Biochimica et Biophysica Acta, 507 (1978) 48—61

BBA 77906

ASSOCIATION OF BACTERIORHODOPSIN-CONTAINING PHOSPHOLIPID VESICLES WITH PHOSPHOLIPID-IMPREGNATED MILLIPORE FILTERS

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(Received June 14th, 1977)

Summary

A photopotential is generated upon illumination of a phospholipid-impregnated Millipore filter to which are bound phospholipid vesicles, containing bacteriorhodopsin isolated from *Halobacterium halobium*. In this study, experiments are described that show how the bacteriorhodopsin-dependent photo effect is influenced by factors that have an effect on the association of bacteriorhodopsin-containing vesicles with the filter. The following conclusions are drawn:

1. The photopotential generated during a short flash of light is directly proportional to the number of bacteriorhodopsin vesicles associated with the filter.

2. The number of bacteriorhodopsin molecules that contribute to the photo effect increases with time according to a hyperbolic relationship, reaching a maximum after prolonged incubation.

3. The increase of the photo effect with time can be ascribed to a time-dependent association of bacteriorhodopsin vesicles with the filter. The maximum value of the photo effect is due to a saturation of the filter with bacteriorhodopsin vesicles.

4. The rate of association of bacteriorhodopsin vesicles with the filter is directly proportional to the vesicle concentration in the aqueous medium. The maximal number of vesicles that becomes associated with the filter after prolonged incubation is independent of this vesicle concentration.

5. Both the rate of association of bacteriorhodopsin vesicles with the filter and the maximal photo effect calculated by extrapolation to infinite incubation time increase with the Ca^{2+} concentration in the medium, and reach a plateau at approximately 40 mM Ca^{2+}. This suggests that Ca^{2+} has an effect on the number of bacteriorhodopsin vesicles that can associate with the filter.

6. The maximal photo effect is decreased by the simultaneous presence in the aqueous medium of untreated bacteriorhodopsin vesicles and of vesicles in which bacteriorhodopsin has been inactivated by treatment at elevated pH or with KBH_4. From this we conclude that inactivated bacteriorhodopsin vesicles

can still associate with the filter in a way comparable to untreated bacteriorhodopsin vesicles. On the other hand, pure phospholipid vesicles only affect the rate of association of bacteriorhodopsin vesicles with the filter but have no effect on the maximal photo effect calculated by extrapolation to infinite time.

Introduction

Of the energy-conserving systems occurring in nature, the chromoprotein bacteriorhodopsin has been studied very extensively during the last few years. One of the reasons for this considerable interest is the ease with which the protein can be isolated. Bacteriorhodopsin is the only protein present in purple membranes, specialized regions of the plasma membrane of some extremely halophilic bacteria (e.g. *Halobacterium halobium*) [1,2], which can be isolated by hypotonic disruption of the cells and subsequent centrifugation [3]. Successful reconstitution of bacteriorhodopsin has been achieved with several artificial membrane systems [4—8].

The ability of bacteriorhodopsin to convert light energy into an electrochemical gradient of protons has been demonstrated both in vivo and in vitro [4,5,9—12]. Measurement of photopotentials generated by bacteriorhodopsin upon illumination has been reported in several systems. In the first place, purple membranes can be directly dispersed in lipid-containing decane solutions, that are used to prepare the classical black-lipid membrane [6,13—15]. Besides, bacteriorhodopsin-containing phospholipid vesicles can be associated with the so-called planar membrane [7,16] and, as we have shown recently, with phospholipid-impregnated Millipore filters [17]. It is the aim of the present study to describe the influence of various factors that influence the association of bacteriorhodopsin-containing vesicles with lipid-impregnated Millipore filters.

Materials and Methods

Purple membranes [3] and soya-bean phospholipids [18] were isolated according to procedures described elsewhere.

Bacteriorhodopsin-containing phospholipid vesicles were prepared as follows: soya-bean phospholipids, dissolved in chloroform, were evaporated to dryness in a round-bottom flask. After addition of purple membranes, suspended in 150 mM KCl, and dilution to the desired protein concentration with 150 mM KCl, the lipids were dispersed by mixing on a Vortex mixer in the presence of small glass beads. The final soya-bean phospholipid concentration was 10 mg/ml. This suspension was sonicated subsequently in a M.S.E. ultrasonifier for 30 (or 60) times 15 s, with 45-s intervals without sonication, at 20 kHz, 4 μm amplitude, cooling in ice and under an argon atmosphere. Pure soya-bean phospholipid vesicles were prepared in an identical way, with the exception that purple membranes were omitted.

Inactivation of bacteriorhodopsin incorporated into phospholipid vesicles was effected by mixing equal volumes of 150 mM potassium carbonate buffer (pH 10) and of bacteriorhodopsin-containing vesicles suspended in 150 mM

KCl (pH 6). After cooling to 0°C, KBH_4 was added to this solution to a final concentration of 15 mg/ml. The solution was kept at 0°C and illuminated with daylight for 10 min, which resulted in an almost complete inactivation of bacteriorhodopsin. During this treatment, the pH was checked several times and, when necessary, readjusted to pH 10 with KOH. Subsequently, the solution was dialysed against a 100-fold excess of 150 mM KCl (pH 6) at 0—4°C for 2 h with six changes of the dialysis medium. Finally, the pH of the suspension was readjusted to pH 6. Partial inactivation of the bacteriorhodopsin was achieved by treating the bacteriorhodopsin vesicles in an identical way at pH 10 in the absence of KBH_4.

The bacteriorhodopsin-dependent photo effect was studied as follows. Millipore filters (pore size 0.15 μm, 0.15 mm thick) were impregnated with soya-bean phospholipids dissolved in hexadecane (150 mg of lipid/ml). After removal of excess lipid solution the filter was clamped between two 20-ml Teflon vessels each containing a circular aperture of 12 mm diameter. Subsequently the compartments on both sides of the filter were filled with 20 ml of a 50 mM $CaCl_2$/75 mM KCl solution, unless stated otherwise. The solutions in both compartments were stirred gently by means of a magnetic stirrer. After an equilibration period of 15 min the experiment was started by the addition of vesicles. Illumination of the filter from one side was achieved using a 250 W projector lamp and fiber optics. Measurement of the photopotential was carried out with a circuit as described by Mueller et al. [19] using two calomel electrodes (Radiometer, Copenhagen, type K 4112), connected with a Keithley 610 C electrometer and a recorder.

Results

Bacteriorhodopsin associated with a phospholipid-impregnated Millipore filter has the ability to generate a photopotential across the filter upon illumination [17]. In most experiments the photo effect was followed by measuring the potential that is generated during a short flash of light, This parameter is designated ψ_{fl}.

In Fig. 1A the time-dependence of ψ_{fl} is shown for a typical experiment. Initially, ψ_{fl} increases rapidly with time tending to reach a maximum at longer incubation time. Analysis of the data showed that the increase of ψ_{fl} with time did not follow (pseudo) first-order or second-order kinetics. It was found empirically, by plotting $1/\psi_{fl}$ versus 1/time, that ψ_{fl} shows a hyperbolic dependence on incubation time.

One possible explanation for the fact that ψ_{fl} reaches a maximum at longer incubation times might be that the bacteriorhodopsin vesicles lose the ability to bind to the filter. If so, a second addition of vesicles should result in a relatively steep increase of ψ_{fl} with time. However, Fig. 2 (curve A) shows that only a very slight increase takes place. This experiment suggests that ψ_{fl} reaches a maximum because the surface of the filter is saturated with bacteriorhodopsin vesicles. However, we cannot exclude a priori that only a limited number of the binding sites on the filter surface is occupied by bacteriorhodopsin vesicles and that ψ_{fl} reaches a maximum for other reasons, e.g. because ψ_{fl} depends

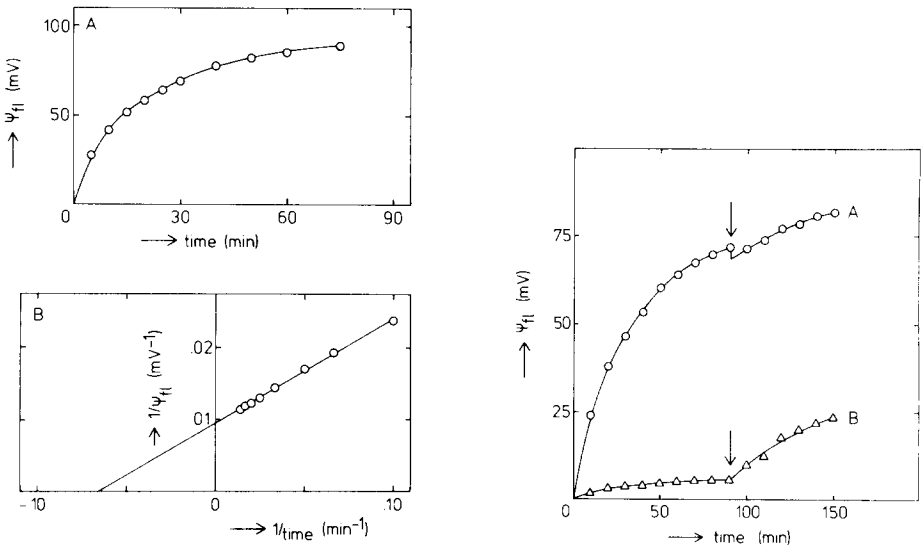


Fig. 1. Time-dependence of ψ_{fl}, the photopotential generated during a flash of light. The experiment was carried out as described in Materials and Methods. At zero-time, bacteriorhodopsin-containing soya-bean phospholipid vesicles were added to one of the Teflon compartments to a final vesicle concentration of 0.10 mg soya-bean phospholipid/ml. Protein/soya-bean phospholipid ratio = 1 : 10 (w/w). A: ψ_{fl}, the photopotential generated during a flash of 1.0 s, is plotted as a function of the incubation time of the filter with the vesicles. Curve B: a double-reciprocal plot of the data of Curve A.

Fig. 2. Effect of a second addition of bacteriorhodopsin-containing vesicles on ψ_{fl}. The experiment was carried out as described in Materials and Methods. In the experiment of curve A bacteriorhodopsin-containing vesicles (protein/soya-bean phospholipid ratio = 1 : 10 (w/w)) were added at zero-time to a final concentration of 0.10 mg soya-bean phospholipid/ml. At t = 90 min, a second portion of bacteriorhodopsin-containing vesicles was added giving a total vesicle concentration of 0.20 mg soya-bean phospholipid/ml. In the experiment of curve B KBH_4-treated, bacteriorhodopsin-containing vesicles were added at zero time to a final concentration of 0.10 mg soya-bean phospholipid/ml. At t = 90 min, untreated bacteriorhodopsin-containing vesicles were added to a final concentration of 0.10 mg soya-bean phospholipid/ml. In the experiments of curves A and B the same batch of bacteriorhodopsin-containing vesicles was used. Part of the batch was used for treatment of the vesicles with KBH_4 as described in Materials and Methods. ψ_{fl} was followed as a function of time by measuring the photopotential generated by a flash of 1 s at the times indicated.

hyperbolically on the number of bacteriorhodopsin vesicles associated with the filter.

To demonstrate that the filter becomes occupied with bacteriorhodopsin vesicles to a large extent after prolonged incubation, the following experiment was performed. Bacteriorhodopsin vesicles were treated with KBH_4 at pH 10, essentially as described by Peters et al. [20]. As will be discussed in the Discussion, this treatment does not change drastically the ability of the vesicles to associate with the filter. Therefore, after identical incubation periods at the same vesicle concentration, approximately the same number of inactivated bacteriorhodopsin vesicles will be bound to the filter, as in the experiment where untreated bacteriorhodopsin vesicles were used. In the experiment of Fig. 2, curve B, a Millipore filter was incubated on one side with inactivated bacteriorhodopsin vesicles for 90 min; this condition leads to a high ψ_{fl} with untreated bacteriorhodopsin vesicles (Fig. 2, curve A). The ψ_{fl} generated by filter-associated inactivated bacteriorhodopsin vesicles was very small. The addi-

tion of untreated bacteriorhodopsin vesicles in the same compartment after this incubation period of 90 min resulted in only a small increase of ψ_{fl} with time (Fig. 2, curve B). This implies that in the experiment of curve B the rate of association of untreated bacteriorhodopsin vesicles with the filter was much less than in the experiment of curve A. This difference is most easily explained by assuming that in the experiment of curve B the filter surface was occupied to a large extent by inactivated bacteriorhodopsin vesicles.

Several studies have indicated that the association of bacteriorhodopsin vesicles with planar membranes [16] or phospholipid-impregnated Millipore filters [17] is practically irreversible. To show that the increase of ψ_{fl} with time is indeed related to an increase in the number of filter-associated bacteriorhodopsin vesicles an experiment was performed in which an excess of KBH_4-inactivated bacteriorhodopsin vesicles was added to the compartment that had already contained untreated bacteriorhodopsin vesicles for a short period of time. Fig. 3 shows that the addition of a five-fold excess of inactivated bacteriorhodopsin vesicles 10 min after the addition of untreated bacteriorhodopsin vesicles resulted in a strong inhibition of the rate of increase of ψ_{fl}. This suggests that the rate of association of untreated bacteriorhodopsin vesicles with the filter is diminished due to a competition of inactivated vesicles for the same binding sites on the filter.

In summary, from the experiments of Figs. 2 and 3 it can be concluded, firstly, that the increase of ψ_{fl} with time after the addition of active bacteriorhodopsin vesicles on one side of the filter is due to association of these vesicles

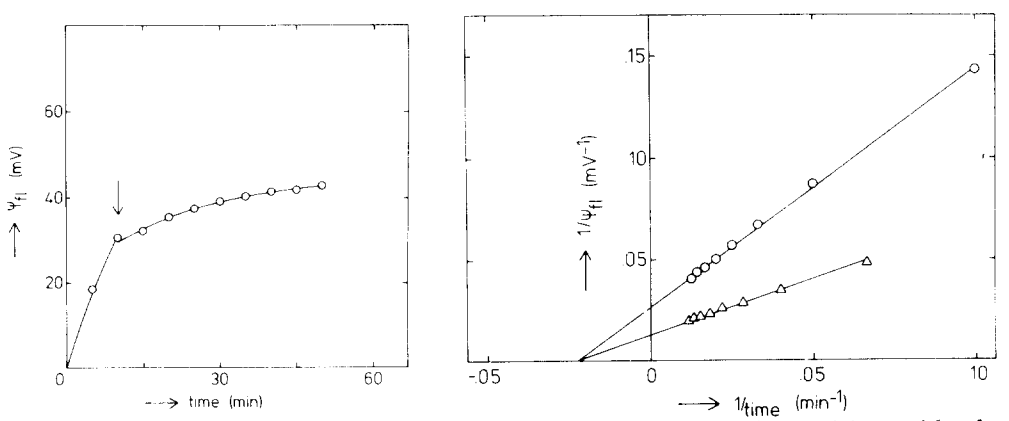


Fig. 3. Competition between untreated and KBH_4-treated bacteriorhodopsin-containing vesicles for binding. The experiment was carried out as described in Materials and Methods. At zero-time, bacteriorhodopsin-containing vesicles (protein/soya-bean phospholipid ratio = 1 : 10 (w/w)) were added to a final concentration of 0.10 mg soya-bean phospholipid/ml. At t = 10 min KBH_4-treated bacteriorhodopsin-containing vesicles were added to a final concentration of 0.50 mg soya-bean phospholipid/ml. Both the untreated and KBH_4-treated bacteriorhodopsin-containing vesicles used in this experiment were from the same original batch of vesicles. ψ_{fl} was measured as described in the legend of Fig. 2.

Fig. 4. The effect of duration of the flash of light on the time-dependence of ψ_{fl}. The experiment was done as described in Materials and Methods. At zero-time, bacteriorhodopsin-containing vesicles (protein/soya-bean phospholipid ratio = 1 : 10 (w/w)) were added to a final concentration of 0.20 mg soya-bean phospholipid/ml. The time-dependence of ψ_{fl} was measured by illuminating the Millipore filter alternately with flashes of 0.25 s (○———○) and 1.0 s (^———^).

with the Millipore filter and, secondly, that ψ_{fl} reaches a maximum after prolonged incubation because all binding sites on the filter become occupied with bacteriorhodopsin vesicles. These conclusions can be drawn irrespective of the exact mathematical relationship between ψ_{fl} and the number of filter-associated bacteriorhodopsin vesicles, which will be one of the aspects to be considered now.

Fig. 1B showed that ψ_{fl} is a hyperbolic function of the incubation time, and this can be expressed as follows:

$$\psi_{fl} = \frac{at}{t + t_{\frac{1}{2}}} \tag{1}$$

where a is a constant and $t_{1/2}$ the incubation time when ψ_{fl} reaches half its maximal value. From plots of $1/\psi_{fl}$ versus $1/t$ the values for a and $t_{1/2}$ can be calculated from the intersections on the y-axis and x-axis, respectively. Fig. 4 shows the effect of the duration of the flash of light on the time-dependence of ψ_{fl}. In this experiment, the Millipore filter was illuminated alternately by equally intense flashes of 0.25 and 1.0 s duration. In both cases, ψ_{fl} is a hyperbolic function of time. Fig. 4 shows also that the duration of the flash has an effect on the constant a, but not on $t_{1/2}$. In a similar experiment it was found that the constant a also depended on the intensity of the flash, whereas $t_{1/2}$ was not affected (data not shown). Finally, from a comparison of ψ values generated during flashes of 0.25 and 1.0 s (Fig. 4) it can be concluded that already within 1.0 s the magnitude of the photopotential deviates from linearity with time.

Fig. 5 shows how the time-dependence of ψ_{fl} varies with the concentration of bacteriorhodopsin vesicles. Since plots of $1/\psi_{fl}$ versus 1/time yield straight lines that all intersect the y-axis at the same point, we conclude that under the

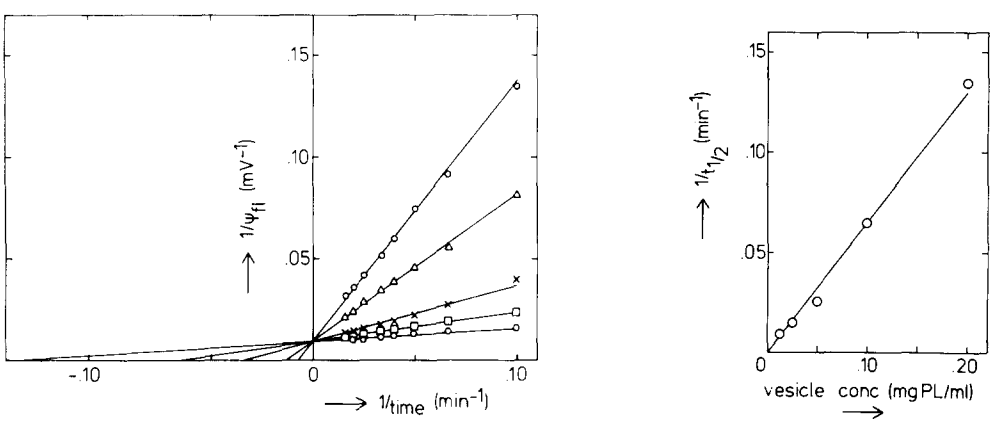


Fig. 5. The effect of the concentration of bacteriorhodopsin-containing vesicles on the time-dependence of ψ_{fl}. Experimental details are given in Materials and Methods. Bacteriorhodopsin-containing vesicles were added at zero-time to a final soya-bean phospholipid concentration of 0.0125 (○———○), 0.025 (△———△), 0.05 (X———X), 0.10 (□———□) and 0.20 mg/ml (O———O). ψ_{fl} was measured as described in the legend of Fig. 2. Protein/soya-bean phospholipid ratio of the vesicles = 1 : 10 (w/w).

Fig. 6. Dependence of $1/t_{1/2}$ on the concentration of bacteriorhodopsin-containing vesicles. $t_{1/2}$ is the incubation time when ψ_{fl} has reached half its maximal value. The data presented in this figure were calculated from the experiment of Fig. 5. PL, phospholipid.

experimental conditions used the maximal value for ψ_{fl} is independent of the vesicle concentration in the medium. In contrast, $t_{1/2}$ varies with vesicle concentration. Fig. 6 shows that $1/t_{1/2}$ depends linearly on the concentration of bacteriorhodopsin vesicles in the aqueous medium. From the direct proportionality of $1/t_{1/2}$ with the vesicle concentration, it can be concluded that the rate of association of the bacteriorhodopsin vesicles with the Millipore filter is directly proportional to the vesicle concentration in the medium.

In Fig. 7, the ψ_{fl} values measured at different times (taken from Fig. 5) are plotted as a function of the vesicle concentration. At short incubation times, ψ_{fl} is directly proportional to the concentration of bacteriorhodopsin vesicles. When the incubation is extended for longer periods of time, ψ_{fl} tends to a maximum at higher vesicle concentrations (Fig. 7). Double-reciprocal plots of ψ_{fl}, from measurements after a minimal incubation period of 10 min, versus vesicle concentration yielded straight lines which intersected the y-axis at the same point, corresponding to a maximal ψ_{fl} which was identical to that calculated from Fig. 5.

The finding that the rate of association of bacteriorhodopsin vesicles with the filter is directly proportional to the concentration of bacteriorhodopsin vesicles in the aqueous medium, whereas ψ_{fl} at infinite incubation time does not depend on vesicle concentration, made it of interest to study how these two parameters are influenced by the presence of other types of vesicles in the incubation medium.

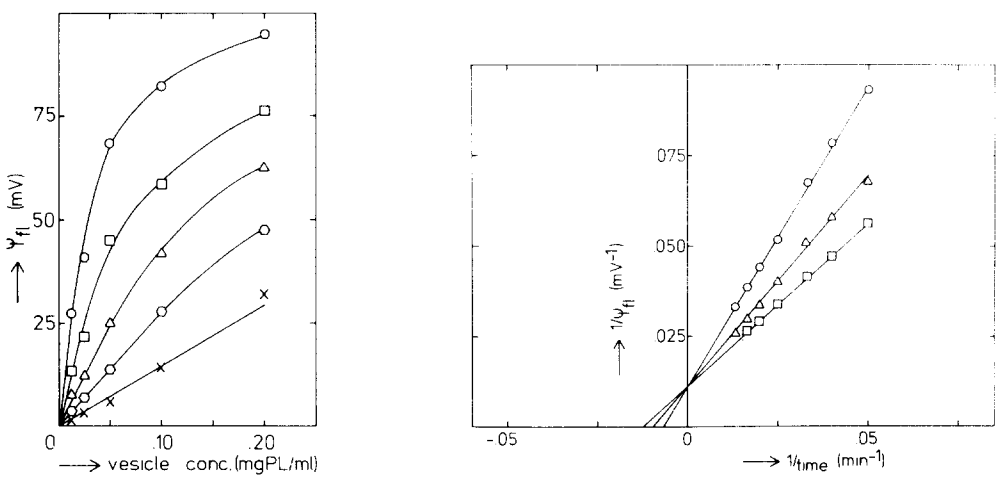


Fig. 7. Dependence of ψ_{fl} on the concentration of bacteriorhodopsin-containing vesicles. The data of this figure are the same as those presented in Fig. 5. ψ_{fl} was measured 2 (X———X), 5 (○———○), 10 (△———△), 20 (□———□) and 50 min (○———○) after addition of bacteriorhodopsin-containing vesicles. PL, phospholipid.

Fig. 8. Time-dependence of ψ_{fl}, due to the association of bacteriorhodopsin-containing vesicles with a phospholipid-impregnated Millipore filter, in the presence of various concentrations of pure soya-bean phospholipid vesicles. The experiment was done as described in Materials and Methods. At zero-time bacteriorhodopsin-containing vesicles and pure phospholipid vesicles were added simultaneously. Bacteriorhodopsin-containing vesicles (protein/soya-bean phospholipid ratio = 1 : 10 (w/w)) were added to a final concentration of 0.025 mg soya-bean phospholipid/ml. The final concentration of pure soya-bean phospholipid vesicles was 0 (□———□), 0.025 (△———△) and 0.10 mg phospholipid/ml (○———○). ψ_{fl} was measured as a function of time, as described in the legend of Fig. 2.

Fig. 8 shows how pure soya-bean phospholipid vesicles affect the time-dependence of ψ_{fl}: it can be seen that the increase of ψ_{fl} with time is retarded by pure phospholipid vesicles. However, extrapolation to infinite time yields identical values for the maximal ψ_{fl}. Since it has been shown that under the experimental conditions used ψ_{fl} reaches a maximum due to a saturation of the filter with bacteriorhodopsin vesicles (Fig. 2), it should be concluded that the presence of pure phospholipid vesicles has no effect on the number of bacteriorhodopsin vesicles that becomes eventually associated with the filter. However, the rate of association of bacteriorhodopsin vesicles is decreased by simultaneous incubation with pure phospholipid vesicles.

In addition to the effect of pure soya-bean phospholipid vesicles, we also studied the effect of inactivated bacteriorhodopsin vesicles on the time-dependence of ψ_{fl}. When we studied the effect of KBH_4 on bacteriorhodopsin vesicles it was observed that incubation of bacteriorhodopsin vesicles at elevated pH without KBH_4 already resulted in a partial inactivation of the protein, as was concluded from the irreversible disappearance of most of the purple colour. Fig. 9 shows the results of an experiment in which we compared the time-dependence of ψ_{fl} of untreated and pH 10-treated bacteriorhodopsin vesicles. It is seen that the maximal value of ψ_{fl}, given by the intersection of the straight lines on the y-axis, differs largely for the two types of bacteriorhodopsin vesicles. The finding that the maximal value of ψ_{fl} is much less in the case of pH 10-treated bacteriorhodopsin vesicles was not unexpected. Under conditions where the maximal value of ψ_{fl} is determined by saturation of the filter (Fig. 2) the association of less active bacteriorhodopsin vesicles should generate a lower maximal ψ_{fl}. Of interest is the observation that the time required to reach half the maximal ψ_{fl} is not significantly different for the two types of vesicles. Therefore, it appears that the partial inactivation at elevated pH only has

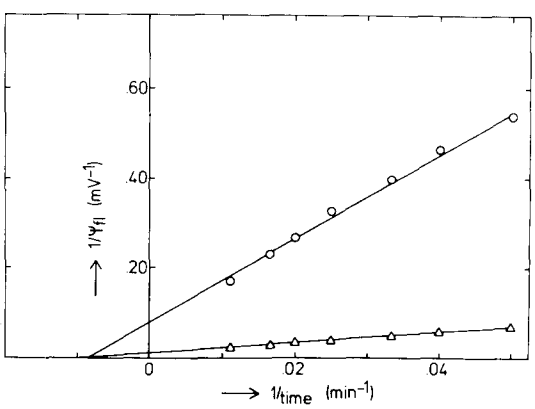


Fig. 9. The effect of treatment of bacteriorhodopsin-containing vesicles at pH 10 on the time-course of ψ_{fl}. Preparation of bacteriorhodopsin-containing vesicles and treatment of part of the suspension were performed as described in Materials and Methods. The time-course of ψ_{fl} was measured as described in the legend of Fig. 2 after addition of untreated (△———△) or pH 10-treated (○———○) bacteriorhodopsin-containing vesicles at zero-time to a final soya-bean phospholipid concentration of 0.10 mg/ml. Protein/soya-bean phospholipid ratio of bacteriorhodopsin-containing vesicles = 1 : 10 (w/w).

significant consequences for the value of the constant a of Eqn. 1 but not for $t_{1/2}$. The most obvious conclusion from these observations is that treatment of bacteriorhodopsin vesicles at pH 10 does not affect the properties, involved in the association of the vesicles with the filter. However, in that case it is assumed that there is a direct proportionality between ψ_{fl} and the number of filter-associated vesicles. An important corollary of the above assumption can be tested by measuring the time-dependence of ψ_{fl} in experiments where mixtures of untreated and pH 10-treated bacteriorhodopsin vesicles are used. This can be rationalized as follows.

Starting from the assumption that ψ_{fl} is directly proportional to the number of filter-associated bacteriorhodopsin vesicles, it can be concluded from the experiment of Fig. 9 that untreated and pH 10-treated bacteriorhodopsin vesicles have the same affinity for phospholipid-impregnated Millipore filters. This implies that in experiments where the medium contains both types of vesicle the ratio of untreated to pH 10-treated bacteriorhodopsin vesicles which are associated with the filter will be the same as the ratio of these vesicles in solution:

$$N_1^f/N_2^f = N_1^s/N_2^s \tag{2}$$

where N_1^s and N_2^s represent the concentration of untreated and pH 10-treated vesicles in the aqueous suspension, respectively, whereas N_1^f and N_2^f represent the concentration on the filter of these two types of vesicles, respectively. This equation holds for each incubation time. Further, the assumption that in experiments with only one type of bacteriorhodopsin vesicle ψ_{fl} is directly proportional to the number of filter-associated vesicles implies that the potentials are additive. So, when two types of vesicles differing in the ability to generate a photopotential are associated with the filter, it can be stated that

$$\psi_{fl} = k_1 N_1^f + k_2 N_2^f \tag{3}$$

where k_1 and k_2 are constants that take into account the ability of the vesicles to generate a photopotential. When the total vesicle concentration is kept constant ($N_{tot} = N_1^s + N_2^s$) it follows that

$$\psi_{fl} = k_3 ((k_1 - k_2) N_1^s + k_2 N_{tot}) \tag{4}$$

$$\psi_{fl} = k_3 (k_1 N_{tot} - (k_1 - k_2) N_2^s) \tag{5}$$

where k_3 is the ratio of the bacteriorhodopsin vesicles associated with the filter to the bacteriorhodopsin vesicles in solution. So, in experiments with two types of bacteriorhodopsin vesicles that differ in the ability to generate a photopotential, it follows from Eqns. 4 and 5 that ψ_{fl} is proportional to the concentration of bacteriorhodopsin vesicles of one type in the medium, provided that the affinity of the vesicles for the filter is the same for both types and that the total concentration of vesicles is kept constant. This condition can be tested by using untreated and pH 10-treated bacteriorhodopsin vesicles since the incubation at elevated pH does not affect significantly the affinity of the vesicles for the filter (Fig. 9). From Fig. 10, which shows the results of such an experiment, it can be seen that the experimental data are in reasonable agreement with the prerequisite outlined above. A relationship between ψ_{fl} and the

number of filter-associated bacteriorhodopsin vesicles of one type, N^t, which is more complicated than a direct proportionality is not very likely for the following reason. If a non-linear relationship between ψ_{fl} and N^f exists, it has to be concluded from Fig. 9 that treatment of bacteriorhodopsin vesicles at pH 10 influences the affinity of the vesicles for the filter. However, the affinity would then be changed such that the time required to reach half the maximal ψ_{fl} would remain constant (Fig. 9), which would be coincidental. Furthermore, it would be very unlikely that under these conditions a linear relationship would be obtained in the experiment of Fig. 10, between ψ_{fl} and the concentration of one type of vesicle in the incubation medium. Therefore, although it has not been proven directly, the experiments of Figs. 9 and 10 can be used as an indication that ψ_{fl} is directly proportional to N^f, the number of filter-associated bacteriorhodopsin vesicles.

Independent of the above appraoch, there is a second line of evidence

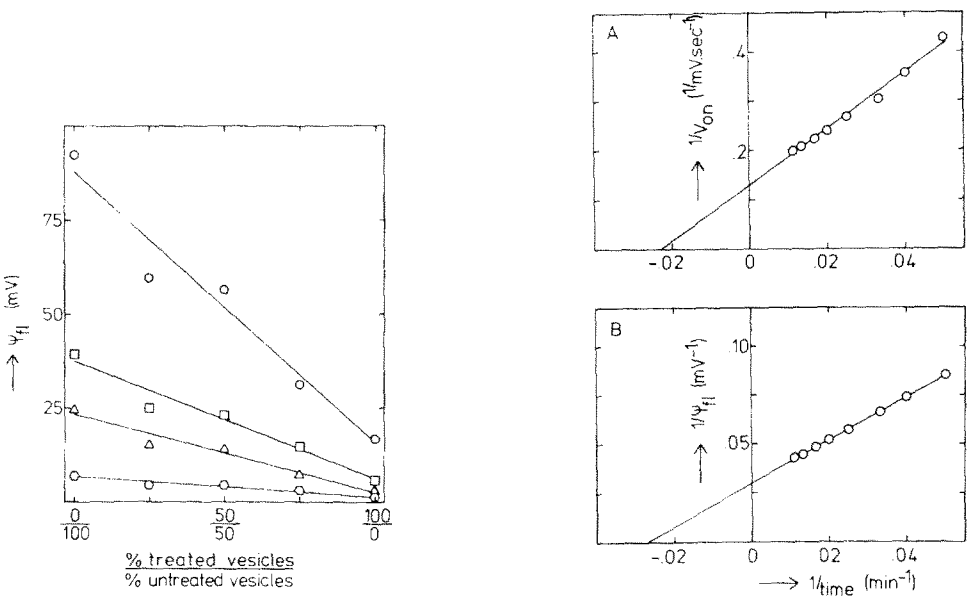
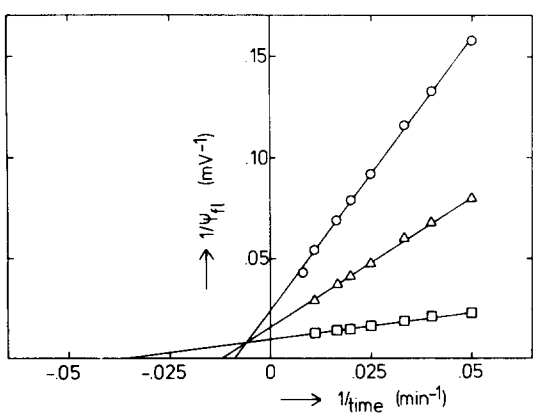


Fig. 10. Relationship between ψ_{fl} and the relative proportions of untreated and pH 10-treated bacteriorhodopsin-containing vesicles. The experiment was done as described in Materials and Methods. At zero-time, untreated and pH 10-treated bacteriorhodopsin-containing vesicles were added simultaneously to a final total vesicle concentration of 0.10 mg soya-bean phospholipid/ml. The vesicles used in this experiment were the same as those used in the experiment of Fig. 9. The data presented are the values of ψ_{fl} that were measured 10 (○———○), 40 (△———△) and 90 min (□———□) after addition of the vesicles. The maximal ψ_{fl}, reached at infinite time (○———○), was calculated from double-reciprocal plots similar to those of Fig. 9. ψ_{fl} was measured as described in the legend of Fig. 2.

Fig. 11. Comparison of the time-dependences of V_{on} and ψ_{fl}. The experiment was performed as described in Materials and Methods. Bacteriorhodopsin-containing vesicles (protein/soya-bean phospholipid ratio = 1 : 10 (w/w)) were added at zero-time to a final concentration of 0.20 mg soya-bean phospholipid/ml. V_{on}, the initial rate with which the photopotential is generated (A), and ψ_{fl} (B) were determined at various times after addition of the vesicles by illuminating the filter with light of low intensity. In this experiment ψ_{fl} represents the photopotential generated by a flash of 10 s. The data of plots A and B were from the same incubation, since it was possible to determine both V_{on} and ψ_{fl} from the time-course of the photopotential generated during one flash.

indicating a direct proportionality between ψ_{fl} and N^t. In analogy to enzyme kinetics, it can be assumed that the initial rate at which the photopotential is generated, V_{on}, is directly proportional to the number of functional bacteriorhodopsin molecules, or to the number of filter-associated bacteriorhodopsin vesicles. Since the generation of the photopotential is a very rapid process, V_{on} can be measured using ordinary techniques only if the Millipore filter is illuminated with light of low intensity. Fig. 11A shows the time-dependence of V_{on} for a typical experiment. It appeared that also V_{on} depends hyperbolically on the incubation time. In Fig. 11B the time-dependence of ψ_{fl} is presented for the same experiment. In this experiment the duration of the flash was relatively long (10 s) because of the low intensity of the light used. From a comparison of Figs. 11A and 11B it can be concluded that the time required to reach half the maximal value of V_{on} and ψ_{fl}, respectively, is identical. This means that, in the equations describing the time-dependences of V_{on} and ψ_{fl}, $V_{on} = a't/(t + t_{1/2})$ and $\psi_{fl} = at/(t + t_{1/2})$, the factor $t_{1/2}$ has the same value. Combining the observation that V_{on} and ψ_{fl} have very similar time-dependences with the statement that V_{on} is directly proportional to the number of filter-associated bacteriorhodopsin vesicles, it can be concluded that ψ_{fl} also is directly proportional to the number of filter-associated bacteriorhodopsin vesicles. In other words, the increase of ψ_{fl} with time parallels the increase of filter-associated bacteriorhodopsin vesicles with time.

In the experiments described so far, the concentration of Ca^{2+} was kept constant. Fig. 12 shows the time-dependence of ψ_{fl} at different Ca^{2+} concentrations. From this figure it can be seen that Ca^{2+} has an effect on both the maximal ψ_{fl}, (constant a of Eqn. 1) and on the time needed to reach half the maximal potential ($t_{1/2}$). Fig. 13 shows how the maximal ψ_{fl} and $t_{1/2}$ depend on the rhodopsin after reconstitution of the protein in lipid vesicles. It was observed that



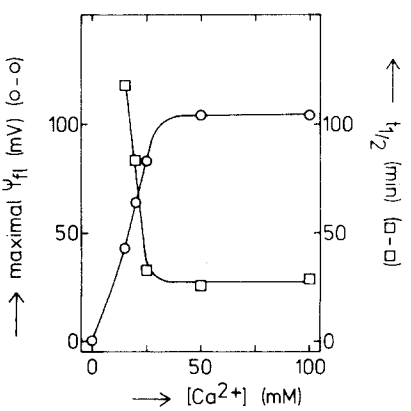


Fig. 12. The effect of variation of the Ca^{2+} concentration in the incubation medium on the time-dependence of ψ_{fl}. The experiment was done as described in Materials and Methods. The final concentration of bacteriorhodopsin-containing vesicles (protein/soya-bean phospholipid ratio = 1 : 10 (w/w)) was 0.10 mg soya-bean phospholipid/ml. The compartments on both sides of the filter contained 15 mM $CaCl_2$, 127 mM KCl (○———○), 20 mM $CaCl_2$, 120 mM KCl (△———△) and 100 mM $CaCl_2$ (□———□). ψ_{fl} was measured as described in the legend of Fig. 2.

Fig. 13. The effect of the concentration of Ca^{2+} on the maximal ψ_{fl} and on $t_{1/2}$. The maximal ψ_{fl} and $t_{1/2}$ were calculated from plots where $1/\psi_{fl}$ was plotted versus 1/time, similar to those shown in Fig. 12.

Ca^{2+} concentration in the incubation medium. Above a concentration of approx. 40 mM Ca^{2+}, both the maximal ψ_{fl} and $t_{1/2}$ reach a plateau and remain constant upon further increasing the Ca^{2+} concentration. From this experiment it can be concluded that the rate of association of bacteriorhodopsin vesicles with the filter depends on the Ca^{2+} concentration. Moreover, the maximal ψ_{fl} is also dependent on the concentration of the divalent cation. This may suggest that Ca^{2+} ions have an effect on the maximal number of bacteriorhodopsin vesicles that can be bound to the filter.

Discussion

In this paper experiments are reported which describe the association of bacteriorhodopsin vesicles with phospholipid-impregnated Millipore filters. The association was followed by measuring ψ_{fl}, the photopotential generated during a short flash of light, as a function of time. It is concluded that ψ_{fl} is directly proportional to the number of filter-associated bacteriorhodopsin vesicles (Figs. 10 and 11). Based on this finding, the observation that ψ_{fl} increases hyperbolically with time (Figs. 1, 4, 5 and 12) indicates that under several experimental conditions the association of bacteriorhodopsin vesicles with the filter also depends hyperbolically on incubation time. This result was rather unexpected since in a simple model where binding of the vesicles to the filter involves one binding site at the filter surface per vesicle, the rate of association should be second order or, when vesicles are in excess, pseudo first order. When, however, the association is a more complex phenomenon, it can be derived mathematically that under certain conditions the number of vesicles bound to the filter increases hyperbolically with incubation time. Our results concerning the kinetics of the association process do not agree with those obtained with the "planar membrane system", in which it was found that the photo effect increased linearly with time up to at least 60% of its maximal value [16]. This difference may be due to the use of different experimental systems.

From our experiments it further can be concluded also that the rate of vesicle association with the filter under otherwise comparable conditions is directly proportional to the concentration of bacteriorhodopsin vesicles (Figs. 6 and 7), whereas the final level of association is not dependent on vesicle concentration (Fig. 5). The rate of vesicle association also depends on the concentration of Ca^{2+} in the incubation medium (Figs. 12 and 13). The finding that the maximal value of ψ_{fl} also varies with Ca^{2+} concentration (Figs. 12 and 13) may be ascribed to an effect of Ca^{2+} on the number of vesicles that can be bound to the filter. However, Ca^{2+} may also have a direct effect on the ability of the reconstituted bacteriorhodopsin to generate a photopotential. The following experimental observation suggests that this direct effect of Ca^{2+} is probably not very large. When the vesicles were associated with the filter at a Ca^{2+} concentration of 50 mM, a subsequent decrease of the Ca^{2+} concentration did not have a very pronounced effect on the photopotential. However, direct measurement of the association of bacteriorhodopsin vesicles with the filter is necessary to answer this question more definitely.

A second point which needs some attention is the lability of bacterio-

rhodopsin after reconstitution of the protein in lipid vesicles. It was observed that incubation of bacteriorhodopsin vesicles, prepared by sonication procedures, at pH 10 led to a relatively rapid loss of the purple colour, which, after readjustment to pH 6, was found to be largely irreversible. Measurement of the time-dependence of ψ_{fl} indicated that partial inactivation of the protein at pH 10 did not significantly affect the association of the vesicles with the filter (Fig. 9). Similar experiments were carried out with bacteriorhodopsin vesicles treated at pH 10 in the presence of the reducing agent KBH_4. Illumination of purple membranes under these conditions results in the formation of a new pigment with an absorption maximum at 360 nm [20,21]. We observed that KBH_4 treatment of bacteriorhodopsin vesicles in the light resulted in a complete loss of the ability of the vesicles to generate a photopotential in the Millipore-filter system. Simultaneous incubation of untreated and KBH_4-treated bacteriorhodopsin vesicles demonstrated that this was not due to a loss of the ability of the vesicles to associate with the filter. The experiment indicated that the KBH_4-treated bacteriorhodopsin vesicles had only a slightly decreased affinity for the filter compared to untreated bacteriorhodopsin vesicles.

A detailed explanation of the experiment in which we studied the effect of pure phospholipid vesicles on the time-dependence of ψ_{fl} generated by bacteriorhodopsin vesicles associated with the filter (Fig. 8) is hampered by the fact that it is unknown how the vesicles are associated with the filter. In this respect, it is important to note that there is no lag period in the appearance of ψ_{fl} after addition of bacteriorhodopsin vesicles to the incubation medium. This indicates that effective binding of the vesicles with the filter is the rate-limiting step and that subsequent processes with filter-bound bacteriorhodopsin vesicles are very rapid or do not occur at all. Furthermore, it is important to note that the association of bacteriorhodopsin vesicles with planar membranes and Millipore filters is practically irreversible [16,17].

A surprising finding of this study was that pure soya-bean phospholipid vesicles only produce a decrease in the rate at which ψ_{fl} increases with time, without affecting the maximal value of ψ_{fl} at infinite incubation time (Fig. 8). This may be due to one of the two following factors. In the first place, the binding of pure phospholipid vesicles with the filter may be reversible, in contrast to the binding of bacteriorhodopsin vesicles, which is supposed to be irreversible [16,17]. This will yield a filter that contains only bacteriorhodopsin vesicles after long incubation times. Secondly, it may be that both pure phospholipid vesicles and bacteriorhodopsin vesicles fuse with the filter. Under these conditions, the phospholipids that constituted the vesicle bilayer may dissolve in the bulk hexadecane of the filter, and the maximal value of ψ_{fl} is determined by a situation where the filter-water interface contains a maximal number of bacteriorhodopsin molecules. Experiments are in progress to obtain more insight how the bacteriorhodopsin vesicles are associated with the filter.

Acknowledgements

The present investigation was carried out with financial aid from the Netherlands Organization for the Advancement of Pure Research (Z.W.O.) under the auspices of the Netherlands Foundation for Chemical Research (S.O.N.). We are grateful to Drs. K.J. Hellingwerf for isolating purple mem-

branes, and to Dr. R. Casey and Dr. P.W. Postma for critically reading the manuscript.

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