Analysis of Passive and Light-Driven Ion Movements in Large Bacteriorhodopsin Liposomes Reconstituted by Reverse-Phase Evaporation. 1. Factors Governing the Passive Proton Permeability of the Membrane†

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ABSTRACT: Proton–hydroxyl passive permeability has been measured in large bacteriorhodopsin liposomes reconstituted by reverse-phase evaporation. Proton fluxes generated by external acid pulses have been monitored by using the fluorescence of the pH-sensitive probe pyranine trapped inside liposomes. When K^+ was the only other permeant ion, low and strongly temperature dependent proton–hydroxyl passive permeability was found (permeability coefficient, 1.25×10^{-5} cm·s^{-1} at 20 °C). It was found to be limited either by proton diffusion or by K^+ counterion diffusion, depending upon the size of the imposed proton gradient. In the presence of Cl^- ions, a greater proton permeability was observed, presumably due to diffusion of HCl molecules. Consistently, chloride pulses induced proton fluxes in liposomes due to HCl diffusion, which was shown to be much more rapid than Cl^- diffusion as a charged species. The effect of incorporated bacteriorhodopsin upon permeability was found to be slight, except at low lipid to protein ratios.

Many important bioenergetic processes involve proton translocation across the membrane catalyzed by specific membrane-associated proteins. According to the hypothesis of Mitchell, the intermediate form of free energy ensuring energy transduction is an electrochemical proton gradient $\Delta\tilde{\mu}_{H^+}$[1] (Mitchell, 1966). Bacteriorhodopsin (BR), the chromoprotein of the *Halobacterium halobium* membrane [for review see Dencher (1983)], is one of the most extensively characterized electrogenic proton pumps. This light-driven proton transporter has been used in many instances as a model $\Delta\tilde{\mu}_{H^+}$ generator. In particular, the use of reconstituted liposomes containing bacteriorhodopsin appears to be a valuable approach for the study of $\Delta\tilde{\mu}_{H^+}$ formation across the membrane (Hellingwerf et al., 1979; Westerhoff et al., 1981; Ramirez et al., 1983). We have recently described a reconstitution procedure for BR using reverse-phase evaporation. This method yields large (average diameter, 200 nm) liposomes of homogeneous composition well suited for proton transport studies (Rigaud et al., 1983; Seigneuret & Rigaud, 1985). In the present paper and the following one (Seigneuret & Rigaud, 1986), we examine in detail factors governing active and passive proton movements in these BR liposomes.

This paper is aimed to a systematic study of the proton passive permeability of reconstituted BR liposomes. Indeed, ion passive permeability of the membrane is an important parameter in proton-linked bioenergetic processes since it partially determines the range of attainable $\Delta\tilde{\mu}_{H^+}$ values. In this context, several studies have addressed the question of passive proton leaks in liposomes (Nichols & Deamer, 1980; Nozaki & Tanford, 1981; Rossignol et al., 1982; Krishnamoorthy & Hinkle, 1984). These studies have produced a broad range of values of proton permeability coefficients, depending upon the experimental approach used. Indeed, the passive proton permeability is likely to be specific to the liposomal system used since it may depend upon the nature of other ions present, the internal volume of the vesicles, and the presence of an incorporated protein. Therefore, in the present work, we have undertaken a systematic study of the passive proton permeability of BR liposomes under nonenergized conditions. Passive proton–hydroxyl fluxes have been measured by monitoring the fluorescence of the pH-sensitive probe pyranine (Clement & Gould, 1981; Seigneuret & Rigaud, 1985) entrapped inside the liposome, after short external pH pulses. It is shown that proton permeability can be varied in a wide range as a function of the ionic composition of the medium, temperature, presence of ionophores, and lipid–protein ratio. In the following paper, it will be shown that knowledge of this passive permeability is essential for understanding and controlling the kinetics and amplitudes of light-induced $\Delta\tilde{\mu}_{H^+}$ formation by bacteriorhodopsin reconstituted in these liposomes.

Materials and Methods

Chemicals. Phosphatidylcholine was extracted from egg yolk according to Singleton et al. (1965). Phosphatidic acid was prepared from the former as described by Allgyer and Wells (1979). Pyranine was obtained from Eastman Kodak. Valinomycin and FCCP were purchased from Sigma.

Reconstitution of BR. Purple membrane was prepared according to Oesterhelt and Stoeckenius (1974). Reconstituted BR liposomes were prepared by reverse-phase evaporation as described previously (Rigaud et al., 1983) using a mixture of egg phosphatidylcholine and phosphatidic acid (mole ratio, 9:1) and a lipid to protein weight ratio of 80 unless otherwise stated. Buffers used were 20 mM Pipes–KOH, 20 mM Pipes–Tris, or 20 mM KH_2PO_4–KOH, pH 7.1, supplemented with either 130 mM K_2SO_4, 150 mM KCl, or 150 mM choline chloride, and contained 200 μM pyranine. After sizing by filtration through 0.4- and 0.2-μM polycarbonate membranes, liposomes were either dialyzed or passed through a small Sephadex G-25 column to remove external pyranine.

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[1] Abbreviations: pyranine, 8-hydroxy-1,3,6-pyrenetrisulfonate; BR, bacteriorhodopsin; FCCP, carbonyl cyanide *p*-(trifluoromethoxy)-phenylhydrazone; Pipes, 1,4-piperazinediethanesulfonic acid; $\Delta\tilde{\mu}_{H^+}$, transmembrane electrochemical proton gradient; Tris, tris(hydroxymethyl)aminomethane.

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Fluorescence Measurement of Proton Fluxes. Fluorescence measurements were performed on a Perkin-Elmer MPF 44A spectrofluorometer equipped with a magnetic stirrer and a temperature control system. Excitation was performed at 460 nm, and emission was recorded at 510 nm. BR liposomes containing entrapped pyranine were diluted to a final concentration of 0.4 mM phospholipid. External pH pulses were performed by adding concentrated acid to liposome samples directly in the fluorescence quartz cuvette, and the kinetics of fluorescence intensity variation were monitored.

Net Proton–Hydroxyl Fluxes and Permeability Coefficient Calculations. In most instances, small acid pulses of 0.5 pH unit starting from an external pH value of 7.1 were performed. In such a range, pyranine fluorescence intensity is linear with pH (Seigneuret & Rigaud, 1985) so that its variations can be directly related to liposome internal pH changes. Net proton–hydroxyl fluxes were calculated from the slope of the fluorescence intensity kinetics by using

$$J_{net} = (dpH_i/dt)(B_iV_i/A) \quad (1)$$

where pH_i is the internal pH, B_i is the internal buffering power, and V_i and A are the internal volume and membrane surface area of the liposomes. V_i was measured as described previously (Rigaud et al., 1983), and A was calculated by assuming a mean packing area per phospholipid of 70 Å^2 (Nozaki & Tanford, 1981). B_i of the buffers was shown to be linear over the pH range studied. J_{net} can be related to permeability coefficients for protons and hydroxyl, P_{H^+} and P_{OH^-} through (Nichols & Deamer, 1980; Rossignol et al., 1982)

$$J_{net} = P_{H^+}[(H^+)_i - (H^+)_o] + P_{OH^-}[(OH^-)_o - (OH^-)_i] \quad (2)$$

It was checked by external pH potentiometric measurements performed under identical conditions that the external buffering power was sufficient to ensure constant values of $(H^+)_o$ and $(OH^-)_o$ during proton–hydroxyl equilibration. J_{net} values were determined both from the initial slope ($\Delta pH = 0.5$) and the slope at midpoint ($\Delta pH = 0.25$) of the fluorescence intensity kinetics by using eq 1. From the two fluxes the values P_{H^+} and P_{OH^-} were calculated with eq 2. Results were expressed as the net proton–hydroxyl permeability coefficient (Rossignol et al., 1982):

$$P_{net} = P_{H^+} + P_{OH^-} \quad (3)$$

RESULTS

Fluorescence Measurements of Proton–Hydroxyl Permeability. Our previous results have shown that pyranine fluorescence can be used as a good reporter of bulk internal pH of BR liposomes (Seigneuret & Rigaud, 1985). The probe does not readily leak out of the vesicles, and fluorescence intensity pH dependence is indistinguishable from that in aqueous solution provided that negatively charged liposomes are used.

Figure 1 shows a typical experiment in which concentrated H_2SO_4 is injected into a BR liposomes suspension at 20 °C in K_2SO_4–KH_2PO_4 medium in order to lower external pH from 7.1 to 6.6. A biphasic decrease of the fluorescence intensity is observed. An initial rapid component that is limited by the sample mixing time and that comprises only 5% of the total response is followed by a much slower decrease ($t_{1/2}$ = 46 min).

There are several possible explanations for the initial fast response. In an earlier pyranine study of proton equilibration in small unilamellar vesicles, Clement and Gould (1981) suggested that it was due to a rapid, partially electrically uncompensated proton entry. However, in our case, the relative proportion of the rapid and slow components was constant

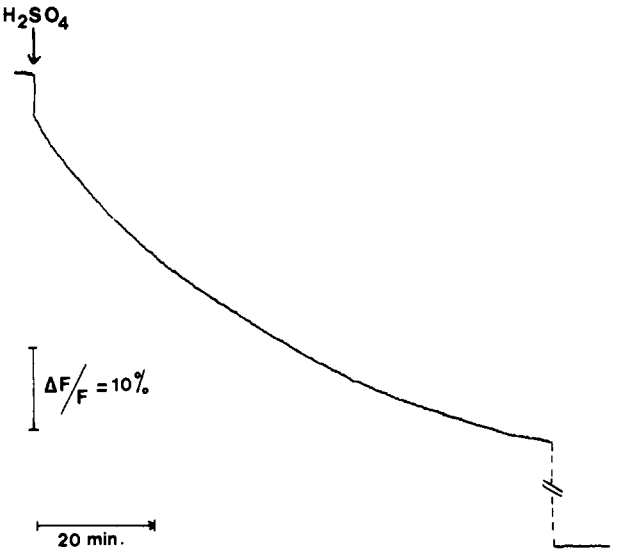

FIGURE 1: Fluorescence response of pyranine trapped inside BR liposomes in 130 mM K_2SO_4 and 20 mM KH_2PO_4–KOH, pH 7.1, after a 0.5 pH unit H_2SO_4 external pulse at 20 °C.

regardless of the initial and final pH values, of the permeant or impermeant nature of other ions present, and of the presence or absence of ionophores (valinomycin or FCCP). On the other hand, the rapid component was decreased after a further passage of the preparation over a Sephadex G-25 column but was increased after overnight storage of the preparation at 5 °C. This suggests that this component could simply be due to small amounts of pyranine present in the external medium. We have previously determined that pyranine leakage out of BR liposomes was of the order of 2.5% per day (Seigneuret & Rigaud, 1985). Furthermore, as shown in the following paragraph, under the conditions of Figure 1, the proton influx is electrically unlimited over the whole kinetics so that the interpretation of Clement and Gould does not hold up in our case (note that, in their experiments, the rapid component could amount to up to 60% of the total).

The slow kinetic component can most likely be attributed to internal pH decrease due to passive diffusion of protons into the liposomes along the pH gradient. Indeed, addition of nigericin, valinomycin/FCCP, or Triton X-100 elicited a very fast decrease of fluorescence down to the final level (not shown).

Factors Limiting Proton–Hydroxyl Fluxes in K_2SO_4 Medium. An important question is whether this acid pulse induced inward proton flux represents unrestricted proton diffusion across the membrane or whether it is electrically limited by the diffusion of counterions (most likely K^+). This can be tested by investigating the effect of either a proton carrier such as FCCP or a K^+ carrier such as valinomycin.

Figure 2 shows that adding FCCP to the BR liposomes suspension either before or after the 0.5 pH unit acid pulse greatly accelerates the slow kinetic component of the fluorescence response. The effect appears to be maximum for an FCCP concentration of 0.2 μM at which a 4-fold increase of the initial rate of proton entry is observed. On the other hand, addition of valinomycin instead of FCCP at concentrations up to 0.15 μM has no effect upon the rate of proton equilibration (Figure 3A). That valinomycin effectively increases K^+ permeability at these concentrations is shown in experiments described in the following paragraph.

These data indicate that, for BR liposomes, after an acid pulse of 0.5 pH unit, the rate of proton equilibration is governed by unrestricted proton diffusion across the membrane

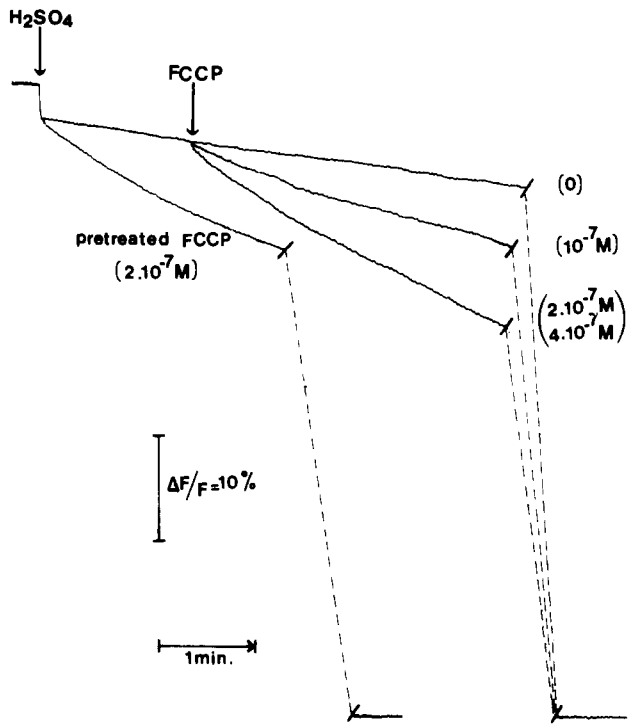

FIGURE 2: Effect of FCCP upon the fluorescence response of pyranine trapped inside BR liposomes in 130 mM K_2SO_4 and 20 mM KH_2PO_4–KOH, pH 7.1, after a 0.5 pH unit H_2SO_4 external pulse at 20 °C. The dashed lines represent the median portions of the fluorescence responses, which have been truncated for the sake of figure sizing.

and is not limited by K^+ counterion diffusion. Under these conditions, it is justified to calculate a true proton–hydroxyl permeability coefficient (see Materials and Methods). From the data of Figure 1, one finds $P_{net} = 1.25 \times 10^{-5}$ cm·s^{-1} at 20 °C. Thus, proton diffusion through BR liposomes appears to be a rather slow process in K_2SO_4 medium.

Similar results were obtained when SO_4^{2-} ions were replaced by phosphate or Pipes (not shown). This suggests that anion permeability and buffer leakage are negligible in these experiments.

Effect of K^+ Permeability. In the presence of FCCP, inward initial proton fluxes in BR liposomes can be increased 4-fold (Figure 2). Under such conditions, it is likely that the proton permeability is increased up to a value where it becomes limited by the diffusion of the only permeant counterion K^+. This allows an estimation of the intrinsic permeability of BR liposomes to K^+ as 4 times higher than that to protons.

In order to demonstrate that proton permeability in the presence of FCCP is effectively limited by counterdiffusion of K^+, the effect of valinomycin was tested. As shown in Figure 3A, for BR liposomes treated with 0.2 μM FCCP, valinomycin does increase the rate of acid pulse induced proton flux in a dose-dependent fashion. The maximum effect is observed at a valinomycin concentration of 0.05 μM, which corresponds to 1.25×10^{-4} mol of valinomycin per mole of phospholipid (i.e., 30–40 valinomycin molecules per vesicle). Thus, at this valinomycin concentration, the FCCP-catalyzed proton permeability does not seem to be any more limited by K^+ permeability. Note that at such concentrations, no effect of valinomycin is found in the absence of FCCP.

The combining effects of valinomycin and FCCP can also be observed by varying the FCCP concentration in valinomycin-pretreated liposomes (Figure 3B). As can be seen, the effect of increasing concentrations of FCCP elicited much higher rates of proton equilibration than what was observed in the absence of valinomycin. This again indicates that proton

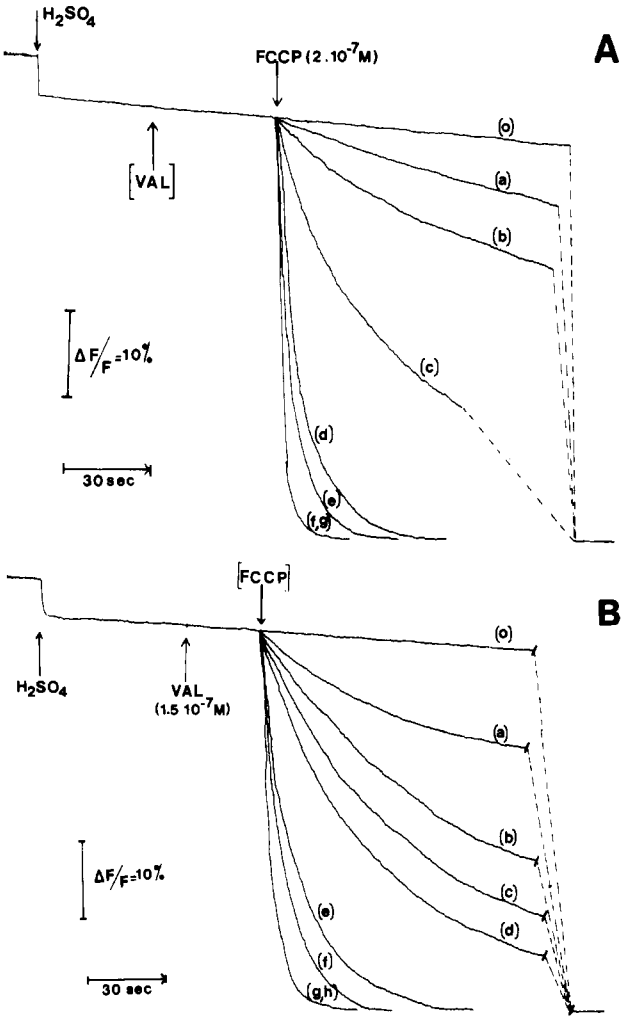

FIGURE 3: Effect of FCCP and valinomycin upon the fluorescence response of pyranine trapped inside BR liposomes in 130 mM K_2SO_4 and 20 mM KH_2PO_4–KOH, pH 7.1, after a 0.5 pH unit H_2SO_4 external pulse at 20 °C. (A) First addition: 0 (o), 0.001 (a), 0.002 (b), 0.005 (c), 0.01 (d), 0.02 (e), 0.05 (f), or 0.1 (g) μM valinomycin. Second addition: 0.2 μM FCCP. (B) First addition: 0.15 μM valinomycin. Second addition: 0 (o), 0.005 (a), 0.01 (b), 0.015 (c), 0.02 (d), 0.05 (e), 0.1 (f), 0.2 (g), or 0.4 (h) μM FCCP.

diffusion in the presence of FCCP is partially limited by K^+ permeability.

Effect of Valinomycin on Proton–Hydroxyl Fluxes. Many authors have attempted to study the effect of valinomycin-mediated K^+ permeability upon proton diffusion in liposomes, using very different concentration ranges for the antibiotics (Nichols & Deamer, 1980; Deamer & Nichols, 1983; Clement & Gould, 1981). It was thus important to test the effect of valinomycin concentration upon passive proton permeability. In Figure 4, the initial proton flux elicited by a 0.5 pH unit acid pulse is plotted as a function of the valinomycin concentration for BR liposomes prepared in K_2SO_4 medium or choline chloride medium (buffered with Pipes–Tris). The flux is independent of valinomycin concentration up to 0.2 μM. On the other hand, higher concentrations elicit a dramatic increase of initial proton fluxes up to 20-fold. This does not appear to be related to specific K^+ permeability effects since this is also observed in choline chloride medium. Thus, the effect may rather be accounted for by either a proton carrier effect of valinomycin (Lev & Buzhinsky, 1967; Walz, 1980) or a nonspecific disrupting effect on bilayer organization. Note that a concentration of 0.2 μM corresponds to a valinomycin to lipid molar ratio of 5×10^{-4}, i.e., about 100 antibiotic

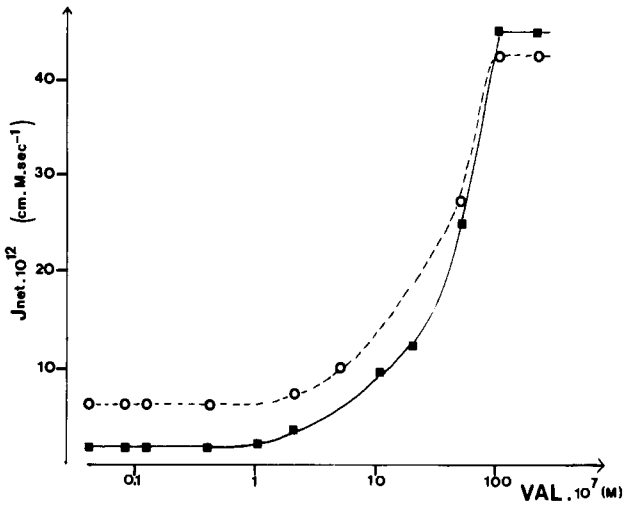

FIGURE 4: Effect of valinomycin upon the initial proton flux for BR liposomes in 130 mM K_2SO_4 and 20 mM Pipes–Tris, pH 7.1 (closed squares), and in 150 mM choline chloride and 20 mM Pipes–Tris, pH 7.1 (open circles), after a 0.5 pH unit H_2SO_4 external pulse at 20 °C. Initial fluxes were calculated from the initial slopes of the internal pyranine fluorescence response as described in Materials and Methods.

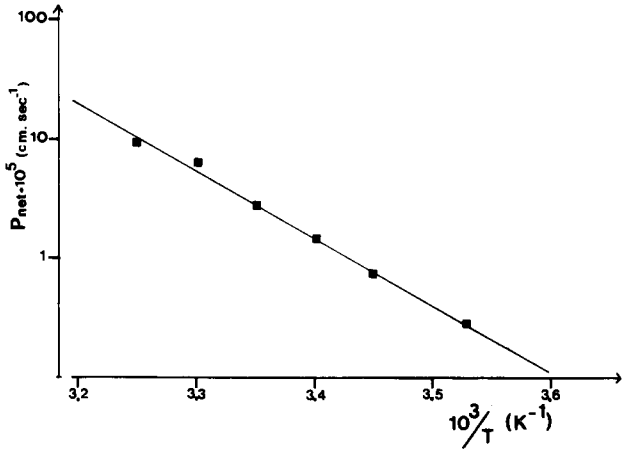

FIGURE 5: Arrhenius plot of proton–hydroxyl permeability coefficient for BR liposomes in 130 mM K_2SO_4 and 20 mM Pipes–KOH, pH 7.1. Permeability coefficients were calculated from the internal pyranine fluorescence responses after a 0.5 pH unit H_2SO_4 external pulse as described under Materials and Methods.

molecules per vesicle in our experimental conditions.

Effect of Temperature on Proton–Hydroxyl Permeability. Figure 5 shows an Arrhenius plot of proton–hydroxyl permeability coefficient measured for BR liposomes in K_2SO_4 medium. A high temperature dependence is found, corresponding to an activation energy of 22 kcal·mol^{-1}. This means that permeability coefficient is doubled every 5 °C.

Effect of the Amplitude of the pH Gradient upon Proton–Hydroxyl Permeability. It is interesting to determine whether the factors determining proton permeability characteristics of BR liposomes are dependent upon the amplitude of the pH gradient created by the H_2SO_4 pulse. The effect of external acid pulses of increasing size upon proton equilibration in K_2SO_4 medium is shown in Figure 6A. It can be seen that the initial rate of the fluorescence response increases when the amplitude of the imposed pH gradient is increased from 0.5 to 1 pH unit but that it does not increase further for higher pH gradients. A reasonable interpretation is that for pH gradients higher than 1 pH unit, the proton flux becomes sufficiently high to become limited by K^+ counterion permeability. This is confirmed by the fact that FCCP, which does

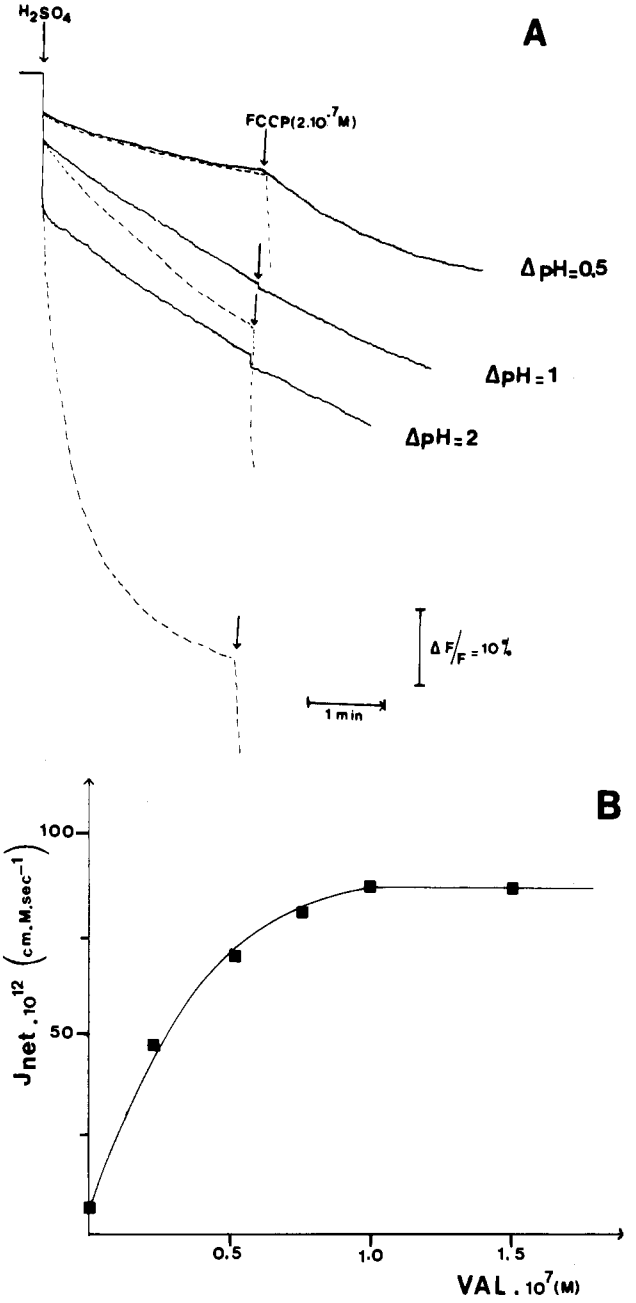

FIGURE 6: (A) Effect of the amplitude of the H_2SO_4 pulse induced pH gradient upon the fluorescence response of pyranine trapped inside BR liposomes in 130 mM K_2SO_4 and 20 mM Pipes–KOH, pH 7.1, pretreated (dashed curves) or not pretreated (solid curves) with valinomycin (0.1 μM) at 20 °C. (B) Effect of valinomycin upon the initial proton flux induced by a 1.5 pH unit external H_2SO_4 pulse for BR liposomes in 130 mM K_2SO_4 and 20 mM Pipes–KOH, pH 7.1. Initial proton fluxes were calculated from the initial slope of the internal pyranine fluorescence response as described under Materials and Methods.

increase the rate of proton equilibration in the case of a 0.5 pH unit acid pulse, has no longer any effect on proton fluxes elicited by higher pH gradients (Figure 6A). This suggests that these are limited by other factors than proton diffusion. On the other hand, valinomycin has a strong accelerating effect on these latter fluxes, and the extent of acceleration is dependent upon the size of the gradient (Figure 6A). This does prove that, in such cases, proton permeability is limited by K^+ diffusion. The effect of valinomycin concentration is shown in Figure 6B for the initial rate of proton influx induced by a 1.5 pH gradient. A concentration of 0.1 μM is necessary to abolish the restricting effect of K^+ diffusion.

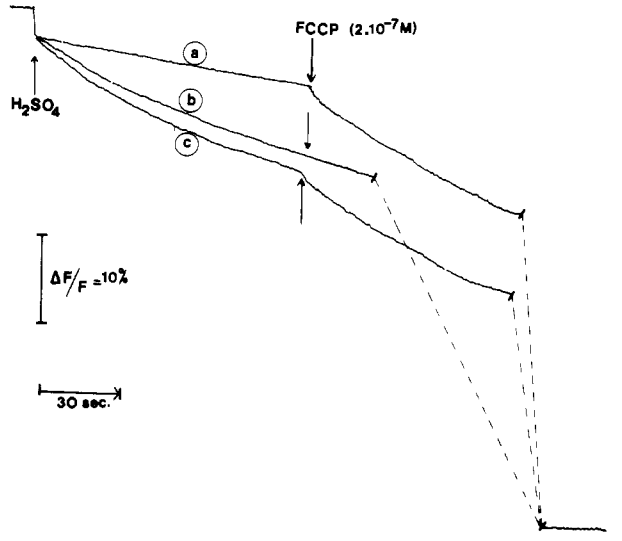

FIGURE 7: Fluorescence response of pyranine trapped inside BR liposomes in 130 mM K_2SO_4 and 20 mM Pipes–Tris, pH 7.1 (a), 150 mM choline chloride and 20 mM Pipes–Tris, pH 7.1 (b), or 150 mM KCl and 20 mM Pipes–Tris, pH 7.1 (c), after a 0.5 pH unit H_2SO_4 external pulse at 20 °C.

Influence of Cl^- Ions upon Proton Permeability. In preliminary experiments, we found that significantly more rapid proton equilibration could be observed with BR liposomes in K_2SO_4 medium when the external pH was lowered with HCl instead of H_2SO_4. For 0.5 pH unit pulses, a 4-fold increase of the initial rate of the slow component of the fluorescence response was found (not shown). This suggests that protons can move more rapidly across the membrane along the gradient when Cl^- is present as a co-ion. However, it has been shown above that proton equilibration across BR liposomes was limited by proton diffusion itself rather than by compensatory diffusion of other ions, in the case of a 0.5 pH unit pulse. An explanation is that there exists a particular mechanism for electrically silent codiffusion of H^+ and Cl^-, possibly in the form of a rapid movement of HCl molecules as already suggested (Nichols & Miller, 1974; Nozaki & Tanford, 1981).

To test this hypothesis, rates of proton equilibration after a 0.5 pH unit H_2SO_4 pulse were compared in BR liposomes prepared in K_2SO_4, KCl, or choline chloride medium. As shown in Figure 7, rates were greatly dependent upon the presence of K^+ and/or Cl^-. The slower proton flux was obtained in K_2SO_4 medium (initial flux, 2×10^{-12} M·cm·s^{-1}) where only K^+ is moving as counterion for proton. In choline chloride medium, the proton permeates more readily (initial flux, 6×10^{-12} M·cm·s^{-1}) though no K^+ is present. This confirms the hypothesis that electroneutral codiffusion of H^+ and Cl^- is a more rapid process (even in the absence of a Cl^- gradient). In KCl medium, the initial proton flux is even more rapid, being equal to the sum of the two preceding ones (8×10^{-12} M·cm·s^{-1}). Thus, in the latter case, both H^+–Cl^- codiffusion and H^+–K^+ exchange appear to operate. Additional information is obtained by observing the effect of FCCP on these proton fluxes. Figure 7 shows that the ionophore readily accelerates proton entry for BR liposomes in K_2SO_4 and KCl media, consistently with the fact that H^+–K^+ exchange is limited by H^+ diffusion. On the other hand, no effect of FCCP is found on proton entry in choline chloride medium. This shows that codiffusion of H^+ and Cl^- as separate ions is a very slow process in BR liposomes, being limited by Cl^- diffusion and not by proton diffusion. The high rates of proton permeability in chloride medium thus do have to be attributed to another mechanism in which a proton can only diffuse

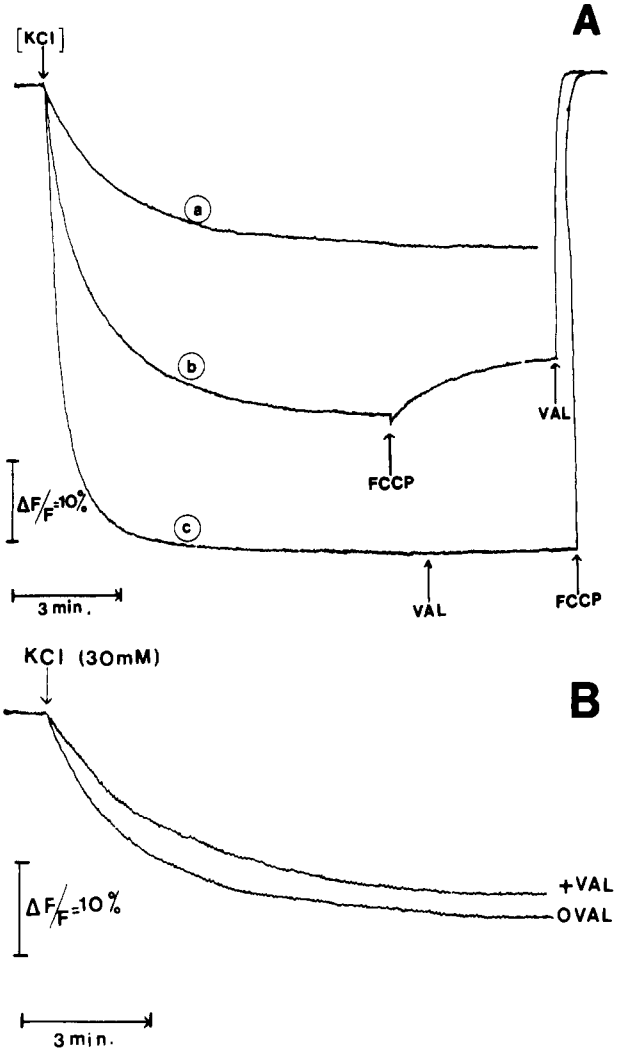

FIGURE 8: Fluorescence response of pyranine trapped inside BR liposomes in 130 mM K_2SO_4 and 20 mM Pipes–KOH, pH 7.1, (A) after a 30 (a), 60 (b), or 90 (c) mM external KCl pulse or (B) after a 30 mM KCl external pulse in the absence (0 Val) or presence (+Val) of 0.1 μM valinomycin at 20 °C.

together with a Cl^- ion. Again, the occurrence of HCl electroneutral diffusion seems likely (this process will be termed "HCl diffusion" for brevity).

Cl^- Permeability. Experiments described above indicate that HCl diffusion is the dominant mechanism of proton equilibration in chloride medium. This raises the question of whether this process has an obligatory nature for Cl^- permeation also or whether Cl^- movements as a charged ion are significant. To illustrate this point, BR liposomes in K_2SO_4 medium were submitted to an external pulse of KCl (Figure 8A). Such a pulse does lead to a decrease of internal pyranine fluorescence, indicating that equilibration of the Cl^- gradient produces an inward proton flux through HCl diffusion. The rate and extent of this proton entry increased with the magnitude of the KCl pulse (pulses of 30, 60, and 90 mM promoted maximum pH gradients of 0.2, 0.4, and 0.6 pH unit, respectively). After the pH gradient had reached its maximum value, it very slowly decayed, presumably due to slow H^+–K^+ exchange. This reequilibration was accelerated by FCCP but not by valinomycin, consistent with the limiting nature of proton diffusion. The presence of both valinomycin and FCCP elicited reequilibration within seconds (Figure 8A).

These data suggest that Cl^- gradients equilibrate partially through HCl diffusion. On the other hand, the experiment described in Figure 8B seems to indicate that a slight part of

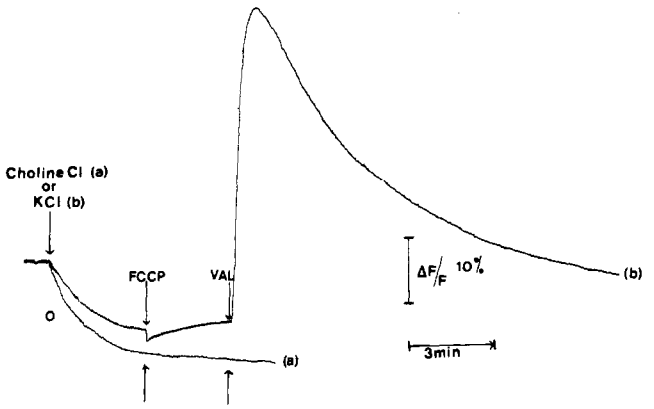

FIGURE 9: Fluorescence response of pyranine trapped inside BR liposomes in 150 mM choline chloride and 20 mM Pipes–Tris, pH 7.1, after a 30 mM choline chloride (a) or 30 mM KCl (b) external pulse at 20 °C. Added concentrations of valinomycin and FCCP were 0.1 and 0.2 μM, respectively.

Cl^- permeates as the charged species. When the KCl pulse experiment is repeated for liposomes pretreated with valinomycin, a slightly but significantly lower internal pH decrease is observed. This suggests that part of the Cl^- ions which permeated through HCl diffusion in the absence of valinomycin are now able to cross the membrane in their charged form, using the valinomycin-accelerated K^+ as co-ions. On the other hand, the latter process appears to be quite slow in view of the small difference observed and considering the high magnitude of the Cl^- gradient.

In control experiments, it was shown that a K_2SO_4 pulse induced no change in the fluorescence of BR liposome trapped pyranine. Thus, the effects observed with KCl pulses are only related to Cl^- permeation. When FCCP was added after the K_2SO_4 pulse, an increase of fluorescence was observed which was accelerated by the further addition of valinomycin (not shown). This resulted from equilibration of the K^+ gradient in exchange with H^+. Thus, unlike Cl^-, K^+ can only move as the charged species in BR liposomes.

Similar chloride pulse experiments were also performed with BR liposomes prepared in choline chloride medium. Figure 9 compares the effects of 30 mM KCl and choline chloride pulses. Both pulses induced a fluorescence decrease consistent with HCl diffusion promoted by the Cl^- gradient. However, a 30% lower effect is observed for the KCl pulse. This suggests that in this case part of the Cl^- can again diffuse together with K^+ and is thus removed from the HCl diffusion pathway. Ionophores had very different effects in the two cases. FCCP increased the decay rate of the KCl pulse induced proton gradient, and further addition of valinomycin elicited a dramatic transient internal alkalinization (maximum pH: 7.4). This is due to dissipation of the large K^+ gradient by H^+–K^+ exchange followed by dissipation of the thus created proton gradient by HCl diffusion. On the other hand, FCCP (and of course valinomycin) had no effect on the proton gradient formed by the choline chloride pulse. This does confirm that diffusion of H^+ with Cl^- as charged species is limited by Cl^- diffusion. Thus, all experiments confirm that charged Cl^- permeation, though it exists, is a slow process.

Influence of BR to Lipid Ratio upon Proton Permeability. All studies described above were performed with BR-reconstituted liposomes since one of our goals was to characterize the influence of passive permeability upon light-induced proton uptake (see following paper in this issue). It was thus important to know to what extent proton permeability is dependent upon the presence of the protein in the membrane.

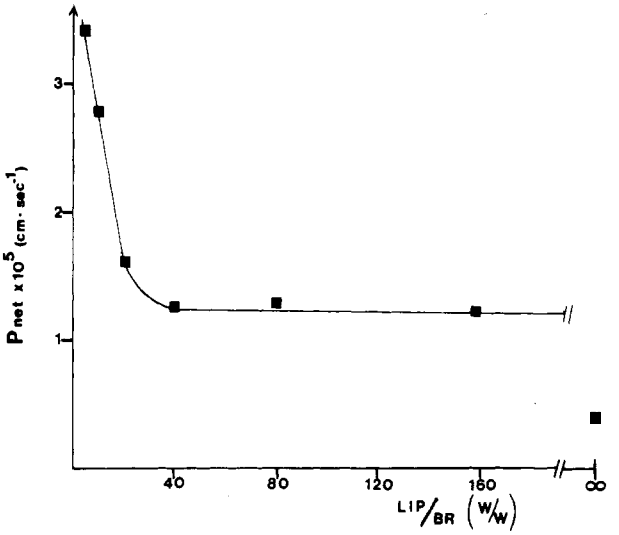

FIGURE 10: Effect of lipid to protein ratio (w/w) upon the proton–hydroxyl permeability coefficient of BR liposomes in 130 mM K_2SO_4 and 20 mM Pipes–KOH, pH 7.1, at 20 °C. Permeability coefficients were calculated from the internal pyranine fluorescence responses after a 0.5 pH unit H_2SO_4 external pulse as described under Materials and Methods.

We have therefore measured proton equilibration following a 0.5 pH unit H_2SO_4 pulse for liposomes reconstituted with different amounts of BR. The evolution of the proton–hydroxyl permeability coefficient with lipid to protein weight ratio is shown in Figure 10. Interestingly enough, for protein to lipid weight ratios ranging from 160 to 40, the coefficient is constant and is only increased 2-fold as compared to pure lipid liposomes (lower protein to lipid ratios were not tested since the homogeneity of the preparation might become uncertain). On the other hand, higher amounts of protein promoted a more and more dramatic increase in permeability coefficient up to 10-fold for a lipid to protein ratio of 5. Note that the onset of this increase of permeability (lipid to protein ratio of 40) corresponds to 1 BR molecule per 2000 phospholipid molecules (i.e., 200 BR molecules per liposome).

Discussion

The first result brought upon by the present study is that, in BR liposomes, proton–hydroxyl permeability coefficients are of the order of 10^{-5} cm·s^{-1}. Proton permeability is very sensitive to temperature, having an activation energy of 22 kcal·mol^{-1} [a similar value has been reported by Rossignol et al. (1982)]. The presence of BR in the bilayer does not increase drastically proton permeability, except below a lipid to protein ratio of 40. Interestingly enough, this ratio corresponds to the onset of a limited aggregation of BR in the membrane (T. Gulik, M. Seigneuret, and J.-L. Rigaud, unpublished results). These aggregates might provide special pathways for proton diffusion. It may be remarked that the reverse-phase evaporation reconstitution procedure does not involve the use of detergent. Remaining traces of detergent seem to increase significantly proton permeability (see following paper in this issue).

The observed range of proton permeability for BR liposomes appears to be critical since, in K_2SO_4 medium, it leads to proton fluxes that can be limited either by proton self-diffusion or by K^+ counterion diffusion, depending upon the magnitude of the imposed proton gradient. Nevertheless, proton permeation appears to occur through a K^+–H^+ exchange process as separate ions. On the other hand, the higher apparent proton permeability observed in the presence of chloride salts can only be explained by an electroneutral obligatory codif-

fusion of H^+ and Cl^-. The simplest explanation of this catalytically linked permeation of the two ions is that they diffuse together in the form of HCl molecules. This possibility has already been suggested by other authors (Hauser et al., 1973; Nichols & Miller, 1974; Toyoshima & Thompson, 1975; Nozaki & Tanford, 1981). It may however be remarked that this process was found to increase proton permeability only 3- or 4-fold so that it remains in the same range. The occurrence of HCl diffusion is confirmed by the observation that a Cl^- gradient (generated by an external chloride salt pulse) dissipates by inducing a proton flux, thereby forming a pH gradient. Thus, HCl diffusion is the dominant permeation mechanism not only for protons but also for Cl^-. In comparison, diffusion of charged Cl^- is a slow process. Taken together, our data appear to indicate the following sequence for permeant species: $HCl > K^+ > H^+ > Cl^-$. Of course, such a sequence is dependent upon the properties of BR liposomes. In particular, the negatively charged membrane is likely to be more easily permeated by cations than by anions.

There exists a large variability in the values of proton–hydroxyl permeabilities found in liposome systems (Nichols & Deamer, 1980; Nichols et al., 1980; Clement & Gould, 1981; Nozaki & Tanford, 1981; Rossignol et al., 1982; Deamer & Nichols, 1983; Krishnamoorty & Hinkle, 1984) since values ranging from 10^{-11} to 10^{-4} cm·s^{-1} have been reported. Our values of $\sim 10^{-5}$ cm·s^{-1} are intermediate between such extremes. It may first be remarked that the bilayer barrier properties for H^+ (and other ions) are likely to be influenced by the characteristics of the liposome system. These include liposome size, membrane fluidity, surface charge, and lipid purity (for example, preparation of liposomes by sonication may induce lipid oxidation or hydrolysis). Additionaly, our results suggest that a careful analysis of experimental conditions is necessary. Ionic composition of the medium (i.e., presence of chloride ions) must be taken into account, and it is important to determine whether the measured proton permeability is limited by proton or counterion diffusion. In this regard, our study emphasizes the advantage of using small pH gradients with which counterions' limiting effects are less likely. Moreover, while the identification of limiting ions is generally obtained from the use of ionophores, our study suggests caution for the use of the latter. We have clearly shown that valinomycin can become a protonophore at high concentration. Increase of proton permeability observed with such concentrations may be falsely attributed to K^+ counterion effects. The valinomycin to lipid ratio has to be considered rather than absolute concentration. The method used for proton flux measurements is also important. Pyranine fluorescence has the advantage of high sensitivity (even with small pH gradient) while membrane binding of the probe can be avoided (Seigneuret & Rigaud, 1985).

Finally, these results indicate that BR liposomes prepared by reverse-phase evaporation constitute a straightforward system to study proton transport phenomena. Besides their large size, unilamellarity, and homogeneity (Rigaud et al., 1985), these appear to have a low proton basic permeability. Application to the study of light-induced proton transport by BR is described in the following paper (Seigneuret & Rigaud, 1986).

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Registry No. H^+, 12408-02-5; HO^-, 14280-30-9; K, 7440-09-7; Cl^-, 16887-00-6.

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