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# Methods for Orienting Purple Membrane in Vitreous Ice

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Cross-sectional views of purple membrane embedded in a thin film of vitreous ice could be observed by electron cryo-microscopy by utilizing the spontaneous orientation of the membrane fragments normal to the air/water interface in the final stage of specimen preparation immediately prior to vitrification. It was found that the edge irregularities of purple membrane fragments restricted their self-orienting, and two chemical procedures were used to smooth the membrane edge in order to improve the orientation. Deoxycholate-treated purple membrane oriented slightly better than the untreated preparation. Purple membrane which had been treated under a modified fusion condition for a short period also showed good orientation. A non-chemical procedure, that of size-restriction by filtration, also improved the membrane orientation although clearly no edge-modification was involved in this procedure. In all three procedures, the spontaneously oriented membranes in their vitrified salt-free media displayed significant bending, although the procedure of size-restriction by filtration without any chemical treatment led to the least deviation from planarity. Finally, two modified approaches to these procedures are recommended for yielding oriented, undistorted membrane fragments suitable for electron data collection to address the 'missing cone' problem in the structure analysis of purple membrane.

KEY WORDS: Purple Membrane / Membrane Orientation / Electron Cryo-microscopy / Deoxycholate / Fusion / Membrane Filtration

## 1. Introduction

Purple membrane (PM***) is a relatively large patch in the cytoplasmic membrane of an extremely halophilic bacterium, *Halobacterium halobium*. PM contains lipid molecules and a single species of membrane protein, bacteriorhodopsin (bR), which has the function of pumping protons from the cytoplasmic side of the bacterium to the extracellular side by using the energy of visible light[1]. Each bR molecule contains one retinal moiety, which absorbs the light for proton pumping and binds to lysine 216 of the protein moiety, bacterio-opsin, through a Schiff base.

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*** Abbreviations used: PM, purple membrane; bR, bacteriorhodopsin; DOC, deoxycholate.

In PM, bR and lipid molecules form a two-dimensional crystal composed of an hexagonal lattice[2]. The two-sided plane group of PM is p3 and its lattice constant is 62.45 Å[3,4]. The three-dimensional structure of bR has been analyzed at near-atomic resolution using electron cryo-microscopy with an advanced image processing system[4]. Amino acids located in the middle of the membrane thickness along the direction normal to its surface showed clear density in the three-dimensional reconstructed map of scattering potential insofar as the shape of their side chains could be identified. However, the density of amino acids near the membrane surface was not as high compared with that of the amino acids at the middle, and hence could not be located or identified with the same accuracy. Additionally, even though the density map has a near-atomic resolution, the density derived from the lipid bilayer was not shown. These limitations of the structure analysis were caused at least in part by the missing cone problem[3]. This is a systematic lack of data in a specific zone of reciprocal space. In electron microscopy, the data for three-dimensional reconstruction are collected by tilting the specimen which is usually adsorbed on a thin supporting carbon film. However, for tilting angles higher than about 60 degrees, the recording of both diffraction and high-resolution image data has been hindered by metal mesh which supports this thin carbon film. It has thus prevented collecting structural data within the corresponding cone in reciprocal space. This lack of structural data results in deficiencies in a reconstructed scattering-potential map in real space as described above[4,5]. The missing cone problem could be overcome if the membrane specimen could be oriented at will and be observed from any direction. Our previous work showed a way to overcome this problem by combining two established techniques, namely rapid freezing and electron cryo-microscopy[6]. Using this combined approach, the cross-sectional views of several kinds of membranes could be observed because of their spontaneous orientation with their surfaces perpendicular to the air-water interface. Data within the missing cone could then be collected from these cross-sectional views.

This orientation depended strongly not only on the conditions of membrane suspension medium but also on the exact shape of the membrane fragments. In the case of PM, the improvement achieved allowed oriented specimens to be prepared with good reproducibility. We report here the details of the specimen preparation techniques for PM orientation.

## 2. Materials and methods.

### 2.1. Preparation of purple membrane.

Purple membrane was prepared from *Halobacterium halobium* JW 5 according to Seiff et al[7]. The membranes from strain ET 1001 and $R_1M_1$ were prepared according to the standard method[8]. The isolated PM was washed 5 - 10 times with pure water prepared by Barnstead NANO pure II. Sometimes, the steps that included a sucrose density gradient were omitted. The removal of sugar and detergent, a possible contaminant, was very important for obtaining the best results.

Usually, PM was stored as a suspension in 0.04 % $NaN_3$ and washed with pure

water just before specimen preparation for electron microscopy. Sometimes they were suspended in 25 mM Hepes (pH 7.4), 75 mM NaCl. Basically, PM was washed by repeated cycles of centrifuging to obtain a pellet followed by re-suspending either in pure water or in the Hepes/NaCl buffer above.

A CF 25 Centriflo ultrafiltration cone (Amicon) was used for an alternative washing procedure. The membranes were washed by a repeated cycle of concentration by centrifugation and dilution by addition of pure water, instead of re-suspending the pellet.

**2. 2. Deoxycholate treatment.**

Before using for sample preparation, deoxycholic acid (DOC) (Wako pure chemical industries, LTD.) was recrystallized in order to be decolorized as described in Ref. 9. Recrystallized DOC was dissolved in water by adjusting its pH to 8.5 by addition of NaOH. The isolated PM was suspended in 50 mM Bicine-NaOH (pH 8.5). A DOC solution was added to the PM suspension such that its concentration was 0.2 % or 0.5 % and the concentration of bR was 1.0 mg/ml. The concentration of bR was evaluated from the absorbance at 568 nm using a molar extinction coefficient $\varepsilon = 63{,}000\ M^{-1}cm^{-1}$ for the light-adapted state of bR[10]. The mixture was allowed to stand for 5 hours at room temperature with protection from light exposure. After 5 hours, it was diluted by ten times by adding 50 mM Bicine-NaOH (pH 8.5). Then the suspension was dialyzed against 20 mM Bicine-NaOH (pH 8.5) for 14 hours in order to remove the detergent, and finally the PM was washed with pure water. After the centrifugation step for washing, the membrane pellet was resuspended with gentle pipetting or gentle stirring because harsh methods of re-suspension caused the membrane fragments to curl and lose their planarity.

**2. 3. Short-period fusion.**

The condition used for the fusion reaction was basically that according to Baldwin and Henderson[11], or Ceska and Henderson[12], but the membrane treatment was performed with some significant modifications in regard to the salt concentration and the reaction time. The reaction mixture was not buffered with any salts whereas it was buffered with 100 mM potassium phosphate (pH 5.2) in Ref. 11. Two species of detergent, octylglucoside and dodecyltrimethylammonium chloride, were added to yield final concentrations of 6 mM and 200 $\mu$M, respectively. The final concentration of bR was 3 mg/ml. The mixture was allowed to stand for 1.5 hours or 24 hours at room temperature (about 20°C). (After several weeks, PMs in the reaction mixture were used for data collection in ref. 12.) Then the membrane suspension was diluted with pure water and washed by repeated centrifugation and re-suspension. During the washing procedure, as described above, the membranes were suspended or stirred only by gentle methods in order to avoid membrane distortion. After 3 to 5 cycles of washing, the resulting suspension was used for electron cryo-microscopy.

**2. 4. Size selection of purple membrane.**

To restrict the largest diameter of PM, which we wished to remain less than the thickness of the vitreous-ice layer on our specimen grid, the isolated PM was sometimes passed through a Nucleopore membrane (Pleasanton, CA, diameter of pores: 0.2, 0.4 and 0.6 $\mu$m) or a Millipore membrane filter (Millipore, diameter of pores: 0.22 and 0.46 $\mu$m). Both were combined with disposal syringes. PM was filtrated before washing by centrifugation cycles.

**2. 5. Specimen preparation for electron microscopy.**

Oriented specimens suspended in vitreous ice were prepared with a Reichert-Jung KF 80 rapid freezing apparatus[6]. A microgrid supported on a copper-mesh grid (400-mesh) was washed prior to its use as a specimen support by soaking in distilled water for at least a day in order to remove any remaining detergents which had been used in a series of procedures for making the microgrid[6]. After washing, it was air-dried before using for specimen preparation. The membrane suspension was applied to a microgrid which had been made hydrophilic by glow discharging. The excess amount of suspension was blotted off with a piece of filter paper and immediately the grid with its remaining thin aqueous film plus oriented PM was plunged into liquid ethane maintained at 110 K. The grid was then transferred to the electron microscope specimen stage keeping its temperature below 100 K to prevent de-vitrification of the thin ice film.

Specimens for electron diffraction and for observing the membrane shape were prepared by the glucose embedding method[4, 11, 12]. Carbon film for specimen support was made by evaporation onto a freshly cleaved mica surface. It was floated off onto water and a grid for specimen preparation was prepared by picking up the carbon film on a copper mesh grid. The resulting carbon/copper grid was aged for several days before use because a freshly prepared carbon film is relatively hydrophilic and disorders the membrane crystals adsorbed on it[4]. The suspension was applied to the grid and after 30 seconds to 1 minute, the drop of the suspension was blotted off with a piece of filter paper. Immediately the grid was washed with a drop of 1 % glucose solution, followed by removal of the drop by the same way as the first blotting, and the resulting grid was then finally treated in one of two ways.

For electron diffraction, the grid was then immediately plunged into liquid ethane in order to vitrify the thin aqueous film of glucose solution which remained around and over the PM after the second blotting. The grid was then transferred into the electron cryo-microscope column and diffraction data were collected from it at 1.5 K.

Alternatively, for observing the membrane shape, the grid was air-dried for about 10 minutes after the second blotting before transferring it into the electron microscope column. These latter specimens were observed at room temperature.

**2. 6. Electron microscopy.**

The procedures for recording images of oriented PM in vitreous ice were those according to Sakata et al[6]. Images were taken with a JEOL 4000 SFX electron cryo-

microscope[13] and were recorded while the specimen-stage temperature was between 4.2 K and 20 K by using the Minimum Dose System[14]. The accelerating voltage was 400 kV and images were recorded using an electron dose of about 40 electrons/Å$^2$ and at a magnification of 40,000 × or 60,000 ×. Either Fuji FG or Kodak SO-163 film was used for image recording. The average optical density of the developed films was about 0.8. Sometimes images were recorded using the defocused diffraction mode. Since the specimen could be observed with high contrast in this mode, unoriented membranes could be detected more readily than in the ordinary image mode.

Electron diffraction patterns were recorded from the DOC treated PM to evaluate its lattice constant. The specimen was prepared by the standard glucose embedding method, but as described above, the specimens were not allowed to air-dry and were instead maintained in the frozen-hydrated state. The diffraction patterns were taken at a stage temperature of 1.5 K and the camera length was 398 mm. The accelerating voltage was 200 kV. Kodak SO-163 film was used for diffraction recording. The photographic developing condition was that according to Ref. 12. The lattice constant of PM was evaluated by comparing the positions of its diffraction spots to those of the diffraction pattern of microcrystalline ice. For this purpose, a limited number of ice microcrystals were allowed to condense on the PM specimen during the final stage of operation in the rapid freezing apparatus before then loading the specimen grid plus ice microcrystals into the electron microscope.

A JEOL 100 CX electron microscope was used in order to survey the membrane shape. The specimen was prepared by the standard glucose embedding method as described above. The concentration of glucose solution used for the final grid-wash was between 0.5 % and 2.0 %. The specimen was observed at room temperature. Images were recorded at a magnification of 5,000 × to 10,000 × with an accelerating voltage of 100 kV and an electron dose of about 0.62 electrons/Å$^2$. Fuji FG film was used for image recording. The area for image recording was searched with a dose rate of about 1.0 electrons/Å$^2$/sec, which was too high to observe biological molecules at room temperature without incurring serious irradiation damage. However, it was confirmed that the electron dose described here did not affect the edge shape of the membrane.

## 3. Results and discussion.

### 3.1. An overview of the preparation.

As shown in our previous work, the membrane fragment with its hydrophilic surfaces and hydrophobic edge tends to orient with its surfaces perpendicular to the air-water interface. We believe this specific and spontaneous orientation was caused by the repulsion between the hydrophobic membrane-edge and the aqueous environment. Consequently, the oriented membrane then presented its cross-sectional view in projection in the electron microscope[6] (Fig. 1). During investigation of the PM orientation, it was found that PM did not always accurately present this cross-sectional view and that it depended on both the condition of the

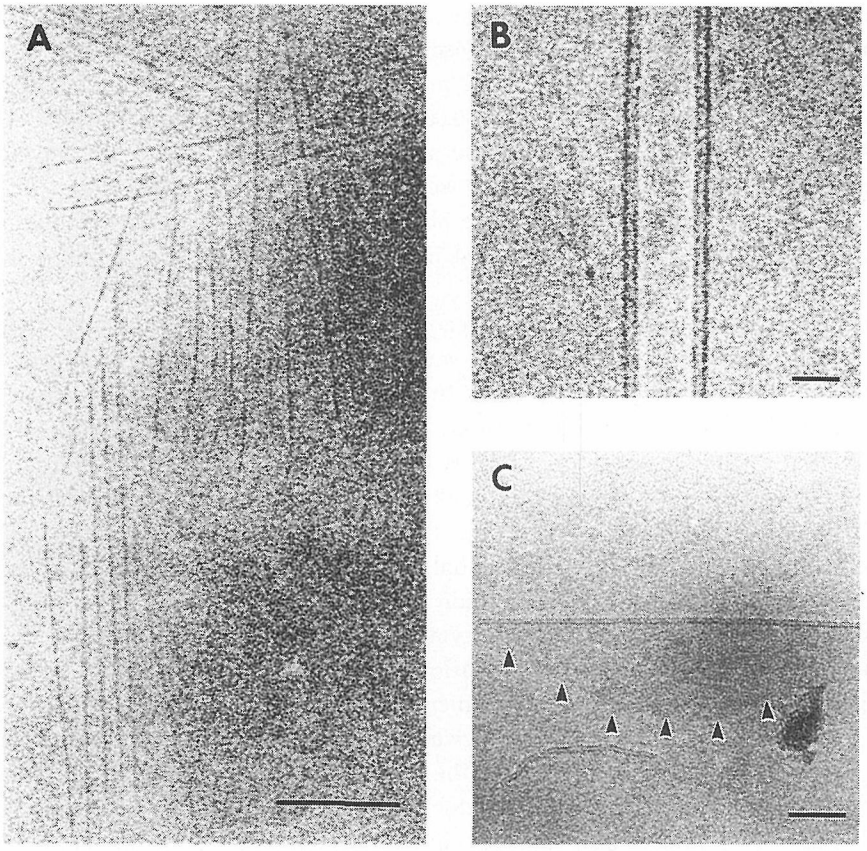


Fig. 1. Oriented purple membrane. (A) Purple membranes (PM) at low magnification. A line contrast shows a PM. Scale bar : 200 nm. (B) PM at high magnification. There are two PMs shown here. Scale bar: 20 nm. (C) PM with a curled region. A PM of diameter larger than the ice thickness shows its bilayer contrasts plus a curled membrane region. The edge of the curled region is indicated by a series of arrow heads. Scale bar: 50 nm. All PMs shown here were prepared only by washing by centrifugation. The strain of PM shown here is JW5.

suspension solvent and the membrane shape. Contamination by surfactants or sugars prevented the membrane from orienting, as described in Ref. 6. Since even trace amounts of them disturbed the orientation, washing PM with pure water for their removal was essential for obtaining consistently good results. Washing by centrifugation proved to be the most effective approach for removing these trace contaminants.

Additionally, the orientation depended on the shape of the membrane. A membrane with a rough, irregular fringe, as shown in Fig. 2 A, rarely showed this orientation. We have not yet been able to determine the factor responsible for causing this edge unevenness or roughness. It seemed that the general shape of PM depended on the preparation batch. When a membrane with an irregular fringe was

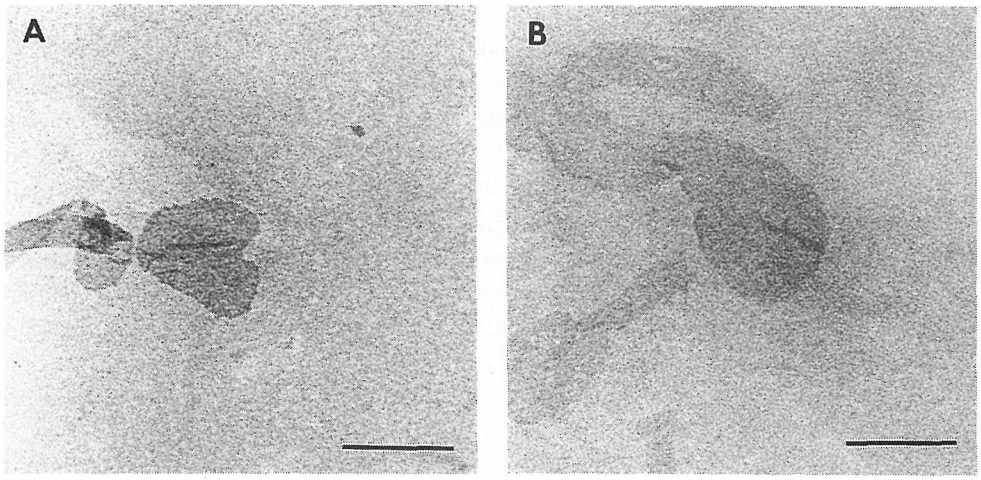


Fig. 2. The shapes of purple membranes. (A) PMs before treatment with the short-period fusion. (B) PMs subjected to the short-period fusion. Their appearances do not change significantly. The strain of PM shown here is ET1001. Scale bar : 0.5 $\mu$m.

adsorbed to the air–water interface with the exposure of its hydrophobic edge to the air, the contact surfaces between the fringe and the interface had a reduced area relative to that if the fringe had been smooth. Accordingly, it produced only a relatively weak force orienting the membrane perpendicular to the water surface. Consequently, such PM preparations rarely exhibited an angle of 90 degrees between the membrane surface and the air–water interface. Therefore, two procedures were tried to smooth the edge of PM in order to increase the force which caused the orientation. Both involved the use of detergents for the removal, or at least the reduction, of the edge roughness.

### 3. 2. DOC treatment.

Deoxycholate (DOC) was used for smoothing the PM fringe. By extreme DOC treatment, it was known that the lattice constant of PM was reduced[15] due to lipid extraction[15, 16]. Therefore the PM edge-smoothing by DOC treatment was performed under relatively mild conditions so as not to extract lipid molecules from unit cells throughout the crystalline bulk of the membrane fragment but to remove the lipid only at the membrane fringe : the concentration of DOC (0.2 or 0.5 %) was lower (7 % for lipid extraction[15]) and the incubation time (5 hours) was shorter (8 hours for lipid extraction[15]). Lattice contraction did not seem to occur by this DOC treatment and this was confirmed by measurement of the lattice constant of DOC-treated PM from its electron diffraction data. Comparing these two DOC treatments, which were performed with DOC concentrations of 0.2 % and 0.5 %, respectively, PM treated with 0.5 % DOC treatment showed the better orientation. However, even with 0.5 % DOC, the number of oriented PM was only slightly increased from that of the untreated membrane preparation.

### 3. 3. Short-period fusion.

To smooth the PM edge with an alternative detergent, mainly octylglucoside, PM was reacted under a modified condition of PM fusion[11, 12]. Since the purpose of this treatment was removal of the edge roughness, it was not necessary to stand the reaction mixture for a long time as for fusing membranes[12]. Furthermore, it had been found that the growth rate of the fused membrane was low when the salt concentration was low. Therefore the fusing reaction was performed for only a short period and without any buffering materials. The PM that had been treated in this manner for only a short period showed good orientation: the number of oriented PM increased and they showed distinct bilayer contrasts, indicating accurate orientation. PM reacted for a longer time (24 hours) showed better orientation than the shorter one (1.5 hours). However, the membrane shapes did not appear to change significantly during either of these procedures (Fig. 2).

Fully fused PM often shows twinning[11, 12]. Under the condition of short-period fusion used here, twinning did not seem to occur because the reaction period was too short to fuse separate PM fragments. However, in a fusion mixture which contained low salt concentration and was allowed to stand for a longer period of 1 week, it was indeed possible to observe many PMs in the process of fusing (Fig. 3).

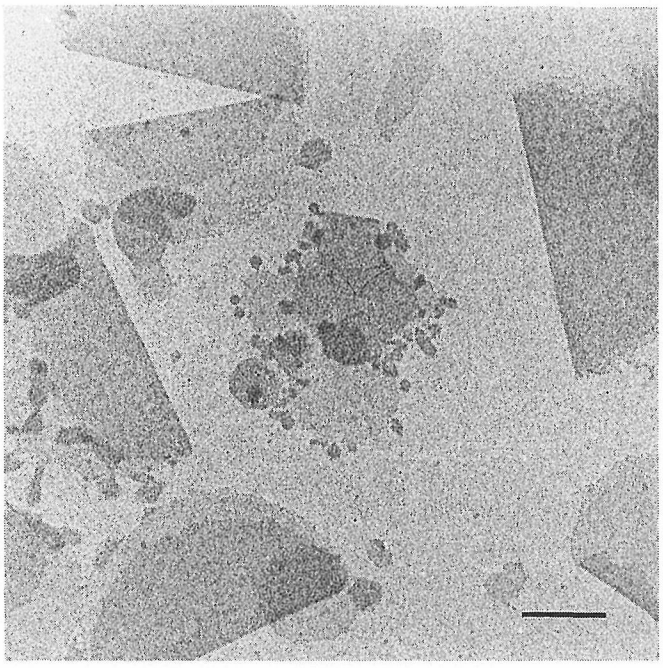

Fig. 3. Purple membranes in the process of fusing. Many small PMs are making contact with a large fused PM around its periphery. PM was suspended in 10 mM MES-NaOH (pH 5.6) with 6 mM octylglucoside and 200 $\mu$M dodecyltrimethylammonium chloride and the final concentration of bR was 3 mg/ml. This view was observed 1 week after the beginning of the fusion reaction. Scale bar: 0.5 $\mu$m.

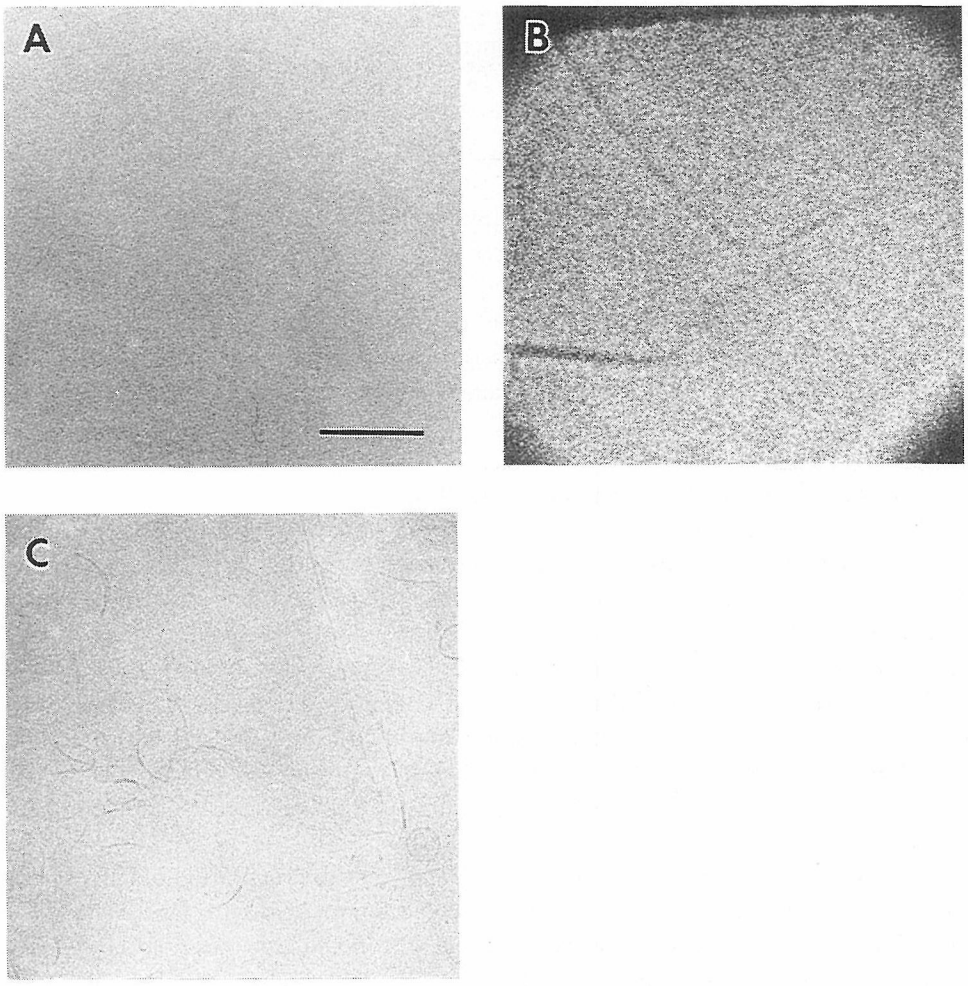


Fig. 4. Ice-embedded purple membrane before and after chemical treatment. (A) PM without any treatment. No bilayer contrast is shown. Scale bar: 100 nm. (B) PMs subjected to short-period fusion. There are many oriented PMs which show line contrasts; however, many of them exhibit bending. The image shown here was obtained in the defocused diffraction mode. (C) PMs treated with DOC. Many PMs exhibit bending as in (B). This image was also obtained in the defocused diffraction mode in order to observe the membranes with heightened contrast.

Unfortunately, many of the oriented PMs prepared in this way were observed as being bent (Fig. 4). Their bilayer contrast showed parallel winding lines of small radius of curvature. Since flat PM oriented exactly normal to the water surface is essential for the collection of accurate data within the missing cone, these distorted PM were not usable for structure analysis.

### 3. 4. Comparison of the DOC treatment and short-period fusion.

When the results of the DOC treatment and those of the short-period fusion were compared, it was found that the DOC treatment could remove only small irregularities at the PM fringe while the short-period fusion could smooth bigger ones. In the case of the DOC treatment, a relatively large irregularity, whose size was larger than that of the bR protein trimer, could remain undisturbed probably because DOC removed only peripheral lipid molecules at the outermost surface of the membrane fringe under the condition of relatively low concentration of DOC and short reaction time.

In the case of the short-period fusion, it seemed that large irregularities at the membrane fringe were able to be smoothed under the conditions used even though the reaction time was short, and PM obtained by this method showed good orientation.

### 3. 5. Orientation of the filtrated purple membrane.

A specimen of PM which had not been subjected to any chemical treatment was also prepared. It was simply washed by repeated centrifugation and resuspension after the initial purification from *Halobacterium halobium*. Using this specimen has an advantage over using the other two chemical procedures in that it rarely introduces any artifacts. In the case of the DOC treatment, for example, there is the possibility that the lattice will shrink if the lipid is extracted; and for the short-period fusion, when the reordering of the components of PM does not reach a stationary state, the crystallinity may be reduced. Therefore, to obtain crystals without any artifacts, it would be best, if possible, to prepare the specimen without any chemical treatment.

However, the number of oriented PM depends on the preparation batch. Since it was expected that PM with small diameter would be oriented with less curvature as their small diameter could be accommodated within the thickness of the ice-film, specimen was prepared by filtrating the raw PM preparation through a membrane filter in order to limit the largest PM diameter in the suspension. This filtration procedure improved the orientation (Fig. 5).

Any given suspension of raw PM had various sizes of membrane with diameters distributed over a relatively narrow range: the majority of membranes had a diameter of between 0.2 $\mu$m and 0.6 $\mu$m[19], which was comparable to the pore diameter of the membrane filter. Before and after filtration with the membrane filter, the concentration of PM with diameter of between 0.2 $\mu$m and 0.6 $\mu$m should not change significantly, and, if anything, might be reduced through losses. However, the filtration procedure actually increased the number of oriented membranes. Superficially this suggests, perhaps, that the relatively small population of membranes of large diameter may in some way have inhibited the orientation of the small membranes, although other explanations are possible.

### 3. 6. Improvements in the preparation of oriented purple membrane.

In this report, we have described three procedures which we have used for im-

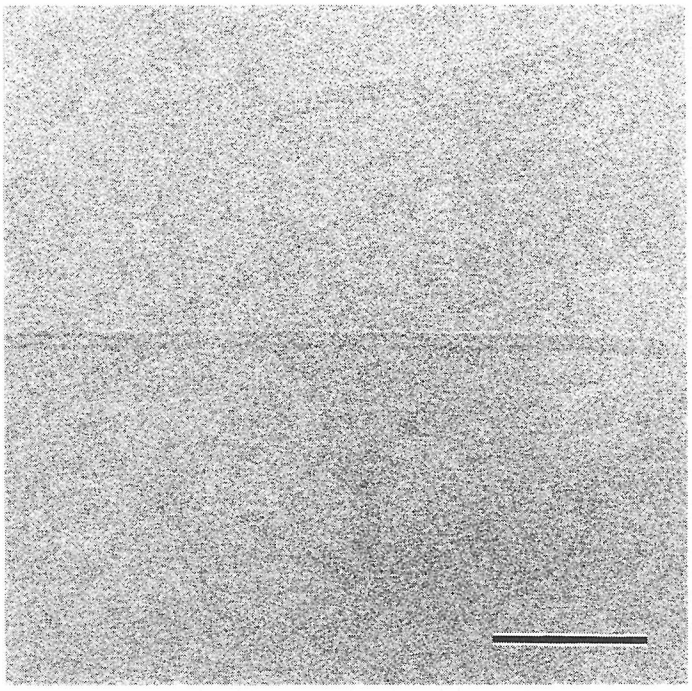

Fig. 5. Purple membranes filtrated with a membrane filter. PMs were suspended in 25 mM Hepes (pH 7.4), 75 mM NaCl. Scale bar: 50 nm.

proving spontaneous PM orientation: the DOC treatment, short-period fusion and filtration by a membrane filter. All of these procedures, however, yielded oriented PM exhibiting varying degrees of bending. The most severe bending of PM resulted from the short-period fusion method, while membranes obtained by the size-selection or filtration procedure showed the least bending.

It seemed that the membranes tended to be bent because PM became less rigid at low salt concentration. It was shown from the results of Glaeser and Downings' specimen preparation method[20] that high salt concentration made PM more rigid. In their method, fused PM, whose diameter was between about 5 $\mu$m and 20 $\mu$m, was used as the specimen and the suspension included 100 mM potassium phosphate (pH 5.2). Whereas their PMs were subjected to relatively severe pipetting in order to disperse them, they yielded electron diffraction single-crystal reflections to 3 Å resolution from their tilted specimens. On the other hand, large PM in the absence of salt, which was obtained by fusing membranes under the standard fusion condition[11] and removing the salt by dialysis, exhibited bending or folding even upon only ordinary relatively gentle pipetting (unpublished data). These data suggest that the high salt concentration makes PM mechanically more rigid.

Since it is difficult to omit all pipettings from specimen preparation for electron cryo-microscopy, the membrane suspension should therefore include salt of high concentration to obtain undistorted PM. Therefore, we suggest that one of the best

ways to prepare flat, oriented PM is as follows: PM is washed and filtrated under the condition of salt concentration of about 100 mM until the final washing immediately prior to cryo-fixation. Just before the specimen is to be prepared for electron cryo-microscopy, the salt concentration is decreased to below 10 mM and this membrane suspension with low salt concentration is used for specimen preparation; the salt concentration should be decreased because PMs aggregate above the threshold concentration of 10 mM NaCl at neutral pH[17].

Another approach to the preparation of flat, oriented PM may be that of obtaining small PM by shifting the timing of the cell harvesting. It is known that both the shape and size of PM change during cell growth[18], and that small PMs can be obtained by harvesting cells at an earlier state than usual. Since it is necessary to prepare small PMs which have high crystallinity for solving the missing cone problem, the conditions for obtaining such PMs should be investigated. By obtaining small PMs without filtration, it would then be possible to eliminate membrane bending caused by the water flow when the PM is filtrated.

Whereas there are some problems which remain to be solved, PM specimens which show good orientation are likely to result from these two methods described above, namely membrane size selection by filtration with high salt concentration, and preparation of small membranes by optimizing the cell harvesting timing.

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## 5. References.

1) D. Oesterhelt and W. Stoeckenius, *Proc. Natl. Acad. Sci. USA*, **70**, 2853 (1973).
2) A. E. Blaurock and W. Stoeckenius, *Nature*, **233**, 152 (1971).
3) R. Henderson and P. N. T. Unwin, *Nature*, **257**, 28 (1975).
4) R. Henderson, J. M. Baldwin, T. A. Ceska, F. Zemlin, E. Beckmann and K. H. Downing, *J. Mol. Biol.*, **213**, 899 (1990).
5) W. Baumeister, M. Barth, R. Hegerl, R. Guckenberger, M. Hahn and W. O. Saxton, *J. Mol. Biol.*, **187**, 241 (1986).
6) K. Sakata, Y. Tahara, K. Morikawa, Y. Fujiyoshi and K. Kimura, *Ultramicroscopy*, **45**, 253 (1992).
7) F. Seiff, I. Wallat, P. Ermann and M. P. Heyn., *Proc. Natl. Acad. Sci. USA*, **82**, 3227 (1985).
8) D. Oesterhelt and W. Stoeckenius, *Methods Enzymol.*, **31**, 667 (1974).
9) S.-B. Hwang and W. Stoeckenius, *J. Membr. Biol.*, **33**, 325 (1977).
10) M. Rehorek and M. P. Heyn, *Biochemistry*, **18**, 4977 (1979).
11) J. Baldwin and R. Henderson, *Ultramicroscopy*, **14**, 319 (1984).
12) T. A. Ceska and R. Henderson, *J. Mol. Biol.*, **213**, 539 (1990).
13) Y. Fujiyoshi, T. Mizusaki, K. Morikawa, H. Yamagishi, Y. Aoki, H. Kihara and Y. Harada, *Ultramicroscopy*, **38**, 241 (1991).
14) Y. Fujiyoshi, T. Kobayashi, K. Ishizuka, N. Uyeda, Y. Ishida and Y. Harada, *Ultramicroscopy*, **5**, 459 (1980).
15) R. M. Glaeser, J. S. Subb and R. Henderson, *Biophys. J.*, **48**, 775 (1985).
16) I. N. Tsygannik and J. M. Baldwin, *Eur. Biophys. J.*, **14**, 263 (1987).
17) Y. Kimura, M. Fujiwara and A. Ikegami, *Biophys. J.*, **45**, 615 (1984).
18) D-C. Neugebauer, H. P. Zingsheim and D. Oesterhelt, *J. Mol. Biol.*, **123**, 247 (1978).
19) Y. Kimura, PhD. Thesis, University of Tokyo (1981).

20) R. M. Glaeser and K. H. Downing, Abstracts, 12 th International Congress for Electron Microscopy, Seattle, 1990, Vol. 1, pp. 98 – 99.