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INCORPORATION OF BACTERIORHODOPSIN INTO LARGE UNILAMELLAR LIPOSOMES BY REVERSE PHASE EVAPORATION

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The reverse phase evaporation procedure was used to prepare large unilamellar liposomes containing bacteriorhodopsin. Electron microscopy showed that proteoliposomes were unilamellar and fairly uniform in size provided the preparation was extruded through calibrated nucleopore membranes : the vesicles have diameters around 200 nm. The spectral properties of the bacteriorhodopsin in the large liposomes resembled those of bacteriorhodopsin in purple membrane. Furthermore, the chromoprotein in the reconstituted vesicles had an inside-out orientation and on illumination, translocated protons efficiently from the external medium into the vesicles in the presence of the ionophore valinomycin. In the absence of the latter, a light-independent transmenbrane potential of about 60 mV was measured from thiocyanate distribution. In the presence of valinomycin, this transmembrane electrical potential was abolished and then a light-dependent transmembrane pH gradient of about 2 pH units could be generated.

It is generally accepted that bacteriorhodopsin, the only protein present in the purple membrane of Halobacterium halobium, operates as a light-dependent H^+ pump (1). This chromomprotein has been reincorporated in phospholipid vesicles by a variety of techniques (2-6) in such a way that net proton movement is observed upon illumination. This was essentially deduced from the light-induced changes occuring in the pH of the extravesicular medium. To calculate the respective amplitudes of the components of the proton motive force ($\Delta\Psi$ and ΔpH), fluorescence (7), ^{31}P NMR (8) and ESR techniques (9) as well as accumulation of radioactive molecules (7) were used. However, quantitation of several parameters is hampered by the relatively small diameters of the vesicles (20-30 nm), since most of these experiments have been carried out with proteoliposomes obtained by sonication. The relatively small aqueous space entrapped by the proteoliposomes seriously restricts their potential use in transport studies and for analysis of the physiochemical properties of the intravesicular space.

Abbreviations: $\Delta\Psi$: transmembrane electrical potential ; ΔpH : transmembrane pH gradient ; 9AA : 9-aminoacridine ; Pipes : 1-4 piperazine diethane sulfonate ; Mes : M-2-hydroxyethyl piperazine-N'-2-ethanesulfonate.

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Recently Szoka and Papahadjopoulos (10-11) reported a method for the production of large unilamellar liposomes by sonication of a phospholipid solution in an organic solvent with an aqueous buffer, followed by solvent evaporation under reduced pressure. Darszon et al (12) combined this technique with the procedure they developed for extracting protein-lipid complexes of membrane proteins into apolar sovents and reported the formation of large single bilayer vesicles containing photochemical active rhodopsin.

In this paper, we report on the reconstitution of purple membrane into large liposomes (200 nm) prepared by reverse phase evaporation. The vesicles formed were characterized with respect to size and structure (electron microscopy), protein orientation (proteolysis) and biological activity (change in external pH, Δ pH and $\Delta\Psi$). A preliminary account of these results was published earlier (13).

MATERIAL AND METHODS

Purple membrane fragments were isolated from Halobacterium Halobium S9 (a gift from Dr Oesterhelt) according to procedures already described (14). Purified egg-yolk phosphatidylcholine (15) and derived phosphatidic acid (16) were used. Pronase, papain and chymotrypsin were obtained from Sigma, [^{14}C] methylamine (38.4 Ci/mol) purchased from the CEN Saclay (France) and [^{14}C]SCN^- (62 Ci/mol) from Amersham.

Preparation of proteoliposomes

The reconstitution procedure was essentially derived from the general procedure described by Szoka and Papahadjopoulos (11). A typical preparation contained 30 µ mol of phospholipid (phosphatidylcholine/phosphatidic : 9/1) in 1.5 ml of diethylether and 0.5 ml of aqueous buffer (150 mM KCl and 1 mM KH_2PO_4 pH 6.8) containing purple membrane. The resulting two-phases system was sonicated under nitrogen for 3 minutes at 5°C. The organic solvent was removed at 20°C by rotary evaporation under reduced pressure (350 mm Hg). When most of the solvent was removed, the material first formed a viscous gel and then an aqueous solution. At this point 1 ml of 150 mM KCl was added and evaporation allowed to proceed for a further 20 minutes to remove all traces of solvent. The preparation (20 µ mol lip/ml) was first extruded through 0.4 µm nucleopore membranes and then through 0.2 µm nucleopore membranes.

Assays

The extravesicular medium can be easily modified by flotation on density gradients (17).

The ratio of internal to total volume of vesicle suspension was determined by titration of the external and the internal phosphate, using the pH stat technique (the internal phosphate being determined after the vesicles have been treated with Triton X100).

Bacteriorhodopsin was digested in purple membrane and in vesicles with pronase, papain and chymotrypsin, as previously reported (18, 19, 20). Gel electrophoresis of digested samples was performed in 12 % polyacrylamide gels. Prior to electrophoresis, samples containing vesicles were delipidated (18).

pH measurements were carried out in a thermostated continuously stirred vessel containing 0.5 ml of vesicle suspension and 1.5 ml of the desired saline solution. The vessel was illuminated by a 150 mW 20 V Xenon lamp equipped with heat filters and a flexible light guide.

ΔpH and $\Delta\Psi$ were measured at 25°C by estimating the respective transmembrane distributions of [^{14}C] methylamine and [^{14}C]SCN^- using a thermostated flow dialysis apparatus. There was usually 1 ml of liposomal suspension (20 µmol lip/ml) in the upper compartment. The lower chamber was perfused at a

rate of 6 ml/min and the dialysate directly connected to a radioactive flow detector (Roche-Kontron). Transmembrane solute distributions were calculated as described by Hellingwerf et al (7). In some experiments, the changes in the transmembrane Δ pH were assayed by measuring the changes in 9-aminoacridine fluorescence intensity (λex : 400 nm ; λem : 455 nm ; [9AA]= 1 μM).

RESULTS AND DISCUSSION

Size, structure and photochemical activity of reconstituted vesicles

The size and organization of phosphatidylcholine-phosphatidic acid vesicles containing bacteriorhodopsin at an 80:1 lipid to protein ratio (w/w) are shown in Fig. 1. A freeze-fracture electron micrograph of a preparation extruded through an 0.4 μm nucleopore membrane shows (Fig. 1A) large vesicles with diameters of up to 600 nm. These vesicles are extremely heterogeneous in size and some appear to consist of a few concentric bilayers. After extrusion of this preparation through an 0.2 μm nucleopore membrane it was seen to contain a more uniform population exclusively composed of unilamellar liposomes with a mean diameter of about 200 nm (Fig. 1B). Negative-staining microcopy confirmed this value (data not shown).

Encapsulation efficiency, i.e. the percentage of aqueous phase entrapped in the liposomes, was about 10 % at 20 μmol lip/ml. Calculations of the expected diameters of the vesicles based on the volume of aqueous phase captured (5 μl/μmol lip) and the phospholipid surface area per molecule (10) give a value in close agreement with the mean diameters observed by electron microscopy, thus confirming that most of the vesicles were necessarily unilamellar.

Systematic studies indicated that the encapsulation efficiency was in a large range independant of the ionic composition of the buffer (phosphate, pyrophospate, Mes, Pipes in the presence of KCl, K_2SO_4 or Na_2SO_4) of its ionic strength (100 mOS to 4000 mOS) as well as of the lipid composition of the vesicles. The extrusion method yielded liposomes the diameters of which approximated the nominal diameter of the nucleopore membrane.

The dark absorption spectrum of bacteriorhodopsin incorporated in the large vesicles, recorded against hydroxylamine-bleached vesicles, exhibited the characteristic maximum absorbance at 562 nm which on illumination showed a red-shift of about 6 nm (Fig. 2). Protein recovery after vesicle reconstitution was between 85-90 % of the original concentration present in the ether extract. These results indicate that virtually all the protein was incorporated into liposomes and that bacteriorhodopsin underwent no major photochemical changes. Therefore, it should be noted that properties of bacteriorhodopsin, which are altered by diethyl ether (21) can be recovered after removal of the solvent.

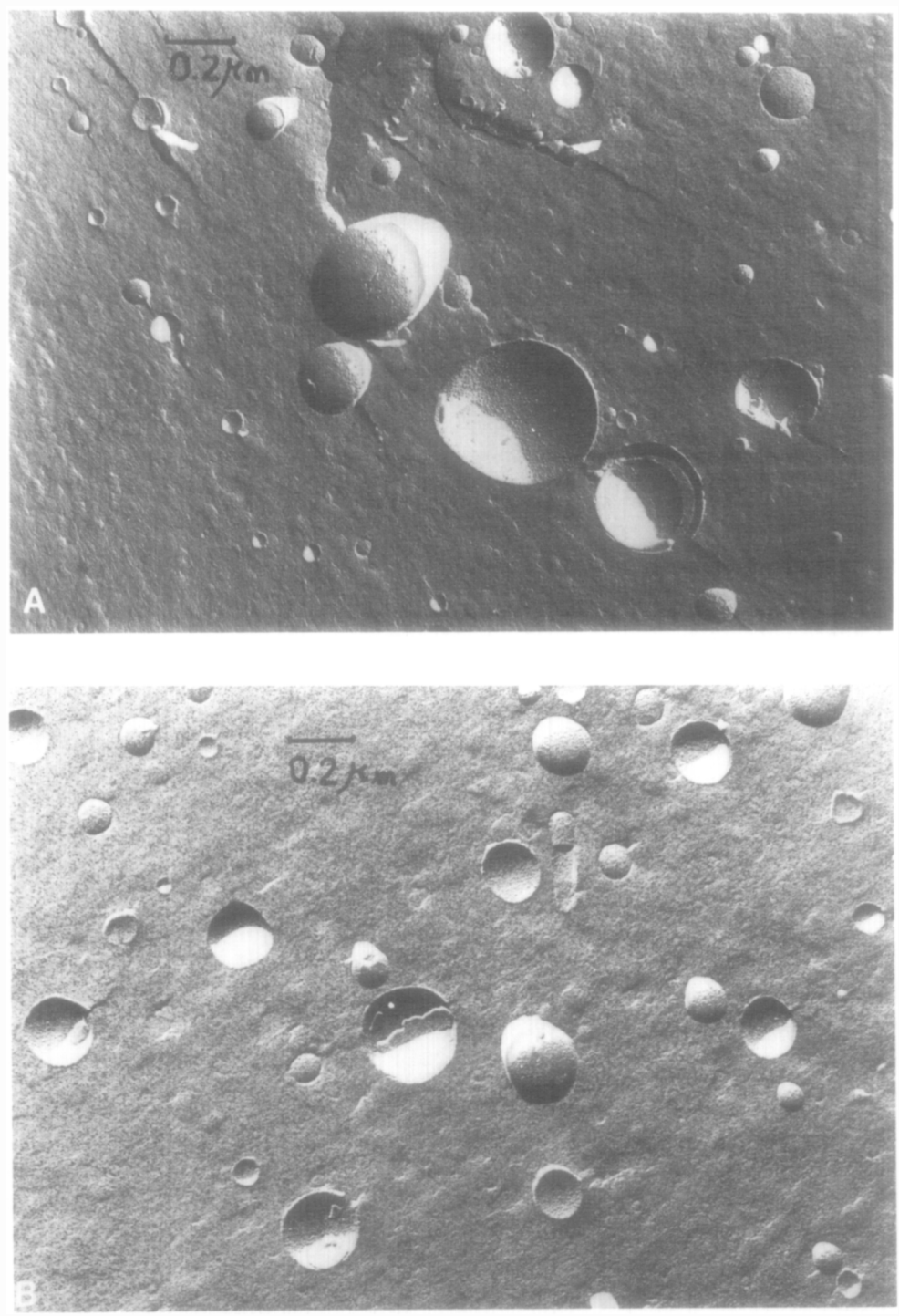
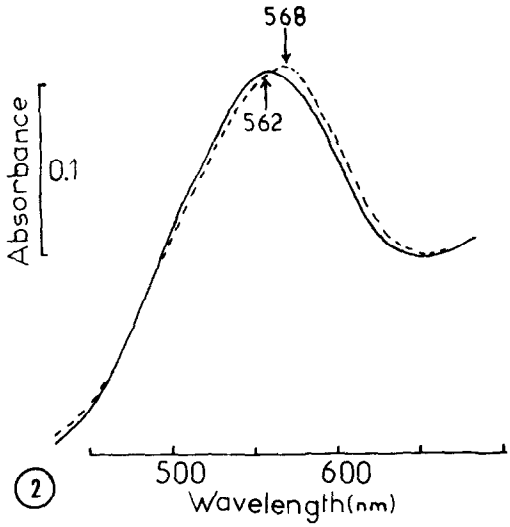


Figure 1 : Freeze-fracture electron micrographs of reconstituted bacteriorhodopsin vesicles. Proteoliposomes were prepared at a lipid/protein ratio of 80:1 (w/w) and sequentially extruded through 0.4 µm (A) and 0.2 µm(B) nucleopore membranes.



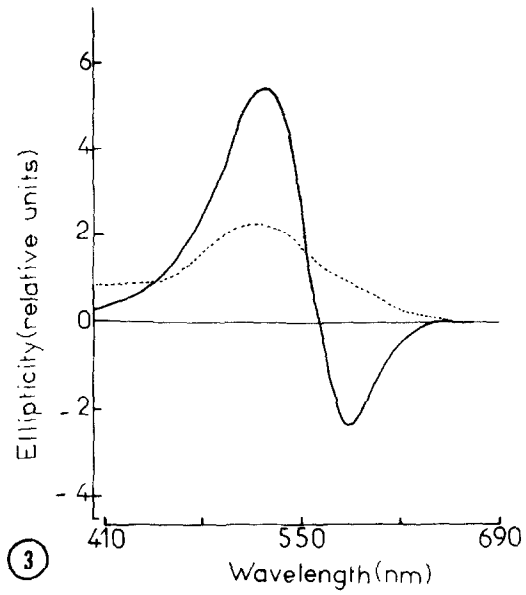


Figure 2 : Visible spectrum of bacteriorhodopsin incorporated into liposomes by reverse phase evaporation. The spectra of bacteriorhodopsin vesicles were recorded against proteoliposomes bleached in the presence of hydroxylamine; (——) light adapted (---) dark adapted.

Figure 3 : Circular dichroic spectra of bacteriorhodopsin in purple membrane (——) and incorporated into large liposomes (---). Because the bacteriorhodopsin containing vesicles exhibited considerable light scattering, proteoliposomes were prepared at a lip/prot ratio of 10:1 (w/w) and spectrum from vesicle suspension without bacteriorhodopsin was substracted.

On an other hand, the 6 nm shift of the absorption maximum of bacteriorhodopsin in the proteoliposomes after illumination (as compared to the 10 nm shift in purple membrane sheets) might be used as a convenient indicator for the monomeric state of the chromoprotein in reconstituted liposomes (6). Such suggestion was corroborated by circular dichroism measurements. From fig. 3 it is clear that the exciton coupling resulting from interactions between neighbouring bacteriorhodpsin molecules present in purple membrane sheets disappears upon reconstitution in large liposomes. These results clearly indicated (22) that bacteriorhodopsin molecules are present in a monomeric form in the reconstituted liposomes at least above the lipid to protein ratio tested (12:1 (w/w)).

Finally it has to be stressed that when the reconstituted proteoliposomes were subjected to sucrose density gradient centrifugation, all the lipid and protein appeared as a single distinct purple band (data not shown).

Bacteriorhodopsin orientation in reconstituted vesicles

For accurate determination of bacteriorhodopsin orientation in the lipid vesicles, proteolysis experiments were performed. Bacteriorhodosin-containing vesicles and purple membrane sheets were reacted with papain under

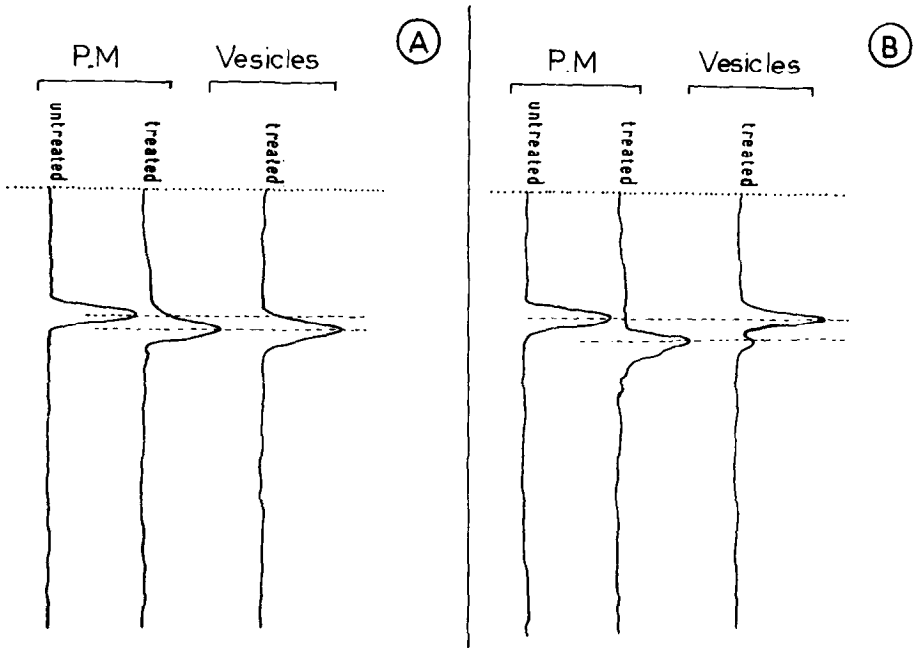


Figure 4 : Proteolysis of bacteriorhodopsin in purple membrane and incorporated into large liposomes. Proteoliposomes were prepared at a lipid/protein ratio of 80:1 (w/w) and filtered through 0.4 and 0.2 µm nucleopore membranes. A) Digestion with papain and gel electrophoresis were performed as specified previously (19) ; B) Digestion with chymotrypsin after bacteriorhodopsin bleaching as described previously (18-20).

conditions where only the COOH terminus was cleaved from the protein (18, 19). The gel-electrophoretic profiles after papain treatment showed that, in both cases, most of the bacteriorhodopsin was cleaved into shorter products (Fig. 4A). The intactness of the vesicles was tested by measuring the light-dependent proton translocation activity exhibited by the vesicles, which was fully retained after 5 hours' incubation with papain. Thus these data indicate an inside-out orientation of the bacteriorhodopsin in reconstituted liposomes compared to the intact cell where the COOH terminus faces the internal space. Although not shown, pronase treatment of the bacteriorhodopsin-containing liposomes confirmed that the carboxyl terminus was exposed on the outside of the vesicles (18).

Finally the orientation of bacteriorhodopsin was also tested using cleavage by chymotrypsin. The chymotrypsin cleavage site of the protein is known to be located on the opposite side to that of the carboxyl terminus (18, 20). Proteoliposomes (whose carboxylic terminus was facing outwards), were treated with chymotrypsin after bleaching of the bacteriorhodopsin. As expected, for an inside-out orientation most of the protein incorporated into the vesicles was unaffected by chymotrypsin (Fig. 4B). The small amount of cleavage observed might imply either that some vesicles disrupted during the

bleaching of bacteriorhodopsin or that the vesicle population contained a minor population of protein with the opposite orientation.

The results of the present proteolysis experiments all consistently show that the carboxyl terminus of bacteriorhodopsin is located on the outside of the vesicles (i.e. inside-out bacteriorhodopsin orientation). The factors controlling the sidedness of bacteriorhodopsin after reconstitution are not well understood. It has been suggested (4, 5, 23) that the orientation of this protein is mainly determined by electrostatic lipid-protein interactions. On the other hand, Van Dijck et al (4) proposed a relation between the size of the reconstituted vesicles and the orientation of bacteriorhodopsin, random protein insertion taking place above a vesicle diameter of 100 nm. From our data on liposomes with a diameter of 200 nm, it can be noted that their results cannot be extrapolated to all reconstitution procedures. The use of reverse phase evaporation and in particular the micelle formation step involved (10) probably imposes an inside-out orientation of bacteriorhodopsin, since its most hydrophilic portion may preferentially be located in the hydrophilic part of the micelle.

Proton translocation by reconstituted vesicles

A typical pH response of reconstituted purple membrane vesicles prepared in a buffer containing 150 mM KCl and 1 mM phosphate potassium at pH 6.8 and resuspended in 150 mM KCl is shown in Fig. 5A. Illumination of the vesicle suspension (15 mW/cm^2) slightly raised the external pH, indicating light-driven proton uptake by the vesicles. When the light was turned off, the pH returned to its original level. It should be stressed that the extent of proton translocation in these vesicles increased up to ten fold in the presence of 1 μM valinomycin and amounted to about 200 nmoles H^+/mg bacteriorhodopsin. Stimulation is also observed in the presence of the lipophilic tetraphenylboron anion (100 µM).

Stimulation of the proton uptake by valinomycin or TPB^- may be connected to the low ionic permeability of the vesicles. In the absence of the ionophore (or permeable lipophilic anion) translocation of very few protons suffices to develop a transmembrane potential which has been reported to be inhibitory (24). The distribution of $[^{14}C]SCN^-$ was used for quantitative measurement of $\Delta\Psi$. Owing to the large internal volume of the reconstituted proteoliposomes, thiocyanate distribution was easily determined in flowdialysis experiments. The SCN^- uptake observed upon illumination obviously indicates a transmembrane $\Delta\Psi$, positive inside, in the absence of valinomycin. From Fig. 5B the amplitude of $\Delta\Psi$ can be calculated and amounted to 60 mV at saturating light intensity (60 mW/cm^2). When the light was turned off, the preformed $\Delta\Psi$ was abolished. More important is the fact that in the presence of 1 µM valinomycin, no SCN^- uptake could be measured (data not shown).

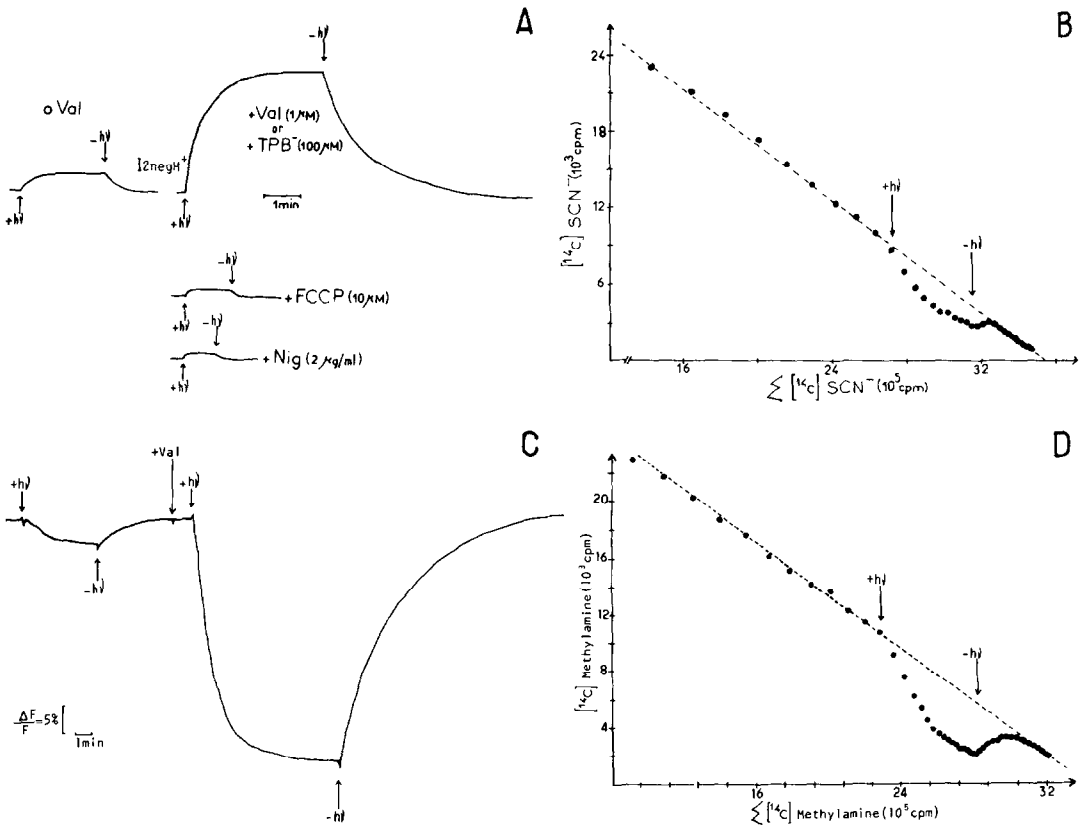


Figure 5 : Light dependent proton movement in reconstituted bacteriorhodopsin vesicles : proteoliposomes were prepared at a lip/prot ratio of 80:1 (w/w) and filtered through 0.4 and 0.2 µm nucleopore membranes.
(A) Changes in external pH. The final bacteriorhodopsin concentration in the assay medium was 50 µg prot/ml. Light intensity : 15 mW/cm^2.
(B) Light-dependent ΔΨ. (^{14}C) SCN^- distribution was measured in the absence of valinomycin by flow dialysis (0.2 mg prot/ml; light-intensity : 60 mW/cm^2).
(C) Light-dependent ΔpH as measured by changes in 9AA fluorescence (5 µg prot/ml; light intensity : 60 mW/cm^2).
(D) Light-dependent pH as measured by (^{14}C) methylamine distribution. Vesicle suspension has been treated with 1 µM valinomycin before light (60 mW/cm^2) was turned out.

Taking changes in 9-aminoacridine fluorescence intensity as an index of transmembrane ΔpH generation (25) also leads to the conclusion that in the absence of valinomycin the light induced pH was small (ΔF/F= 3-4 % (fig. 5C). However in the presence of valinomycin, 9-aminoacridine fluorescence intensity fell drastically (ΔF/F=40 %) when the light was turned on. From the work of Schuldiner et al (25) a transmembrane ΔpH gradient of 3 units can be calculated. Since fluorescence quenching measurements to quantify ΔpH are subjected to much doubt (26), the uptake of the weak base ^{14}C methylamine was used as a quantitatibe measurement of the light-dependent chemical proton gradient in reconstituted proteoliposomes. In agreement with the previously reported fluorescence results, an important ^{14}C methylamine uptake is observed only in the

presence of 1 µM valinomycin when the light was on. From the transmembrane distribution of the weak base a ΔpH of 1.8-2 units can be calculated at saturating light intensity (Fig. 5D). Finally the inhibitory effets of FCCP and nigericin (Fig. 5A) are in accordance with the electrogenic character of the light-driven proton translocation process.

The size of the pH response is a function of light intensity, increasing up to 10 fold between 5 mW/cm^2 and 60 mW/cm^2. At saturating light intensity, steady state proton pumping amounts to 800 nmoles H^+/mg prot. On the other hand the light-dependent proton uptake increases with increasing lipid to protein ratio. Maximal uptake as high as 1000 nmoles H^+/mg prot at 15 mW/cm^2 is obtained when the lipid to protein ratios are in the range 1000 to 8000:1 (w/w). Assuming an homogenous protein distribution in vesicles of 0.2 µm, it can be calculated that around 1 molecule of protein is present per vesicle at this ratio of 8000:1.

It can be concluded that in addition to the homogeneity of size of the vesicles, their relative impermeability to ions and the good orientation of bacteriorhodopsin, the large size of the proteoliposomes described in this paper has great advantages in many respects : (i) for transport studies : accurate determination of the intravesicular volume (1000 fold larger than sonicated liposomes) ; low vesicle concentrations for permeability studies ; more reliable determination of initial rates (a small internal volume would rapidly come in equilibrium with the external fluid) ; (ii) for lipid-protein interaction studies : the lipid/protein ratio can be varied within a wide range ; there is no packing difference between inner and outer vesicle layer ; no detergent is present.

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