# STUDY OF PROTON PUMPS BY PHOSPHOLIPID-IMPREGNATED MILLIPORE FILTERS

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Received 6 November 1979

## 1. Introduction

Model membranes have been extensively used to investigate various properties of biological membranes [1]. In order to get biologically relevant information the physical properties of the model membrane should be as close as possible to those of biological membranes [1,2].

The physical properties of phospholipid vesicles and planar lipid bilayers are close to those of biological membranes. Phospholipid vesicles are too small to permit direct electrical measurements across their membrane; planar lipid membranes, which allow electrical measurements, are usually too fragile and difficult to handle. To overcome this drawback phospholipid-impregnated Millipore filters were introduced [3–5]. It was claimed that the physical properties of these model membranes are close enough to those of biological membranes to allow conclusions about reconstitution of isolated peptides into them [5].

Here we show that the capacitance of phospholipid-impregnated Millipore filters is too low, indicating that these membranes are too thick, to allow the incorporation and study of membrane channels. However, one can attach phospholipid vesicles to the phospholipid-impregnated Millipore filters and record electrical parameters across the filter to investigate proton movement across the vesicles and into the Millipore filters.

## 2. Materials and methods

Bacteriorhodopsin was prepared from *Halobacterium halobium* as in [6]. Chloroplast proteolipid in butanolic solution was prepared as in [6]. Proteolipid enriched preparations were separated by a ficol gradient following sonication in the presence of 1% sodium cholate as in [7]. The proteolipid and the bacteriorhodopsin were reconstituted into preformed 20 mg/ml asolectin vesicles by the freeze–thaw technique in [8].

Millipore filters were soaked in 2% asolectin in *n*-decane. When cellulose filters were used the right impregnation required 12 h incubation [5]. We found that when Teflon (LCWP 01300 10 $\mu$m pore-size) filters were used 1 min incubation was sufficient. The filter was then mounted between two half-cells with 1 cm diam. aperture between them. Leaks were sealed off by vaseline coating on the filter edges. The filter was dried by blowing nitrogen for 10 min and both chambers were filled with aqueous solutions. Bacteriorhodopsin and chloroplast proteolipid containing vesicles were applied to the system either in the aqueous solution or directly onto the dry filter prior to filling of the chambers. In the latter case 5 min equilibration with 100 mM $CaCl_2$ was sufficient to achieve saturation of the photovoltage. A pair of calomel electrodes were used for measurements of the conductance, capacitance and photovoltage across the filter. The capacitance was measured by means of a universal bridge (Wayne-Kerr B-224) using an external oscillator.

## 3. Results

The Millipore membrane-specific conductance was between $2 \times 10^{-10}$ and $10^{-8}$ S/cm$^2$ and its specific capacitance was between 100–200 pF/cm$^2$. These data strongly indicate that the impregnated Millipore filter is a rather thick membrane in the range of a few nm.

When bacteriorhodopsin containing vesicles were

 *Elsevier/North-Holland Biomedical Press*

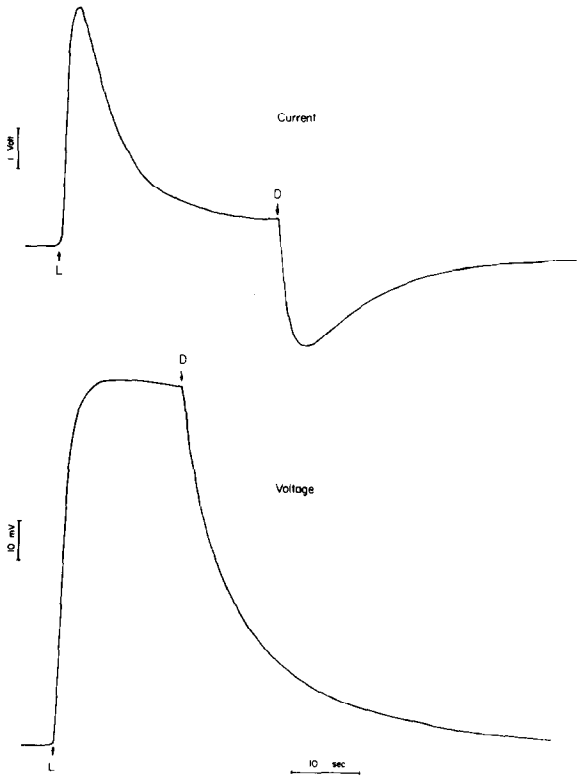


Fig.1. Photovoltage and photocurrent generated by bacteriorhodopsin vesicles attached to phospholipid-impregnated Millipore filter. Bacteriorhodopsin vesicles (50 $\mu$l) containing 1 mg phospholipids and 0.1 mg bacteriorhodopsin were placed on one side of the phospholipid-impregnated Millipore filter in a medium containing 50 mM Tricine (pH 8) and 100 mM $CaCl_2$. After 10 min incubation the excess vesicles were washed out and the two chambers were filled with a solution containing 25 mM Tricine (pH 8) and 50 mM NaCl. The filter in this experiment was 0.5 $cm^2$. The photocurrent was measured under voltage clamp with $10^{10}$ $\Omega$ resistance [2]. Illumination was with a slide projector providing $5 \times 10^5$ ergs . $cm^{-2}$. $s^{-1}$ at the filter.

placed on one side of the filter, in the presence of 100 mM $CaCl_2$, photovoltage (negative on the bacteriorhodopsin side) and photocurrent were developed in the light (fig.1). Incubation for 5 min was sufficient for obtaining maximum effect following which the chambers could be washed several times without losing the light-induced voltage formation. Thus, the possibility that the photovoltage originates from vesicles in suspension seems to be ruled out. It was suggested that bacteriorhodopsin pumps the protons across the impregnated Millipore filter [9]. Figure 2 shows a very simple experiment that rules out this hypothesis. The extent of the amplitude that was generated by the bacteriorhodopsin in the light was independent of the pH gradient across the Millipore filter. There is no biological pump that will not be affected by an uphill concentration gradient of 4 orders of magnitude. Moreover, the imposed pH gradient of 4 units across the filter did not create the expected 240 mV potential. This is probably due to the very low proton conductance across the filter.

Numerous attempts to collapse the photovoltage, by incorporating chloroplast proteolipid, a proton channel [6], into the Millipore filter during the impregnation produced negative results. Moreover, application of chloroplast proteolipid containing vesicles to the compartment opposite to that containing the bacteriorhodopsin vesicles, had no effect on the photovoltage. However, when the proteolipid was incorporated to the same vesicles as the bacteriorhodopsin the photovoltage response was diminished.

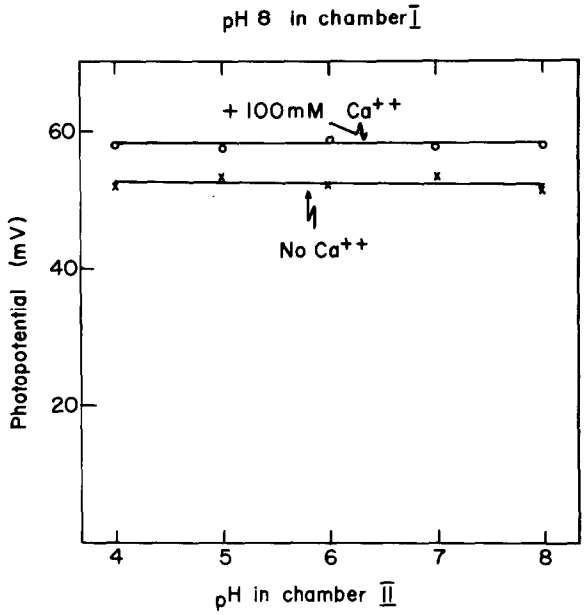


Fig.2. Proton concentration gradients across the phospholipid-impregnated Millipore filter have no effect on the amplitude of the photovoltage generated by attached bacteriorhodopsin vesicles. Experimental conditions as described in fig.1. After 10 min incubation with $CaCl_2$ the excess vesicles were washed out and the chambers were filled with a solution containing 50 mM Tricine–maleate at the given pH, 100 mM NaCl and 100 mM $CaCl_2$ when added. The samples were illuminated by a slide projector providing $5 \times 10^5$ ergs . $cm^{-2}$. $s^{-1}$ at the level of the filter.

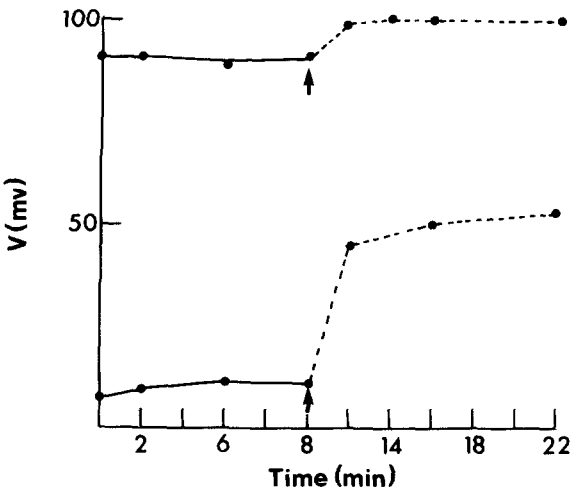


Fig.3. Chloroplast proteolipid collapse the photovoltage when present in the bacteriorhodopsin vesicles. Experimental conditions as in fig.1 except that the medium was at pH 7 and during the reconstitution with bacteriorhodopsin 20 μl light fraction (upper trace) or heavy fraction (lower trace) from the ficol gradient were added (see [7] for conditions). Where indicated (arrow) dicyclohexyl carbodiimide at 10 μM final conc. was added.

Figure 3 shows the extent of the photovoltage evoked by light across the filter with vesicles containing in addition to bacteriorhodopsin one of the two different fractions that were obtained from the ficol gradient [7]. The upper trace shows that incorporation of the fraction which is depleted of the chloroplast proteolipid had almost no effect on the amplitude. The lower trace illustrates that when the fraction enriched with proteolipid was incorporated into the bacteriorhodopsin vesicles, the photovoltage was markedly reduced and that upon the addition of dicyclohexyl carbodiimide it increased 4-fold, up to 50% of the control.

## 4. Discussion

The low capacitance of the phospholipid-impregnated Millipore filters suggests that they are thick membranes. Under no condition could we reproduce the reported high capacitance in a similar system [5]. The fact that the amplitude of the photovoltage was not affected by an uphill ΔpH of 4 units renders the proposal that the protons are pumpted by the bacteriorhodopsin across the filter unlikely. We suggest the following mechanism for the photovoltage generation by bacteriorhodopsin vesicles attached to phospholipid impregnated Millipore filters:

(1) Reconstituted bacteriorhodopsin vesicles fuse to the surface of the phospholipid impregnated Millipore filter;
(2) The thick 'membrane' provides space for the protons that are pumped by the bacteriorhodopsin in the light. This is reflected by the high initial capacitative current (fig.1).
(3) The low steady state current (~30 pA/cm$^2$) is a result of the proton leak through the filter.
(4) In the dark the protons are diffusing out of the filter leading to a negative capacitative current, and the photovoltage is cancelled via conductance (not necessarily proton conductance) in the thin portions of the membrane.

These conclusions are in line with the experiments that showed that the chloroplast proteolipid had to be present in the bacteriorhodopsin vesicles in order to collapse the photovoltage. Nevertheless, the application of phospholipid-impregnated Millipore filters for the study of proton pumps and proton channels is useful (see [4]). The system is very stable, reproducible and easy to handle. It is more sensitive than direct pH measurements and it is not sensitive to external buffers.

## Acknowledgements

This work was supported by a grant from The Israel Commission for Basic Research. We wish to thank Dr M. Montal for critically reading the manuscript.

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