J. Membrane Biol. 60, 45–49 (1981)

The Journal of
Membrane Biology

Interactions of Bacteriorhodopsin-Containing Membrane Systems with Polyelectrolytes

Tilly Bakker-Grunwald* and Benno Hess
Max-Planck-Institut für Ernährungsphysiologie, Rheinlanddamm 201, D-4600 Dortmund, Germany

Summary. The interaction of purple membranes and bacteriorhodopsin-containing phospholipid vesicles with diethyl aminoethyl (DEAE-)dextran and dextran sulfate was investigated. Between pH 1 and pH 7, DEAE-dextran at low concentrations caused aggregation in both membrane systems; at higher concentrations these aggregates disappeared again, probably due to a reversal of net membrane charge. Dextran sulfate did not aggregate the membrane systems at any concentration tested; it rather prevented the aggregation normally occurring at low pH. The effects of the polyelectrolytes on the pH-dependent formation of the 605-nm chromophore were studied in both membrane systems. In purple membrane, DEAE-dextran and dextran sulfate caused shifts in the pH-curves corresponding to changes in local surface potential of +90 mV and −10 mV, respectively. Qualitatively comparable, although smaller changes were observed in bR-containing vesicles. We propose that in vesicular preparations the effect of charged polymers on the potential profile may be limited to the external membrane surface; in that case, our results are consistent with a location of the chromophore close to the cytoplasmic surface.

Several years ago it was proposed that bacteriorhodopsin (bR) in the purple membrane of *Halobacteria* acts as a light-driven proton pump [21]. Since then, the purple membrane system has been subjected to massive research attacks (for a recent review, *see* [27]). In a current model [19, 27], photocycling of the purple complex (bR_{570}) is linked to a conformational change within the protein moiety; this in its turn induces a vectorial shift of protons along a series of proton-binding groups spanning the membrane. As a result, in the intact bacterium net proton transport is accomplished from the cytoplasm to the external medium.

At low pH, a form of the chromophore is generated with an absorption maximum at approximately 605 nm [6, 15–17, 20]. It has been suggested [15] that this species is identical to one of the intermediates of the normal photocycle. The appearance of the acidic form is sensitive to ionic strength and influenced by the addition of ionic detergents [4, 6]. In the present communication we show that the pH-dependence of the 605-nm chromophore can also be manipulated by the polyelectrolytes DEAE-dextran and dextran sulfate. We propose that those polymers may be used to selectively modify the pH-profile on one side of the membrane; our preliminary results are consistent with a location of the chromophore near the cytoplasmic surface.

Materials and Methods

Purple membrane from *H. halobium* NRL (strain R1 M1) was prepared according to [22], and kept in distilled water. 'Inside-out' vesicles were prepared essentially according to [25]; cardiolipin was included to increase the negative surface charge. 50 mg phospholipid (40 mg egg phosphatidyl choline plus 10 mg cardiolipin) were presonicated for 15 min in 5 ml 10 mM KCl. Then 0.6–1.0 ml purple membrane suspension containing 0.25 μmoles bR was added and the mixture was sonicated for another 10 min. The resulting vesicles exhibited an inwardly-directed photosteady-state proton pump of 1.5–2 H^+ per bR (measured in 0.15 M KCl plus 1 μg ml^{-1} valinomycin, pH 5.5–6.0; further conditions as in [5]).

'Right-side-out' vesicles were prepared according to Hellingwerf's [9] modification of the method of Happe et al. [8]. The purple membranes used for this preparation were not subjected to the final sucrose gradient purification step. These vesicles showed an outwardly-directed proton pump of 0.2 H^+ per bR originally added.

Spectra were recorded on either a Beckmann 5230 or an Aminco DW-2 spectrophotometer; in both instruments the positioning of the cuvette housing close to the photomultiplier allowed for the measurement of slightly-turbid suspensions.

* *Present address:* Universität Osnabrück, Fachbereich Mikrobiologie, Postfach 4469, D-4500 Osnabrück, Germany

0022-0631/81/0060-0045 $01.00

Table 1. Minimal amount of DEAE-dextran needed to overcome aggregation of bR-containing systems

	Purple membrane		"Inside-out" vesicles
bR (nmoles ml^{-1})	3.7	10.3	2.9
Phospholipid (μg ml^{-1})	30	83	630
Negative charge (nmoles ml^{-1})	28	77	97
DEAE-dextran (μg ml^{-1})	20	40	250
Positive charge (nmoles ml^{-1})	34	70	425
Positive charge added per negative charge	1.2	0.9	4.4

Suspensions of either purple membrane or "inside-out" vesicles (prepared according to Materials and Methods) in 10 mM KCl were brought to pH 3.5 with 2M HCl. To a series of 2-ml aliquots, microliter quantities of a 2% (w/w) solution of DEAE-dextran (pH 3.5) were added. The smallest amount added (5 μg ml^{-1}) caused heavy floccular aggregation. To each of the next tubes increasing amounts of DEAE-dextran were added up to a point where all visible aggregates disappeared again. The same results were obtained when one fixed sample was "titrated" with DEAE-dextran. For the calculation the following approximations were made: (*i*) we took all DEAE-groups (including the "tandem" groups [24]) as ionized at pH 3.5, leading to a contribution of 1030 net positive charges per DEAE-dextran molecule. (*ii*) We took a ratio of surface negative charges per bR of 7.5 in native purple membrane at pH 3.5 (estimated from [12, 23]), and an average mol wt of 900 for phospholipid (1800 for cardiolipin, each cardiolipin molecule bearing 2 negative charges). (*iii*) For the vesicles, we assumed that 60% of the negative charge contributed by the phospholipid was facing outward.

The concentration of bR was determined from the extinction at 568 nm (assuming a molar extinction coefficient of $63 \times 10^3 M^{-1}$ cm^{-1} [19, 26]; for vesicles, a correction for light-scattering was applied, based on the visible spectrum [9]. For the molecular weight of bR, a value of 26,000 was taken [27].

DEAE-dextran (average substitution grade: one group per 3 glucose residues), dextran sulfate (2.3 groups per glucose residue), both with apparent mol wt of 500,000, and Sephadex G-75 came from Pharmacia. Egg phosphatidyl choline (type V E), cardiolipin and valinomycin were purchased from Sigma. Nigericin was provided by Eli Lilly. ^{3}H-inulin (mol wt, 6,000) was obtained from Amersham.

Results

When DEAE-dextran at low concentrations was added to purple membrane suspensions, extensive aggregation occurred at all pH-values tested (between pH 1 and 7); the suspensions cleared up again at higher polymer concentrations. We attempted to quantitate this effect. As shown in Table 1, at pH 3.5, the amount of polymer required to reverse aggregation was roughly proportional to the amount of purple membrane present. We calculated that, to overcome aggregation, approximately one positive DEAE-group per negative phospholipid charge had to be added (Table 1). In contrast to the DEAE-derivative, dextran sulfate did not induce aggregation; it even prevented the aggregation normally occurring at low pH [17].

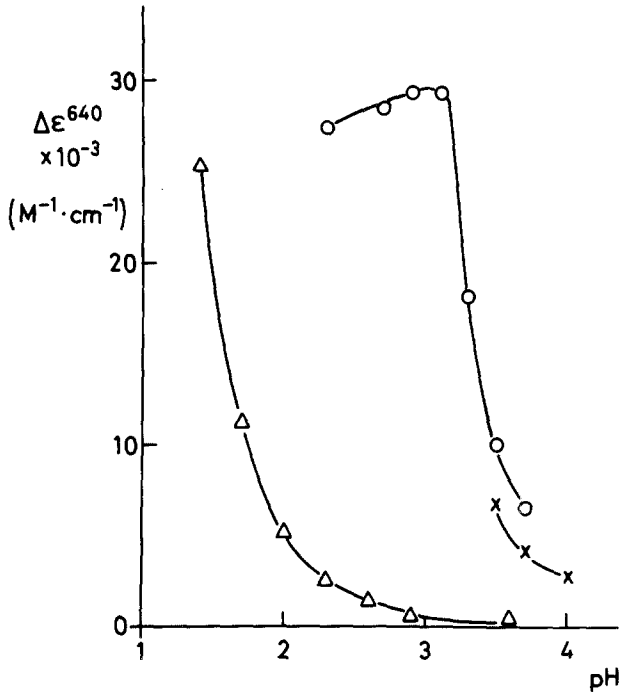

Fig. 1. Effects of DEAE-dextran and dextran sulfate on the pH-dependence of the 605-nm chromophore in purple membrane. Duplicate 2-ml samples of purple membrane suspension in 10 mM KCl, pH 6.5 (containing 24 nmoles bR) were made up with either no polymer (control) or DEAE-dextran or dextran sulfate. The reference sample was kept at pH 6.5 whereas the pH in the other sample was successively adjusted to the indicated values by the addition of μl-quantities of 2 to 10 N HCl (final amount not exceeding 20 nmoles). The appearance of the 605-nm chromophore was estimated from the difference spectrum, which showed a maximum around 640 nm [5, 15–17]. In the absence of polymer, excessive aggregation occurred below pH 3.5; in the presence of either DEAE-dextran or dextran sulfate, samples remained clear throughout the titration. (×–×) control; (○–○) control plus dextran sulfate (0.04 μg ml^{-1}); (△–△) control plus DEAE-dextran (0.2 μg ml^{-1})

Absorption spectra at neutral pH were not altered by either of the two polyelectrolytes, and were identical to those published (*not shown*; [6, 15–17]). Also, circular dichroic spectra recorded in the presence of the polymers showed the characteristic 'bilobe' indicative of trimer interaction [10] (J. Watters, *unpublished observation*). However, the polymers did influence the pH-dependence of the 605-nm form: at the low salt concentration employed (10 mM KCl), DEAE-dextran caused a shift towards lower pH of at least 1½ pH units, whereas the dextran sulfate curve appeared slightly (0.2 pH-units) shifted towards higher pH (Fig. 1; the control curve without added polymer was not measured below pH 3.5 because of excessive aggregation. *See also* [15–17]). The difference was especially striking to the eye at pH 3, where membranes in

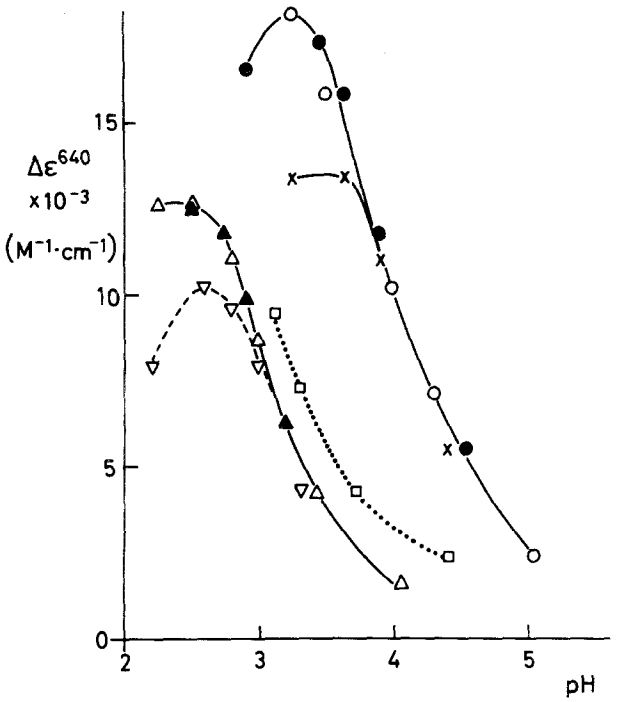

Fig. 2. Effects of DEAE-dextran and dextran sulfate on the pH-dependence of the 605-nm chromophore in "inside-out" phospholipid vesicles. Vesicles were prepared according to Materials and Methods. They were diluted 1:5 with KCl, 10 mM and adjusted to pH 6.5 (final bR-concentration: 6.4 nmoles ml^{-1}). To ensure rapid pH-equilibration of the vesicle interior during titration, valinomycin plus nigericin were added (final concentrations: 1 μg ml^{-1} each). 2-ml samples were titrated in the absence or presence of polymer as described in the legend to Fig. 1. (×–×) control; (□···□) control plus 0.1 M KCl; (○–○) control plus dextran sulfate (2 mg ml^{-1}); (●–●) idem, after a 30-sec sonication; (△–△) control plus DEAE-dextran (6 mg ml^{-1}); (▲–▲) idem, after a 30-sec sonication; (▽---▽), control plus 0.1 M KCl plus DEAE-dextran (16 mg ml^{-1})

the presence of DEAE-dextran looked reddish-purple, whereas in the presence of dextran sulfate they were deeply blue.

We next set out to determine the effects of the polyelectrolytes on bR-containing phospholipid vesicles. First we considered vesicles prepared by sonication at neutral pH: these vesicles presumably incorporate bR in an 'inside-out' orientation [7, 25]. As with purple membranes, DEAE-dextran at low concentrations aggregated these vesicles; but calculated per bR-molecule, much higher amounts of the polymer had to be added to overcome this aggregation phenomenon (Table 1). Again, the amount of positively-charged groups required was of the order of the negative charge contributed by the phospholipids. Thus, it seems probable that in both membrane systems the higher DEAE-concentrations overcame aggregation by reversing the net surface charge.

Figure 2 shows the pH-titration of the 605 nm-chromophore in inside-out vesicles. Also here, addition of DEAE-dextran caused a shift towards lower pH; sonication in the presence of the polymer did not enhance this shift. Increasing the salt concentration by 0.1 M KCl shifted the pH-curve too (*see also* [4, 6]), but not as effectively as DEAE-dextran; moreover, this shift was not additive to that caused by the polymer. Dextran sulfate did not influence the position of the control curve either before or after sonication; however, in both cases it increased the intensity of the 605-nm form at lower pH values. Note that both the slope and the maximal extent of the pH-curves are approximately half those found for purple membrane.

Table 2. ^{3}H-inulin space of "inside-out" vesicles before and after addition of DEAE-dextran

Treatment	^{3}H-inulin space		
	μl (mg bR)$^{-1}$	μl (mg phospholipid)$^{-1}$	% Control
Control (pH 7.0)	3.2	0.41	100
DEAE-dextran added (25 mg ml^{-1}, pH 7.0)	1.5	0.19	46
pH lowered to 2.3	1.2	0.16	39
10-min sonication	0.7	0.09	22

To 2 ml of a vesicle suspension prepared according to Materials and Methods in 10 mM KCl-1 mM Tris-tricine, pH 7.0, ^{3}H-inulin was added to a final activity of 6.2 μCi ml^{-1}, and the mixture was sonicated for 2 min. Extravesicular ^{3}H-inulin was removed on a 40-ml G-75 column. The vesicle fraction was successively subjected to the treatments indicated above, and after each treatment 0.4-ml aliquots were passed over 5-ml G-75 columns. Each treatment (including elution step) took 30 min. From the original sonicate and the vesicle eluates, samples were taken for determination of bR content and radioactivity. Since we found that bR in the presence of DEAE-dextran was bleached upon passage through G-75, bR content was calculated from a protein determination according to [2]. (It should be noted that DEAE-dextran disturbed the (modified) Lowry [1] method.) For the calculation it was assumed that the final vesicular protein/phospholipid ratio was equal to that given in Materials and Methods.

We also set out to test the effects of the polymers on vesicles of opposite orientation ('right-side-out'). However, in this system we were confronted with practical problems which we have not overcome so far. As can be appreciated from Fig. 2 and [6], any effect of the dextrans on the 605-nm absorption will be most obvious at low ionic strength. In view of this, we subjected the right-side-out vesicles (prepared in 0.15 M KCl) to a dialysis step against buffer containing 10 mM KCl–250 mM sucrose. However, when we measured the light-driven H^+ pump in those vesicles, we found that its direction was inverted (*not shown*), which means that the dialysis treatment abolished the preferential 'right-side-out' orientation. Moreover, because of the extreme acid lability of the chromophore in these vesicles [9] we were not able

to observe the 605-nm form. Qualitatively, we noticed that at pH 4.3 DEAE-dextran up to a certain degree protected bR against bleaching, but in view of the fact that the vesicle preparation was clearly of mixed orientation we have as yet not attempted to quantitate this effect.

Since polyelectrolytes are known to cause permeability changes in intact cells [11], we attempted to get an impression of the degree of leakiness they would induce in vesicles. We observed that the light-driven H^+ pump in inside-out vesicles was largely suppressed by either DEAE-dextran or dextran sulfate at the concentrations employed in the experiment of Fig. 2 (*not shown*): this most probably indicates an increased H^+ leak. In order to test whether the polymers also induced permeability towards larger molecules we included ^{3}H-inulin (mol wt 6,000) in the vesicles. Table 2 shows that more than 50% of the enclosed inulin was lost within 30 min after addition of DEAE-dextran.

Discussion

Our results may be most conveniently discussed in terms of a current model [6] in which the retinyledene Schiff base in bR_{570} interacts with a negatively-charged group, B^-, in the vicinity; this interaction is broken upon protonation of B^-, with a concomitant red shift in the absorption maximum. The protonation state of B^- depends upon the *local* H^+ concentration; the latter is determined by both the bulk pH and the local potential at the level of B^-, according to [3]

$$pH_s = pH_b - \frac{F\psi}{2.3\,RT} \qquad (1)$$

where pH_s and PH_b are the negative logarithms of the H^+ concentration near the B^- group and that in the bulk, respectively, and ψ is the local potential experienced by B^-; $RT/F = 25$ mV at 22 °C. If, conversely, we consider the chromophore absorption a probe for the protonation state of B^-, our data show that the local H^+ concentration and thus the potential experienced by B^- can be manipulated by the addition of polyelectrolytes. Thus, from Fig. 1 and Eq. (1) it can be estimated that in the purple membrane system DEAE-dextran shifted the potential at the level of B^- by more than +90 mV and dextran sulfate by approximately −10 mV.

Similar effects on the pH-dependence of the 605-nm chromophore have been brought about by mono-, di- and trivalent salts or addition of ionic detergents [4, 6]. However, the dextrans have one property that fundamentally distinguishes them from either ordinary salts or detergents: their extreme bulkiness, which would be expected to prevent them from equilibrating across phospholipid membranes. We figured that this property might enable us to determine at which membrane surface the B^- group is positioned, or rather: which pH it relates to, that on the cytoplasmic side or that on the outside.

To this end we incorporated bR in closed vesicular structures. Figure 2 shows that also in this system the polymers affected 605-nm chromophore formation. Although the effects were qualitatively similar to those in purple membranes, it should be noted that in vesicles both the slope and the final extent of the 605-nm chromophore formation were only half those observed for purple membranes. Whereas the difference in extent may partly be due to irreversible bleaching of the chromophore, the difference in slope has to be ascribed to a fraction of the vesicular bR that for some reason does not respond to either the external or the internal pH.

Whatever the reason for these quantitative differences, our results show that the polyelectrolytes exhibited their maximal effects already when added at the external surface of inside-out vesicles. This would be in agreement with a cytoplasmic orientation of B^-.

It may be mentioned here that one should not *a priori* expect the functional orientation of a group (as deduced from its titration behavior) to coincide with its physical location: this point may become especially relevant in view of the possible existence of a low-resistance proton-conducting pathway between the membrane surface and the chromatophore (*see* Introduction). However, in this case recent data on the topology of the protein chain tend to confirm the notion that also physically the Schiff base is located close to the cytoplasmic surface [13, 23, 28].

The concept that the potential profile across a membrane may be asymmetrically modified by unilateral addition of an impermeant ion is not new: in model studies on black lipid membranes Ca^{2+} and Mg^{2+} have been used for this purpose [14, 18]. In vesicular preparations, though, divalent cations cause aggregation; as we have shown here, this problem may be circumvented by the use of polyelectrolytes. However, it should be stressed that the presumption that the polymers act unilaterally remains to be proven. For one thing, although DEAE-dextran apparently left the crystalline structure of the purple membrane intact (judging from the circular-dichroic spectrum), it did induce a significant leak of ^{3}H-inulin (Table 2). This points to the presence of big pores, probably due to 'dielectric breakdown' of the membrane. Thus it is feasible that the polyelectrolytes disturb the phospholipid bilayer sufficiently to end up exerting their spectral effects from the inside. Moreover, the dextran

derivatives might induce a randomization of bR orientation. The most straightforward test of these possibilities, involving right-side-out vesicles, proved very difficult experimentally. A theoretical alternative would be to include the polymers in the vesicles and remove them from the outside. However, this approach poses its own problems because of the strong polymer binding to the membrane surface. It thus appears that the concept of polyelectrolytes as unilateral surface potential modifiers will have to be tested on other, better-characterized model systems.

T.B.-G. would like to thank Dr. Evert Bakker for invaluable help and advice, the "7th Floor" for friendship and support, and the Max-Planck-Gesellschaft for providing her with a Stipendium. We thank Dr. Dietrich Kuschmitz for helpful discussions and Mrs. H. Westkamp for expert preparation of purple membrane.

References

1. Bensadoun, A., Weinstein, D. 1976. Assay of proteins in the presence of interfering materials. *Anal. Biochem.* **70**:241
2. Bradford, M.M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**:248
3. Davies, J.T., Rideal, E.K. 1961. Interfacial Phenomena, Chapters II and III. Academic Press, New York/London
4. Edgerton, M.E., Moore, T.A., Greenwood, C. 1978. Salt reversal of the acid-induced changes in purple membrane from *Halobacterium halobium*. *FEBS Lett.* **95**:35
5. Eisenbach, M., Bakker, E.P., Korenstein, R., Caplan, S.R. 1976. Bacteriorhodopsin: Biphasic kinetics of phototransients and of light-induced proton transfer by sub-bacterial *Halobacterium halobium* particles and by reconstituted liposomes. *FEBS Lett.* **71**:228
6. Fischer, U., Oesterhelt, D. 1979. Chromophore equilibria in bacteriorhodopsin. *Biophys. J.* **28**:211
7. Gerber, G.E., Gray, G.P., Wildenauer, D., Khorana, H.G. 1977. Orientation of bacteriorhodopsin in *Halobacterium halobium* as studied by selective proteolysis. *Proc. Nat. Acad. Sci. USA* **74**:5426
8. Happe, M., Teather, R.M., Overath, P., Knöbling, A., Oesterhelt, D. 1977. Direction of proton translocation in proteoliposomes formed from purple membrane and acidic lipids depends on the pH during reconstitution. *Biochim. Biophys. Acta* **465**:415
9. Hellingwerf, K.J. 1979. Structural and Functional Studies on Lipid Vesicles Containing Bacteriorhodopsin. Ph.D. Thesis, University of Amsterdam, The Netherlands
10. Heyn, M.P., Bauer, P.J., Dencher, N.A., 1975. A natural CD label to probe the structure of the purple membrane from *Halobacterium halobium* by means of exciton coupling effects. *Biochem. Biophys. Res. Commun.* **67**:897
11. Kasahara, M. 1977. A permeability change in Ehrlich ascites tumor cells caused by dextran sulfate and its repair by ascites fluid or Ca^{2+} ions. *Arch. Biochem. Biophys.* **184**:400
12. Kates, M., Kushwaha, S.C. 1978. Biochemistry of the lipids of extremely halophilic bacteria. *In*: Energetics and Structure of Halophilic Microorganisms. S.R. Caplan and M. Ginzburg, editors. Vol. 1, pp. 461–479. Elsevier/North Holland, Amsterdam – New York
13. Khorana, H.G., Gerber, G.E., Herlihy, W.C., Gray, C.P., Anderegg, R.J., Nihei, K., Biemann, K. 1979. Amino acid sequence of bacteriorhodopsin. *Proc. Natl. Acad. Sci. USA* **76**:5046
14. McLaughlin, S. 1977. Electrostatic potentials at membrane-solution interfaces. *In*: Current Topics in Membrane Transport, F. Bronner, and A. Kleinzeller, editors. Volume 9, pp. 71–144. Academic Press, New York
15. Moore, T.A., Edgerton, M.E., Part, G., Greenwood, C., Perham, R. 1978. Studies of an acid-induced species of purple membrane form *Halobacterium halobium*. *Biochem. J.* **171**:469
16. Mowery, P.C., Lozier, R.H., Quae Chae, Y.-W.T., Taylor, M., Stoeckenius, W. 1979. Effect of acid pH on the absorption spectra and photoreactions of bacteriorhodopsin. *Biochemistry* **18**:4100
17. Muccio, D.D., Cassim, J.Y. 1979. Interpretations of the effects of pH on the spectra of purple membrane. *J. Mol. Biol.* **135**:595
18. Muller, R.U., Finkelstein, A. 1972. The effect of surface charge on the voltage-dependent conductance induced in thin lipid membranes by monazomycin. *J. Gen. Physiol.* **60**:285
19. Oesterhelt, D., Hess, B. 1973. Reversible photolysis of the purple complex in the purple membrane of *Halobacterium halobium*. *Eur. J. Biochem.* **37**:316
20. Oesterhelt, D., Stoeckenius, W. 1971. Rhodopsin-like protein from the purple membrane of *Halobacterium halobium*. *Nature New Biol.* **233**:149
21. Oesterhelt, D., Stoeckenius, W. 1973. Functions of a new photoreceptor membrane. *Proc. Natl. Acad. Sci. USA* **70**:2853
22. Oesterhelt, D., Stoeckenius, W. 1974. Isolation of the cell membrane of *Halobacterium halobium* and its fractionation into red and purple membrane. *Meth. Enzymol.* **31A**:667
23. Ovchinnikov, Yu.A., Abdulaev, N.G., Feigina, M.Y., Kiseley, A.V., Lobanov, N.A. 1977. Recent findings in the structure-functional characteristics of bacteriorhodopsin. *FEBS Lett.* **84**:1.
24. Pharmacia. 1977. Dextran fractions, dextran sulphate, DEAE dextran: Defined polymers for biological research. Literature Service, Pharmacia Fine Chemicals AB
25. Racker, E. 1973. A new procedure for the reconstitution of biologically active phospholipid vesicles. *Biochem. Biophys. Res. Commun.* **55**:224
26. Rehorek, M., Heyn, M.P., 1979. Binding of *all-trans*-retinal to the purple membrane. Evidence for cooperativity and determination of the extinction coefficient. *Biochemistry* **18**:4977
27. Stoeckenius, W., Lozier, R.H., Bogomolni, R.A. 1979. Bacteriorhodopsin and the purple membrane of halobacteria. *Biochim. Biophys. Acta* **505**:215
28. Walker, J.E., Carne, A.F., Schmidt, H.W. 1979. The topography of the purple membrane. *Nature* **278**:653

Received 29 May 1980; revised 10 September 1980