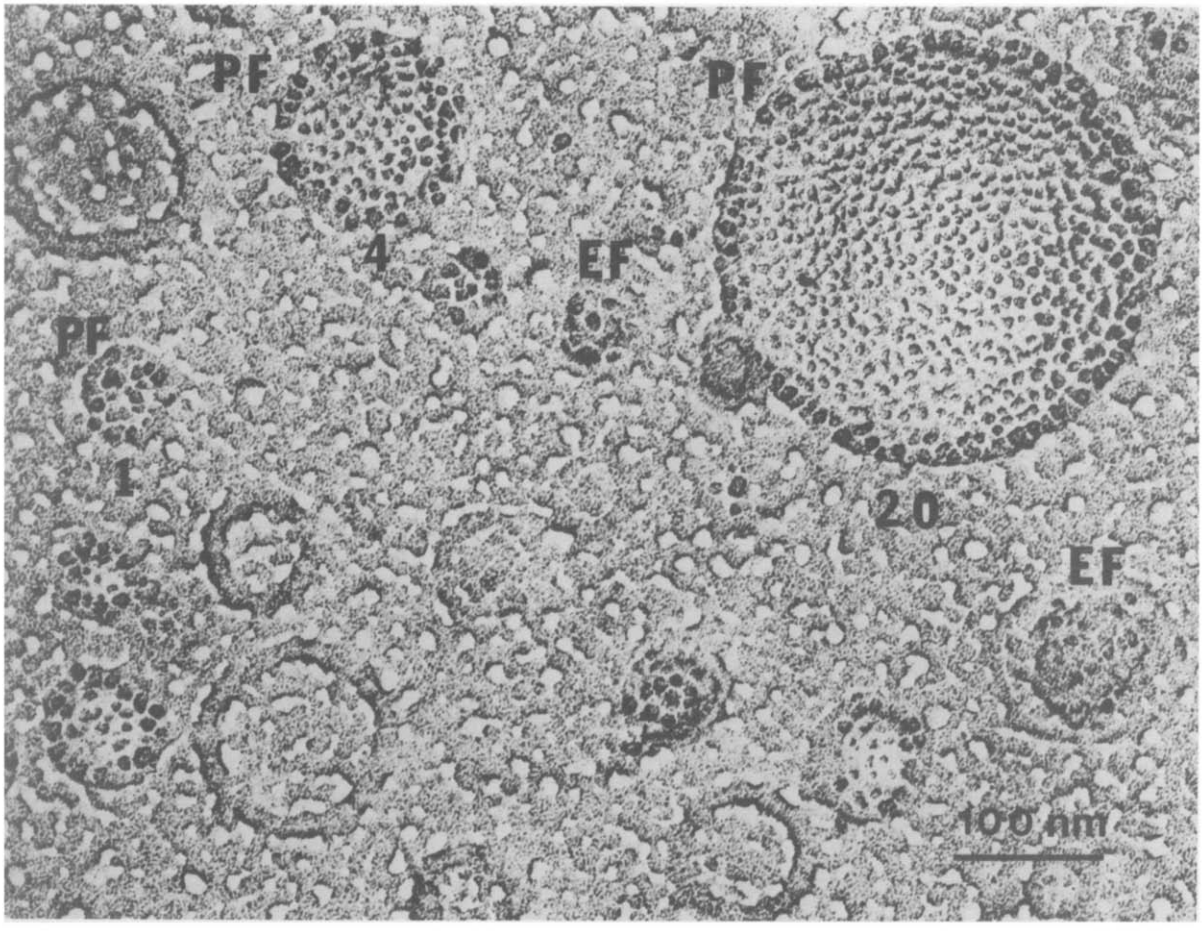

Fig. 4. Rotary shadowed freeze-fractured sample of total fused chromatophores treated with 20% glycerol in 10 mM sodium phosphate buffer, pH 7.0, for 1.5 h before freezing. The PF (concave) faces were densely packed with particles whereas fewer particles were associated with the EF (convex) faces. Numbers (below fracture faces) indicate the estimated, minimal numbers of unfused chromatophores (with an average diameter of 66.5 nm) which fused and gave rise to the larger vesicle. For example, 1 means the vesicle arose from at least one unfused chromatophore and 4 means the vesicle was the fusion product of at least four regular chromatophores. Bar represents 100 nm. Magnification: ×195000.

roides, strain NCIB 8253, have been reported by Lommen and Takemoto [27]. Therefore, the results suggested that fused chromatophores and control chromatophores had the same orientation. Of major importance for future studies was the very large yield of distinct and resolvable fracture faces.

Proteolytic enzyme digestion

In order to determine the membrane orientation of control and fused chromatophores, chromatophore samples were digested with either protease, trypsin or α-chymotrypsin and the polypeptide composition of each sample was characterized by SDS-polyacrylamide gel electrophoresis. Chromatophores were digested with agarose-bound protease or free form protease respectively, as described. The agarose-bound protease, due to its size (70–150 μm in diameter), can only digest polypeptides exposed on the outside of chromatophores whether or not they are completely sealed. The free protease, on the other hand, can digest polypeptides exposed both on the outside and the inside of chromatophores if the chromatophores

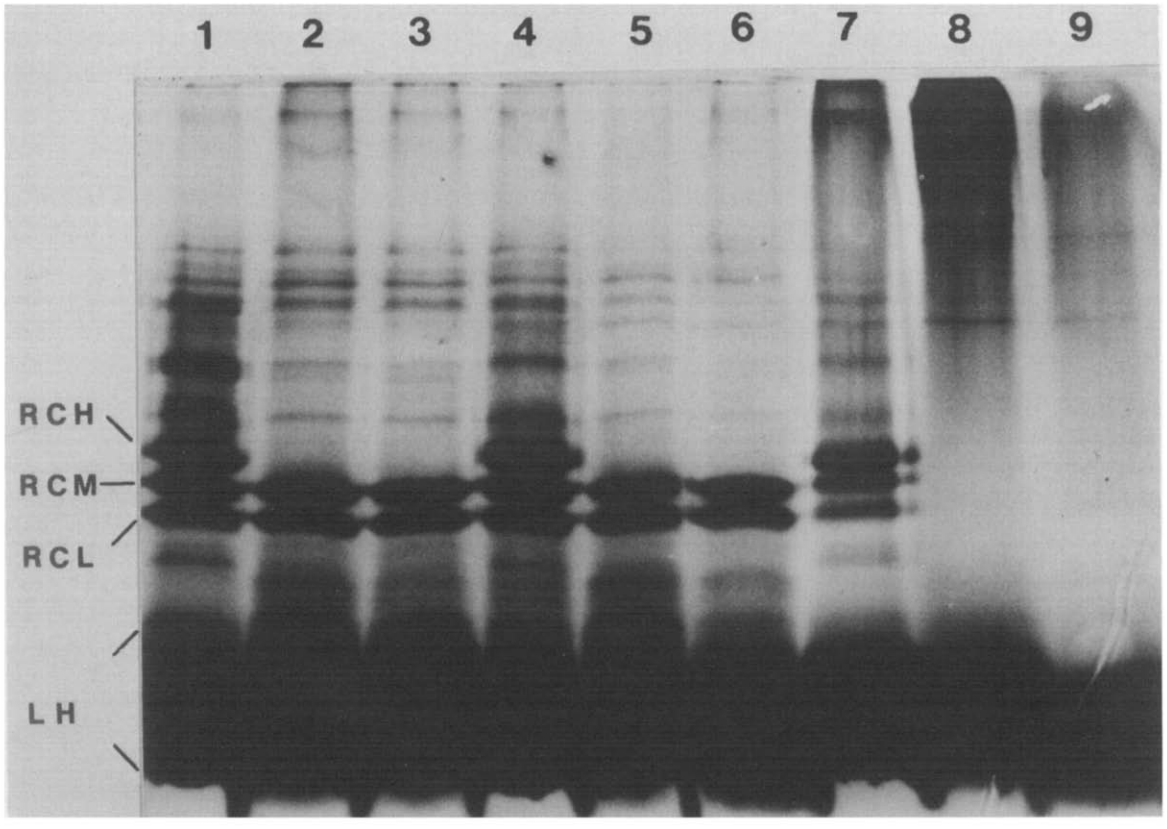

Fig. 5. Coomassie brilliant blue-stained SDS-polyacrylamide gel electrophoresis gel of *Rps. sphaeroides* chromatophores. All samples were solubilized at 100°C for 90 s. Lane 1, control chromatophores; lane 4, fused chromatophores; lane 7, fragmented chromatophores; lanes 2, 5, and 8, control, fused, and fragmented chromatophores treated with agarose-bound protease (2.25 units/mg chromatophore protein) at 37°C for 42 h, respectively; lanes 3, 6, and 9, control, fused, and fragmented chromatophores treated with free protease (0.05 unit/mg chromatophore protein, i.e. 1.25% w/w) at 33°C for 30 min. Shown at the left of lane 1 are locations of reaction-center polypeptides H, M and L (designated RCH, RCM and RCL) and light-harvesting polypeptides (designated LH). 180 μg protein loaded per lane.

are not completely sealed, i.e. if the tear in the membrane is large enough to allow the passage of the protease (see carotenoid bandshift).

The SDS-polyacrylamide gel electrophoresis profiles of control and fused chromatophores before protease digestion indicated that there was no selective removal or loss of polypeptides in fused chromatophores as compared to control chromatophores (Fig. 5, lanes 1 and 4). The SDS-polyacrylamide gel electrophoresis profiles of control and fused chromatophores after protease digestion suggested that control and fused chromatophores had the same orientation, i.e. both were inside-out with respect to the cytoplasmic membrane (Fig. 5, lanes 2, 3 and 5, 6). It is clear that protease treatment resulted in the digestion of RCH, whereas many of the high molecular weight polypeptides as well as RCM, RCL and LH were not significantly affected by protease treatment. However, when fragmented membranes of fused chromatophores were digested with protease, RCM, RCL and most high molecular weight polypeptides were cleaved (Fig. 5, lanes 7 to 9) whereas other polypeptides appear to be unaffected. LH polypeptides may be attacked under these conditions but due to their migration at the front, precise

estimates of digestion are difficult to obtain. The data suggest that RCM and RCL were susceptible to protease digestion when the inside of the chromatophore was made accessible following fragmentation but intact fused chromatophores did not display this sensitivity.

The profile of control chromatophores digested with both types of protease revealed that penetration of chromatophores by protease, if any, was insignificant (Fig. 5, lanes 2 and 3). Similarly if a significant percentage (> 20%) of the fused chromatophores were open vesicles, i.e. allowed access of free form protease rendering both sides of the membrane available for digestion, then SDS-polyacrylamide gel electrophoresis would have shown a quantitatively different profile due to cleavage of polypeptides that were exposed only at the inner surface and whose loss or dimunition from the gel profile would be discernable (data not presented). The results, (Fig. 5, lanes 5 and 6), however argued against this possibility and therefore suggested that, by and large fused chromatophores were closed

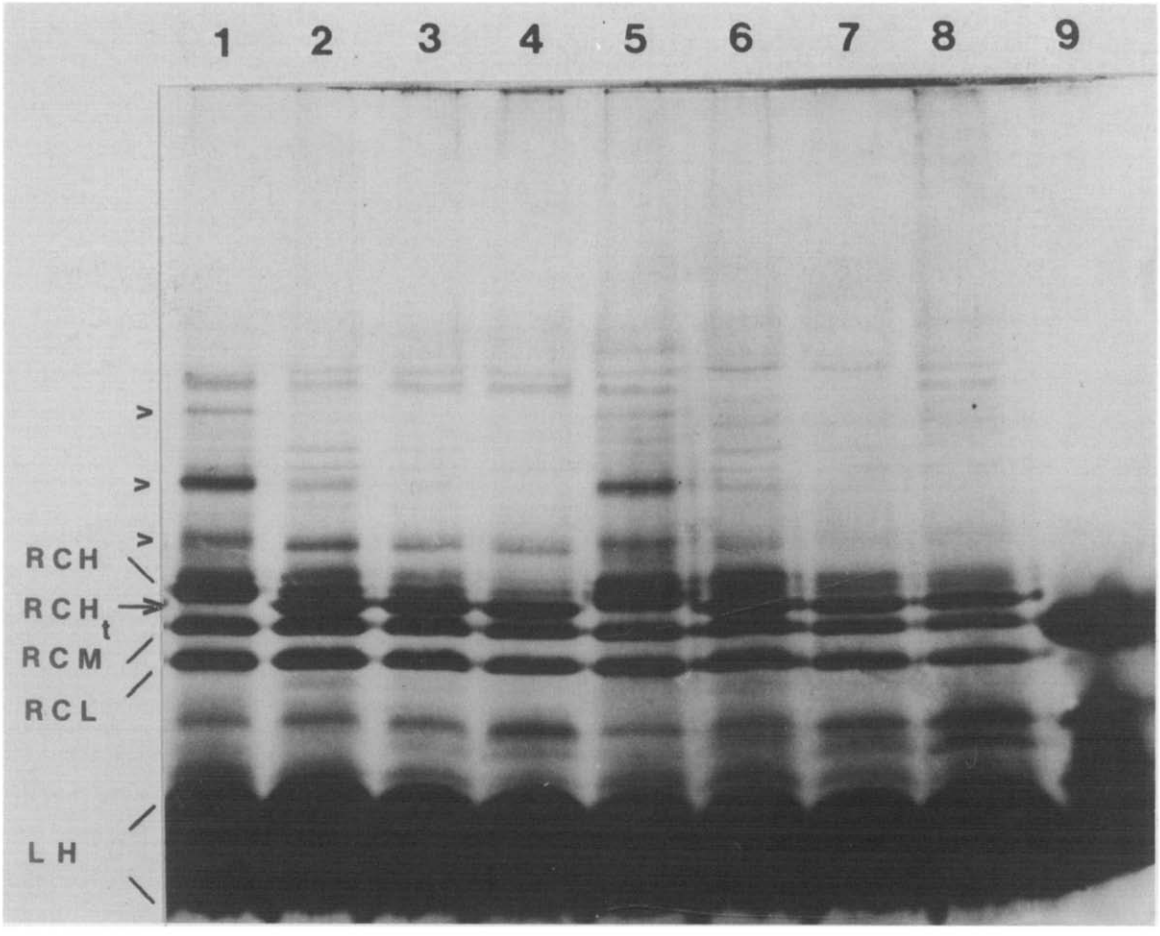

Fig. 6. Coomassie brilliant blue-stained SDS-polyacrylamide gel electrophoresis gel of chromatophores digested with trypsin (3% w/w or 7.5 units/mg protein) at 37°C. All samples were solubilized at 100°C for 90 s. Lanes 1 to 4, control chromatophores treated with trypsin for 0, 2, 8, and 22 h, respectively. Lanes 5 to 8, fused chromatophores treated with trypsin for 0, 2, 8, and 22 h, respectively. Lane 9, trypsin. Arrow indicates the position of RCH_t, the major, new polypeptide appearing after trypsin digestion. Arrowheads indicate the locations of high molecular weight polypeptides disappearing after trypsin digestion. Lanes 1 to 8, 180 μg protein per lane. Lane 9, 60 μg trypsin.

vesicles and the number of vesicles designated as open by electron microscopy was an overestimate. Since it took much less time for free enzyme to promote significant digestion of chromatophores as observed by SDS-polyacrylamide gel electrophoresis, free instead of agarose-bound trypsin and α-chymotrypsin were used for further studies involving proteolytic digestion.

Fig. 6 illustrates the time-dependent digestion of several high molecular weight polypeptides, RCH, and the appearance of a major, new polypeptide migrating immediately below RCH [37], when control and fused chromatophores were treated with trypsin (3% w/w) at 37°C. The SDS-polyacrylamide gel electrophoresis profiles of trypsin-treated control chromatophores were very similar to those of trypsin-treated fused chromatophores. When control and fused chromatophores

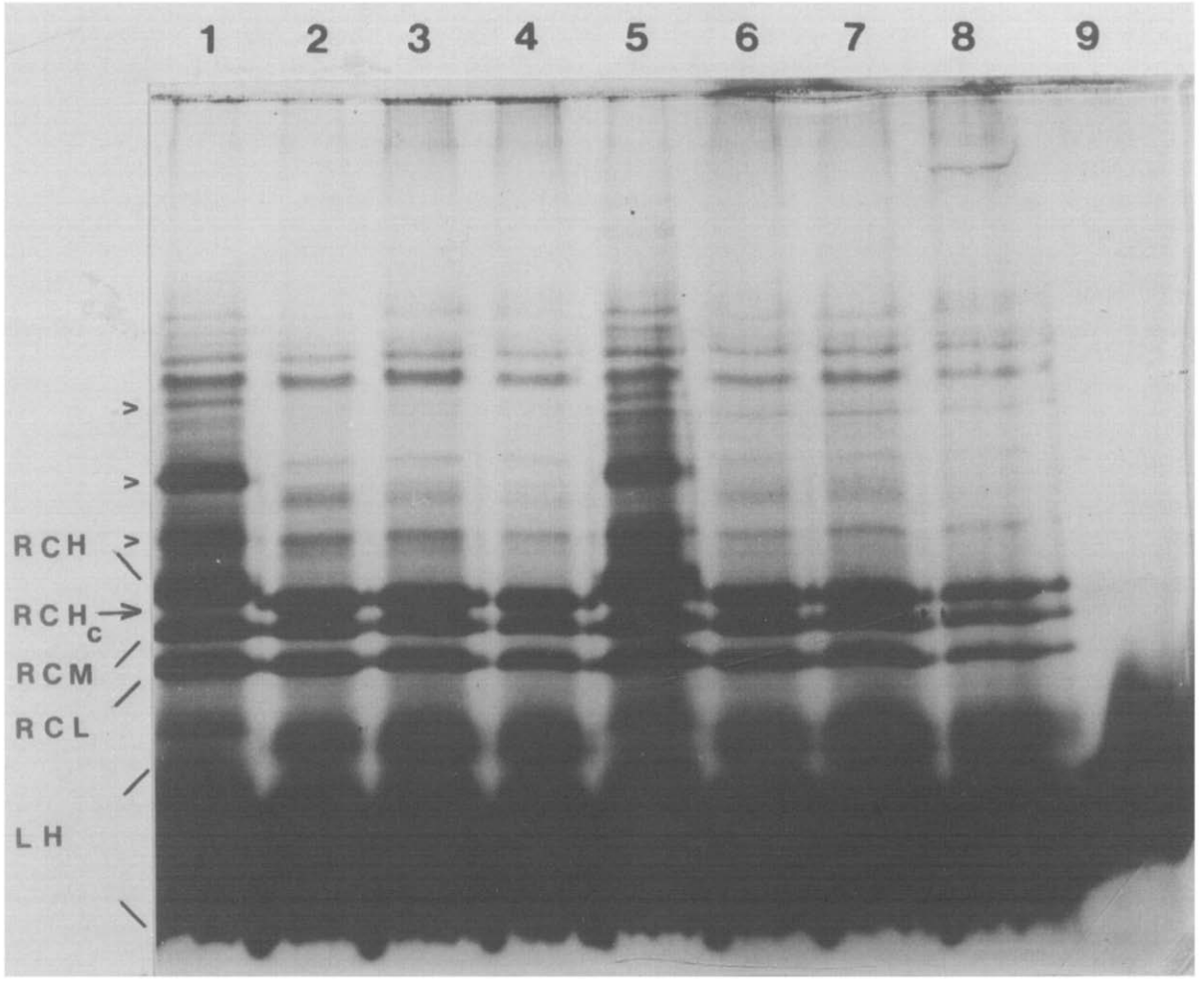

Fig. 7. Coomassie brilliant blue-stained SDS-polyacrylamide gel electrophoresis gel of chromatophores digested with α-chymotrypsin (15% w/w or 7.5 units/mg protein) at 37°C. All samples were solubilized at 100°C for 90 s. Lanes 1 to 4, control chromatophores treated with α-chymotrypsin for 0, 2, 8, and 22 h, respectively. Lanes 5 to 8, fused chromatophores treated with α-chymotrypsin for 0, 2, 8 and 22 h, respectively. Lane 9, α-chymotrypsin. Arrow indicates the position of RCH_c, the major polypeptide appearing after α-chymotrypsin digestion. Arrowheads indicate the locations of high molecular weight polypeptides disappearing after α-chymotrypsin digestion. Lanes 1–8, 180 μg protein per lane. Lane 9, 20 μg α-chymotrypsin.

were incubated with α-chymotrypsin (15% w/w) at 37°C, RCH was cleaved after 2 h of digestion accompanied by the disappearance of a few high molecular weight polypeptides (Fig. 7). α-chymotrypsin digestion also led to the appearance of a major new polypeptide, migrating slightly below RCH, and the appearance of several polypeptides banding between RCL and LH. However, RCM, RCL, and LH were still present after either trypsin or α-chymotrypsin digestion. The data again suggest that control and fused chromatophores have the same orientation. When fragmented membranes prepared from fused chromatophores were digested with trypsin or α-chymotrypsin, RCH, RCM and RCL were all cleaved but LH appeared to remain relatively intact (data not presented) although because of reasons cited earlier precise estimates await a two-dimensional gel analysis employing radioactive material. The data are consistent with the suggestion that RCM and RCL are exposed on the inside of chromatophores with RCH being exposed to at least the outside. Because LH was apparently cleaved by protease and only marginally attacked by trypsin and α-chymotrypsin when fragmented membranes were employed, a portion of these polypeptides probably exists on the inside of chromatophores.

Enzymatic iodination

Further studies on the orientation of control and fused chromatophores employed radioiodination by lactoperoxidase. The autoradiogram in Fig. 8 demonstrates that the same polypeptides in control and fused chromatophores were labeled by ^{125}I. When ^{125}I-labeled control and fused chromatophores were digested with protease (1.25% w/w) at 33°C for 30 min, respectively, all labeled polypeptides were cleaved. The results present further evidence that control and fused chromatophores have the same membrane protein orientation.

Intermembrane phospholipid transfer

The data presented so far are primarily concerned with the polypeptides exposed on the two surfaces of chromatophores. Therefore, another approach, involving phospholipid exchange protein-mediated transfer of phospholipids from chromatophores to recipient liposomes was undertaken

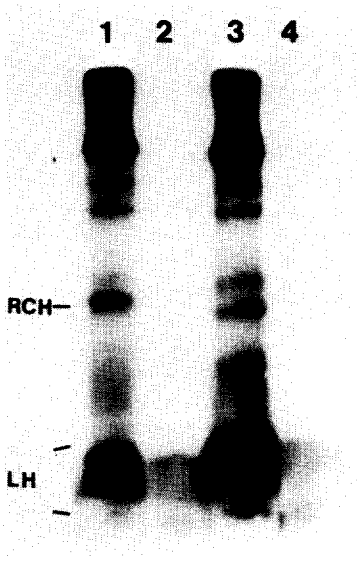

Fig. 8. Autoradiogram of the SDS-polyacrylamide gel of ^{125}I-labeled chromatophores. Lanes 1 and 2, ^{125}I-labeled, control chromatophores before and after protease digestion, respectively. Lanes 3 and 4, ^{125}I-labeled, fused chromatophores before and after protease digestion, respectively. Arrows indicate the position of RCH and LH. Approx. 60 μg protein (with 15000 cpm/μg protein) per lane.

to compare the membrane orientations of the phospholipids of both control and fused chromatophores.

^{32}P-labeled control or ^{32}P-labeled, fused chromatophores were incubated with recipient liposomes in the presence of phospholipid exchange protein from *Rps. sphaeroides* strain 2.4.1 as described under Materials and Methods. At the end of the incubation period, chromatophores were removed by precipitation with chromatophore-specific antibodies followed by centrifugation and the clarified supernatant was extracted for phospholipids. The supernatant contained the recipient liposomes and ^{32}P-labeled phospholipids transferred from ^{32}P-labeled chromatophores to the liposomes [30]. Control and fused chromatophores used as donor, were also extracted for phospholipids. Radioactivities in the bulk, extracted phospholipids were determined and used in the calculation of percent exchange of phospholipids from chromatophores to recipient liposomes. On the average from two experiments, 29.6% of total phospholipids from control chromatophores and

TABLE II

CHROMATOPHORE PHOSPHOLIPID COMPOSITION AND THE DISTRIBUTION OF TRANSFERRED PHOSPHOLIPID SPECIES

Transfer of ^{32}P-labeled phospholipids from ^{32}P-labeled donor chromatophores to recipient liposomes, extraction of phospholipids and resolution of individual species by two-dimensional thin-layer chromatography were as described under Materials and Methods. PG, phosphatidylglycerol; PC, phosphatidylcholine; PE, phosphatidylethanolamine.

Sample analyzed	% Total recovered phospholipid		
	PG	PC	PE
Control chromatophores (Donor substrate)	37.7	23.1	39.3
Fused chromatophores (Donor substrate)	34.3	21.5	44.3
Phospholipid transferred from control chromatophores	47.3	22.4	30.3
Phospholipid transferred from fused chromatophores	46.1	23.6	30.4

30.6% of total phospholipids from fused chromatophores were transferred to recipient liposomes. The values agree very well with similar results obtained by Cohen et al. [30].

Individual phospholipid species from the bulk, extracted phospholipids were resolved by two-dimensional thin-layer chromatography. The phospholipid composition of donor chromatophores and the distribution of the transferred phospholipid species are listed in Table II. Importantly, the data show that the phospholipid compositions of control and fused chromatophores were very similar to one another and the distribution of phospholipid species transferred from control and from fused chromatophores were also similar to one another but different from the starting material. This is precisely in accord with that observed by Cohen et al. [30] who showed that despite the relative abundance of PE in chromatophores, PE is the phospholipid transferred to the lowest extent by phospholipid exchange protein. The ratio of PG transferred to PG in the starting material is 1.25 and 1.34, respectively, for control and fused chromatophores. Similar values for PE are 0.77 and 0.69, respectively.

Carotenoid bandshift

The total carotenoid bandshift spectrum in fused membranes was considerably inhibited in amplitude by comparison with unfused controls. Gramicidin abolished the transmembrane component of this spectrum leaving only the reaction center response. The reaction center carotenoid spectral shift was not significantly different in the fused and unfused preparations. By subtraction of the spectra obtained in the presence and absence of gramicidin, the trans-membrane potential carotenoid bandshift could be obtained. The amplitude of this response was inhibited in the fused preparations, by 80 to 85%. Simple leakiness of the membrane to ions causes an acceleration in the decay of this component of the carotenoid bandshift without affecting the initial amplitude. However, extreme leakiness could cause an apparent loss of amplitude if the decay rate were faster than the response characteristics of the spectrophotometer (20 μs).

Measurements of the flash-induced kinetics of cytochrome *c* and reaction centers indicated that the fused preparations were depleted of soluble cytochrome c_2. This was evidenced by the very slow re-reduction of the reaction center and oxidation of the membrane bound cytochrome c_1, both reactions being mediated by cytochrome c_2. From the amplitudes of the flash induced absorbance changes, fused vesicles contained 0.3–0.34 total cytochrome *c* (c_1 plus c_2) per reaction center. The controls contained 0.67–0.69 total cytochrome *c* per reaction center. Very similar values were obtained from chemically induced reduced-minus-oxidized difference spectra of the total cytochrome *b* and *c* controls. This suggests either a significant leakiness of the fused membrane to macromolecules or that the fusion process involves a transient opening of vesicle structures. Cytochrome *c* added externally to the chromatophores was only oxidized in small amounts following each flash. Quantitatively this indicated that only 9–16% of the reaction centers were accessible to external donors in the fused preparations compared to 4–7% in the control. This prospective is compatible with the protease studies and quite distinct from the 80% indicated, by the electrochromatic bandshift, to be leaky to ions.

Discussion

The increase in the size of chromatophores as clearly revealed by electron microscopy after poly(ethylene glycol) treatment suggests that true membrane fusion, not aggregation, has occurred. The formation of ^{3}H- and ^{14}C-labeled chromatophores with a mean density after fusion between ^{3}H-labeled, normal and ^{14}C-labeled, deuterated chromatophores further supports the conclusion that true fusion of chromatophores took place as a result of poly(ethylene glycol) treatment.

The minimal degree of fusion (60%) calculated from the histograms was based on the assumption that all the small chromatophores (e.g., 50–79.9 nm in diameter) present after fusion were already present in the starting population before fusion. In fact, they could be the fusion product of even smaller chromatophores (e.g., 30–69.9 nm in diameter). (Theoretically, the diameter of fused chromatophore is $\sqrt{(X^2 + Y^2)}$, where X and Y represent the diameter of the two fusing chromatophores, respectively.) Therefore, 60% is an underestimate of the degree of fusion between chromatophores.

The periodicity in the size distribution of chromatophores from the fused lower peak (Fig. 3, panel C) suggests that the fusion process did not yield a continuum of sizes for the fused chromatophores. This phenomenon is more obvious in the fused lower peak than in the fused upper peak since the frequency of larger chromatophores is higher in the former. If the initial products of chromatophore fusions are chromatophore dimers then, the entire population will be enriched for multiples of these units. When these dimers fuse with each other as well as with other monomers still present, more tetramers than trimers will be formed and so on. However, as discussed previously in Results, variability in dehydration during negative staining can introduce the potential for artifacts when measuring vesicle diameters.

Investigations on the topological organization of chromatophore proteins, demonstrate that these membrane proteins are distributed asymmetrically across the membrane [26,27,37–44]. These studies, although somewhat conflicting, focused largely on the three RC polypeptides (i.e., RCH, RCM and RCL) with estimated sizes of 28, 24 and 21 kDa. It was reported that pronase digests RCH but not RCM or RCL in *Rps. sphaeroides* chromatophores [41]. On the other hand, incubation of chromatophores of *Rps. sphaeroides* with antisera against either RCH or RCM-RCL complex indicates that while RCH was exposed to the outside, RCM-RCL complex was accessible to both sides of the membrane [42]. In studies in a related organism, *Rhodospirillum rubrum*, Oleze [43] revealed that RCH is exposed at the surface of chromatophores using both trypsin, and α-chymotrypsin digestion as well as enzymatic iodination. RCM and RCL are not labeled during enzymatic iodination, and they are digested only at extremely high concentrations of trypsin or α-chymotrypsin [43]. Zurrer et al. [44] also reported that only RCH, but not RCM or RCL is exposed at the outside. In summary, these studies indicated that RCH is probably exposed on the outer surface of chromatophores, and RCM and RCL may possibly span the membrane, but are certainly exposed to the inner surface of the chromatophore.

Our data also suggest RCH was exposed at the outer surface of chromatophores since it was readily cleaved by protease, trypsin or α-chymotrypsin. However, neither RCM nor RCL was significantly digested by any of these three enzymes unless the membrane was first fragmented, therefore exposing both sides of chromatophores. This appears to suggest that RCM and RCL were exposed at the internal surface of chromatophores. However, we can not rule out the possibility that membrane structure in fragmented chromatophores might be different from that of native, non-fragmented chromatophores as the result of polypeptide scrambling during fragmentation. The fact that our results more clearly confirm those of others, makes this possibility unlikely. Further the fragmented chromatophore preparation represents the first reported instance where both inner and outer surfaces of the chromatophore were available to chemical treatment in a pure preparation of chromatophores. As to why the fused chromatophores, upon aging, become susceptible to fragmentation is still a matter of conjecture, although several possibilities exist.

Enzymatic iodination of chromatophores with lactoperoxidase and subsequent removal of all labeled polypeptides following protease digestion

presented further evidence that control and fused chromatophores have the same orientation. Fig. 8 shows that RCH but not RCM or RCL was labeled by ^{125}I. This result is consistent with our proteolytic enzyme digestion and chromatophore fragmentation data which suggest that RCH was exposed at the outer surface and RCM and RCL exposed at the inner surface of chromatophores. Bachmann et al. [37] reported that both RCH and RCM were preferentially labeled in comparison to RCL in chromatophores isolated from *Rps. sphaeroides* strain NCIB 8253. However, the use of different strains of *Rps. sphaeroides*, different procedures for membrane preparation and different techniques of radioiodination make comparisons difficult to assess at this time. The label present in the LH-region of the gel seemed to contradict earlier observations that LH was mostly embedded in the membrane. One possible explanation is that LH-associated chlorophylls and/or carotenoids as well as other polypeptides migrating in this region [5,6] were labeled with ^{125}I during the iodination process. However, the extent of labeling is not heavy and even following protease digestion, background labeling was not removed. Observations made for a related organism, *Rhodospirillum rubrum* strain G-9, by Zurrer et al. [44] indicate that RCH and LH were predominantly labeled with ^{131}I employing lactoperoxidase-mediated iodination of chromatophores. However, Oleze [43] showed that certain high molecular weight polypeptides and RCH, but not LH of *Rhodospirillum rubrum*, strain FR1, were labeled with ^{125}I using similar techniques. The reason for the difference among these results is not clear.

Phospholipid composition of chromatophores and transfer of chromatophore phospholipid species to acceptor liposomes mediated by a preparation of phospholipid exchange protein from *Rps. sphaeroides* allowed the assessment of membrane orientation. The results are consistent with earlier observations that while the major chromatophore phospholipid species is PE followed by phosphatidylglycerol, PE always constitutes the smallest percentage of total transferred species [30]. Because, PE is two to three times more prevalent within the outer leaflet of the chromatophore (Ref. 45; Cain, B. unpublished results) we must conclude that the low transfer efficiency of PE is due to the specificity of the phospholipid exchange protein. However, we can still conclude that fused chromatophores had the same orientation as native chromatophores and the perturbation of membrane structure, if any, after fusion was non-detectable.

The observation that digestion of chromatophores by free and agarose-bound protease resulted in the same SDS-polyacrylamide gel electrophoresis profile is consistent with the assumption that free protease did not penetrate the chromatophore membrane during digestion. However, due to the qualitative and semi-quantitative nature of the data provided by SDS-polyacrylamide gel electrophoresis, a limited degree of enzyme penetration ($< 20\%$) of closed, but leaky chromatophores or of open chromatophores could not have been easily detected and quantitated. On the other hand, carotenoid bandshift measurements, being a more sensitive probe for membrane integrity, can detect membrane leakiness to much smaller molecules, e.g. ions. Studies of subchloroplast particles derived from chloroplasts of spinach showed that chloroplast membrane had higher permeability to ions than subchloroplast particles formed following sonication of chloroplast [46,47]. Therefore, the observed difference in membrane leakiness to ions between control and fused chromatophores could be due to their differences in size and not as a direct effect of the fusion procedure. From the studies performed we can further conclude that despite the leakiness of the membranes to ions, they are otherwise intact relative to the unfused, control vesicle population.

In conclusion, we have provided biochemical and morphological evidence that true fusion of chromatophores takes place following poly(ethylene glycol) treatment. We also have presented biochemical and morphological evidence demonstrating that fused chromatophores have the same orientation as native chromatophores, and that the membrane is not perturbed significantly, despite their ion permeability, after poly(ethylene glycol)-induced fusion. Even here, it may prove possible to modify the fusion process such that ion leakiness is no longer a problem. Because, the freeze-fracture patterns of fused chromatophores are quantitatively and qualitatively superior to those obtained from unfused chromatophores a study of

the intramembrane particle distribution as a function of protein content of the vesicles is now possible. Additionally, fused chromatophores may be useful in complementation studies involving photosynthetic mutants as well as in reconstitution studies. Finally, the availability, through fragmentation, of both aspects of purified intracytoplasmic membrane makes topological studies involving antibody absorption [39,42] or protease digestion [37,41] much less ambiguous.

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