Analysis of Passive and Light-Driven Ion Movements in Large Bacteriorhodopsin Liposomes Reconstituted by Reverse-Phase Evaporation. 2. Influence of Passive Permeability and Back-Pressure Effects upon Light-Induced Proton Uptake†

Michel Seigneuret* and Jean-Louis Rigaud
Service de Biophysique, Département de Biologie, CEN Saclay, 91191 Gif-sur-Yvette Cedex, France
Received March 13, 1986; Revised Manuscript Received July 1, 1986

ABSTRACT: Light-induced proton pumping has been measured in large bacteriorhodopsin liposomes (reconstituted by reverse-phase evaporation) by using internally trapped pyranine fluorescence, [^{14}C]methylamine uptake, and pH potentiometric measurements. The effect of proton pumping rate and proton or non-proton passive permeability was studied. In K_2SO_4 medium, a very low light-induced proton uptake was obtained due to the formation of a retroinhibitory electrical potential (back-pressure effect of $\Delta\psi$). In KCl medium a higher proton uptake was observed, suggesting the occurrence of partially compensatory Cl^- movements. In both media, valinomycin increased proton pumping initial rates until the $\Delta\psi$ was overcome, allowing a large pH gradient (2 units) to be formed. Proton back-leakage rates were varied either with valinomycin (by increasing K^+ permeability), with chloride (through HCl diffusion), or with nonsolubilizing concentrations of Triton X-100. In a certain range, the steady-state ΔpH did not depend on the extent of proton leakage. Furthermore, by variation of the actinic light intensity and the lipid to protein ratio of liposomes, it was found that the ΔpH could also become independent of pumping rate. Such effects were interpreted as resulting from retroinhibition of the pump by the proton gradient (back-pressure effect of ΔpH). Thus, in bacteriorhodopsin liposomes, light-induced steady-state proton electrochemical potential is only partially determined by the number of pumping units and proton passive permeability. Back-pressure effects also appear to be strong regulating factors.

Bacteriorhodopsin (BR),[1] the light-driven proton pump of *Halobacterium halobium*, is considered as a good model for the study of various aspects of proton-linked biological energy transduction (Dencher, 1984; Westerhoff & Dancshazy, 1984). In particular, reconstituted liposomes containing BR provide relatively simple systems for the investigation of proton electrochemical gradient ($\Delta\tilde{\mu}_{H^+}$) formation across membranes (Hellingwerf et al., 1979; Ramirez et al., 1983). The main advantage of such reconstituted systems is that many functionally relevant parameters can be varied, affecting either the proton pumping rate or the proton and counterion passive permeability of the membrane. These include ionic composition of the medium, internal buffering power, presence of ionophores, lipid composition, and lipid–protein ratio. The influence of such parameters upon the kinetics and extent of proton uptake by liposomes can thus be studied.

In this regard, a fundamental problem is to know which factors determine the amplitude of the $\Delta\tilde{\mu}_{H^+}$ across the membrane under a given set of experimental conditions. $\Delta\tilde{\mu}_{H^+}$ values are of course determined by the balance between active proton pumping and the counteracting leakage processes due to ion passive permeability of the membrane. However, in the case of BR, the situation is complicated by the fact that the specific activity of the pump does not seem to be constant over the whole proton uptake kinetics. This is interpreted as being due to retroinhibitory effects of the $\Delta\tilde{\mu}_{H^+}$ upon proton transport by BR, the so called "back-pressure" effects [for review, see Westerhoff and Dancshazy (1984)]. Such processes may seriously influence $\Delta\tilde{\mu}_{H^+}$ formation in liposomes.

In the preceding paper (Seigneuret & Rigaud, 1986), we have studied in detail the proton passive permeability of BR-reconstituted large liposomes prepared by reverse-phase evaporation. This proton permeability was shown to be greatly influenced by the nature of the other ions present in the medium and by their own permeability characteristics. Here, using again pyranine fluorescence as well as other techniques, we examine light-induced proton uptake in BR liposomes. The influence of both pumping rates (varied through the effects of light intensity and lipid–protein ratio) and ion passive permeability is studied. It is shown that this passive permeability only partially determines $\Delta\tilde{\mu}_{H^+}$ amplitudes and that back-pressure exerts a strong regulatory effect upon $\Delta\tilde{\mu}_{H^+}$.

Materials and Methods

Chemicals. Purified egg yolk phosphatidylcholine and derived phosphatidic acid were isolated according to Singleton et al. (1965) and Allgyer and Wells (1979), respectively. Pyranine was obtained from Eastman Kodak. Valinomycin was purchased from Sigma and [^{14}C]methylamine (38.4 Ci/mol) from CEN Saclay (France).

Reconstitution of BR. Reconstitution was carried out as described in the preceding paper in this issue. Except when otherwise stated, a lipid to protein ratio of 80:1 (w/w) was used.

Light-Induced Proton Movements. Changes in internal pH were measured as changes in the fluorescence intensity of the pH-sensitive fluorescence probe pyranine trapped within the vesicle internal aqueous compartment as detailed elsewhere (Seigneuret & Rigaud, 1985, 1986). Proton uptake into the BR liposomes was assayed by the pH meter essentially as described earlier (Rigaud et al., 1983). The transmembrane pH difference was determined from [^{14}C]methylamine uptake

† This work was in part supported by grants from CNRS (ATP 90 1445).

[1] Abbreviations: pyranine, 8-hydroxy-1,3,6-pyrenetrisulfonate; BR, bacteriorhodopsin; Pipes, 1,4-piperazinediethanesulfonic acid; $\Delta\tilde{\mu}_{H^+}$, transmembrane proton electrochemical potential gradient; ΔpH, transmembrane pH gradient; $\Delta\psi$, transmembrane electrical potential.

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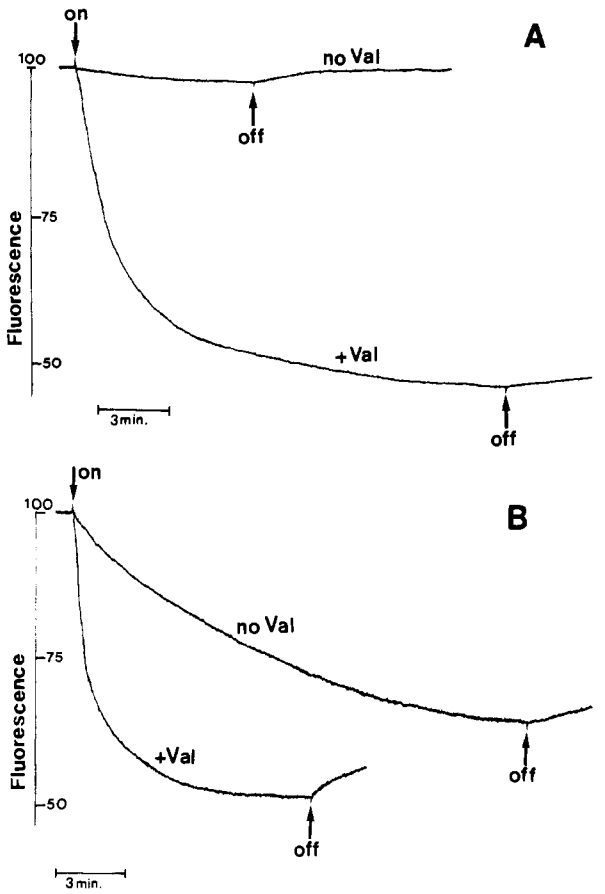

FIGURE 1: Light-induced fluorescence response of pyranine trapped inside BR liposomes in 130 mM K_2SO_4 and 20 mM KH_2PO_4–KOH, pH 7.1 (A), and in 150 mM KCl and 20 mM KH_2PO_4–KOH, pH 7.1 (B), in the absence (no Val) or presence (+Val) of 0.01 μM valinomycin at 20 °C.

by using the flow dialysis apparatus (Colowick & Womack, 1969) with the interpretation method described earlier (Hellingwerf et al., 1979; Rigaud et al., 1983). A 150-W–20-V xenon lamp (Osram) equipped with heat filters and flexible light guide was used as the actinic light source. Where indicated, light intensity was varied with neutral density filters.

RESULTS

Light-Induced Proton Pumping in K_2SO_4 and KCl Media. Internal pH variations due to light-induced proton pumping were measured from the fluorescence of pyranine trapped inside BR liposomes reconstituted in either K_2SO_4 or KCl medium (buffered with 20 mM phosphate, pH 7.1). In all cases, actinic illumination induced a time-dependent fluorescence decrease indicative of internal acidification.[2] Nevertheless, the amplitude of this response depended strongly upon the ionic composition of the medium.

In K_2SO_4 (Figure 1A), a very slow and small decrease was observed (maximum amplitude, 5%) unless valinomycin was added. In the presence of the antibiotic, a large and rapid decrease (maximum amplitude, 55%) occurred, which slowly reversed in the dark as the accumulated protons leaked back into the external medium. These results can be interpreted in view of the passive permeability data obtained in the preceding paper (Seigneuret & Rigaud, 1986). In the absence of valinomycin, all ions present in the medium are relatively impermeant (no permeability to SO_4^{2-} and PO_4^{2-}, slight permeability to H^+ and K^+). Thus, light-driven proton translocation allows a large transmembrane electrical potential ($\Delta\psi$) to develop. Indeed, in previous experiments (Rigaud et al., 1983), such a $\Delta\psi$ could be evidenced from measurements of $S^{14}CN^-$ uptake. The very low proton pumping observed in these conditions is due to a retroinhibitory effect (back-pressure effect) of the $\Delta\psi$ upon the activity of BR. While this effect has already been reported by other authors (Hellingwerf et al., 1979; Westerhoff et al., 1981), it appears to be much stronger in our liposomes since practically no ΔpH is observed. This can be related to the very low basic permeability of these BR liposomes. In the presence of valinomycin, the $\Delta\psi$ is overcome by compensatory K^+ movements, and a large ΔpH (acidic inside) can develop due to the high rate of proton pumping.

On the other hand, in KCl medium (Figure 1B) a relatively important fluorescence decrease was observed (maximum amplitude, 35%) even in the absence of valinomycin. Similar results were obtained in K_2SO_4 medium supplemented externally with KCl and equilibrated (data not shown). In view of what was observed in pure K_2SO_4 medium, it has to be assumed that compensatory Cl^- movements partially abolish the light-induced inhibitory $\Delta\psi$ and consequently allow formation of a significant ΔpH. This is surprising since Cl^- passive permeability as a charged ion has been shown in the preceding paper (Seigneuret & Rigaud, 1986) to be very low. This point will be dealt with later under Discussion. However, despite this Cl^- movement, a significant inhibitory $\Delta\psi$ appears to be present. Indeed, in KCl medium, the presence of valinomycin allowed proton pumping to develop with a much higher initial rate, comparable to what was found in K_2SO_4 medium.

The occurrence of an effect of $\Delta\psi$ upon initial pumping rate must be emphasized. This indicates that the $\Delta\psi$ is generated very rapidly after illumination (i.e., in the very first photocycles) so that it already affects the early phase of proton pumping. Indeed, rapid formation of $\Delta\psi$ has been reported in other liposome systems (Hellingwerf et al., 1979). Thus, initial rates of pumping can be taken as a measure of the remaining $\Delta\psi$.

Influence of Valinomycin Concentration upon Proton Pumping Parameters. In Figure 2 is shown the dependence upon valinomycin concentration of V_{ON} and V_{OFF}, initial slopes of fluorescence variations when actinic illumination is switched on and off (indicative of initial rates of proton pumping and back-leakage, respectively), and of $(\Delta F/F)_{max}$, maximum relative fluorescence response (indicative of the steady-state ΔpH value in the light), in both K_2SO_4 and KCl media.

The effect of low concentrations of valinomycin is to increase initial rates of proton pumping (V_{ON}) in both media. This can be attributed to the more and more efficient decoupling of inhibitory $\Delta\psi$ by K^+ movements. The valinomycin concentration needed to obtain the maximum V_{ON} value is lesser in KCl than in K_2SO_4. Indeed, in the former medium, $\Delta\psi$ is also

[2] In our previous publications, it was shown that both inside-out and right-side-out orientations of the protein are present in our reconstituted liposomes, with relative amounts of 80% and 20%, respectively (Rigaud et al., 1983; Seigneuret & Rigaud, 1985). The two orientations appear to be at least partially segregated in distinct subclasses of liposomes which thus exhibit oppositely directed proton pumping. The observed pyranine fluorescence decrease observed upon pumping is mainly representative of the behavior of the majoritary inwardly pumping liposomes. However, it is difficult to calculate true initial rates of proton pumping and steady-state ΔpH values in such a situation. Nevertheless, initial rates of fluorescence response and steady-state fluorescence decreases constitute relative measurements of these parameters and can be used for comparative purposes. On the other hand, [^{14}C]methylamine uptake measurements monitor selectively the majoritary inwardly pumping liposomes. Steady-state ΔpH values obtained by this technique can be considered accurate.

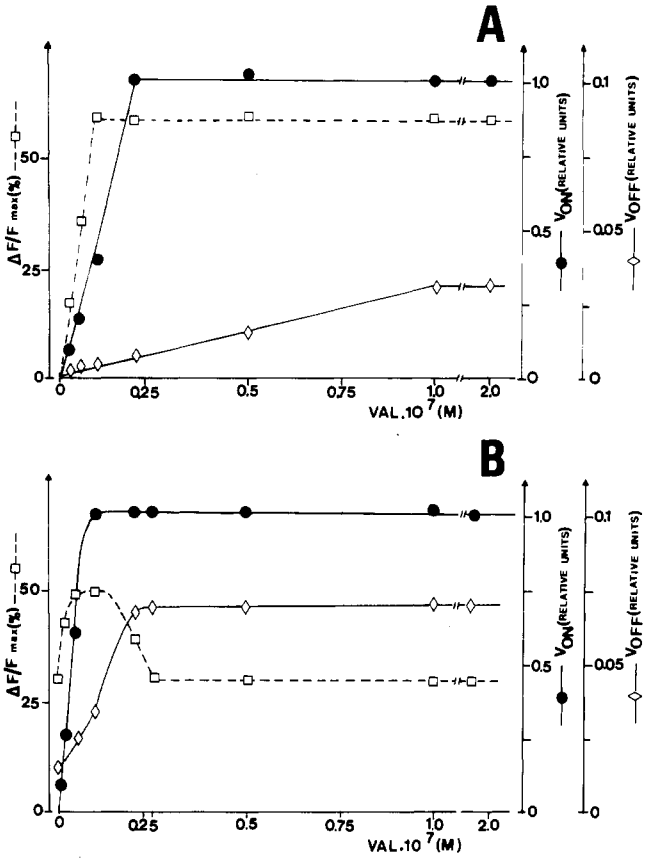

FIGURE 2: Effect of valinomycin upon initial rates of light-induced internal pyranine fluorescence response (V_{ON}, closed circles) and of dark reversal response (V_{OFF}, open diamonds) and upon maximum relative fluorescence decrease [$(\Delta F/F)_{max}$, open squares] for BR liposomes in 130 mM K_2SO_4 and 20 mM KH_2PO_4–KOH, pH 7.1 (A), and in 150 mM KCl and 20 mM KH_2PO_4–KOH, pH 7.1 (B), at 20 °C. Note that, for both V_{ON} and V_{OFF}, similar relative units (i.e., fluorescence intensity units per second) are used throughout this paper. Thus, V_{ON} values can always be compared since the initial pH remains at 7.1. V_{OFF} values can be compared at identical $(\Delta F/F)_{max}$. V_{ON} and V_{OFF} values cannot be compared since the dependence of fluorescence intensity vs. internal pH and the internal buffer capacity are not constant during the whole fluorescence response.

partially decoupled by Cl^- movements.

Due to this increase of pumping rates, the $(\Delta F/F)_{max}$ values (i.e., the stationary ΔpH) also rise with valinomycin concentration up to a maximum value that is roughly similar in K_2SO_4 and KCl media (60% and 50%, respectively). However, it may be remarked that, in both cases, the valinomycin concentration needed to yield this maximum ΔpH is less than (about half) that needed to yield a maximum pumping initial rate (V_{ON} value). This can be accounted for by the fact that, as shown in Figure 2, proton passive efflux rates, measured through V_{OFF}, also increase with valinomycin concentration. Thus, a compensatory effect of pump and leak rates must occur.

This effect of valinomycin on the rate of proton back-leakage can be understood as follows. In K_2SO_4 medium, according to [^{14}C]methylamine uptake experiments (see Table I), the maximum attainable ΔpH is of the order of 2 pH units.[2] According to the preceding paper in this issue with acid ΔpHs of such magnitude, proton permeability is limited by K^+ counterion diffusion. Thus, in the present experiments, it is natural that proton back-leakage be accelerated by valinomycin. Indeed, the valinomycin concentration giving a maximum proton leak rate (0.1 μM) is similar with both light-induced (Figure 2A) and pulse-induced (preceding paper) proton gradients. In KCl medium, greater rates of proton

Table I: Influence of Valinomycin on Steady-State ΔpH of BR Liposomes in KCl and K_2SO_4 Media

sample					
valinomycin (mol/mol of lipid)	medium[a]	ΔpH[b]	max fluorescence decrease (%) pyranine	max fluorescence decrease (%) 9-aminoacridine	total proton uptake[c] (nequiv of H^+)
50	K_2SO_4	1.9	60	81	66.2
	KCl	1.8	51	79	65.4
200	K_2SO_4	2.0	61	85	70.3
	KCl	1.1	30	40	35.6

[a] Exact media are as follows: 130 mM K_2SO_4 and 10 mM Pipes, pH 7.1; and 150 mM KCl and 10 mM Pipes, pH 7.1. [b] Calculated from [^{14}C]methylamine uptake flow analysis measurements at a lipid concentration of 16 mg/mL. [c] Estimated from external pH potentiometric measurements at a lipid concentration of 4 mg/mL resuspended in 130 mM K_2SO_4 or 150 mM KCl and 2 mM Pipes, pH 7.1.

leakage are found (Figure 2B), which are also accelerated by valinomycin. It must be considered (see preceding paper) that in such a medium, besides H^+–K^+ exchange, a more efficient proton permeation mechanism operates, namely, HCl diffusion. Despite these differences in proton leakage, nearly identical proton gradients can be formed in KCl and K_2SO_4 media at valinomycin concentrations around 0.01 μM. As can be seen in Figure 2, though the rate of proton leakage is 2.5 times higher in KCl, the rate of proton pumping is also 2.5 times higher.

When the $\Delta\psi$ is totally decoupled, initial rates of light-induced proton pumping (V_{ON}) become identical for both media and constant with valinomycin concentration. However, when valinomycin is further increased, very different behaviors of the ΔpH are found in KCl and K_2SO_4. While with K_2SO_4, ΔpH remains constant, with KCl, it decreases progressively [$(\Delta F/F)_{max}$ falls from 50% to 35%]. This point is also illustrated in Table I, in which steady-state ΔpH estimated by four different techniques are shown for both KCl and K_2SO_4 media at two valinomycin to lipid ratios.

The ΔpH decrease observed in KCl medium can be accounted for by the parallel increase of proton back-leakage with valinomycin concentration (analyzed above). It is much less straightforward to explain why, in K_2SO_4 medium, the ΔpH remains at its maximum value though the rate of proton back-leakage increases by a factor of 4 and the initial pumping rate is constant. This cannot be accounted for on the basis of a simple pump-leak system. Indeed, in this framework any variation of either initial pump rate or initial leak rate should affect the amplitude of the ΔpH. Rather, this can be taken as reflecting another type of back-pressure effect, already suggested (Hellingwerf et al., 1979; Arents et al., 1981a,b), namely, the retroinhibitory effect of the ΔpH upon bacteriorhodopsin activity. Briefly (see discussion), if the rate of pumping decreases steeply with increasing ΔpH (a feature that cannot be observed from initial rate measurements), then any "reasonable" increase of proton back-leakage leads to a compensatory increase of pumping rate. Thus, the ΔpH is unchanged. While this can happen in K_2SO_4 medium, in KCl medium rates of leakage become too high to be compensated by such a mechanism.

Finally, at higher concentration (0.2 μM), we have been able to observe deleterious effects of valinomycin on initial pumping rate, initial back-leakage rates, and ΔpH (not shown). These can be accounted for by both a protonophore activity of the antibiotic (see preceding paper in this issue) and an inhibitory effect on BR pumping activity and photochemical cycle (Rott & Avi-Dor, 1977; Westerhoff et al., 1981a,b). These effects are observed at valinomycin to BR ratios of 2.5 (mol/mol).

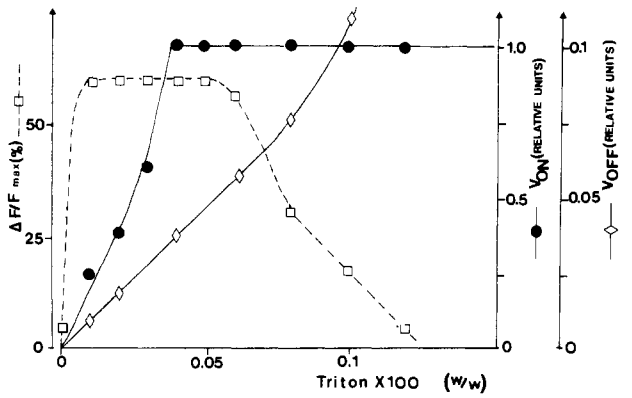

FIGURE 3: Effect of Triton X-100 upon initial rates of light-induced internal pyranine fluorescence response (V_{ON}, closed circles) and of dark reversal response (V_{OFF}, open diamonds) and upon maximum relative fluorescence decrease [$(\Delta F/F)_{max}$, open squares] for BR liposomes in 130 mM K_2SO_4 and 20 mM KH_2PO_4–KOH, pH 7.1, at 20 °C. Detergent concentrations are expressed in grams of Triton X-100 per gram of phospholipid.

Effect of Detergents upon Proton Pumping Parameters. Results obtained above indicate that increasing the permeability of BR liposomes either to protons or to K^+ has various effects upon proton uptake. We thus investigated the effect of another ion permeability increasing agent, namely, Triton X-100. Figure 3 shows the effect of increasing concentrations of detergent upon apparent proton pumping (V_{ON}) and back-leakage (V_{OFF}) rates and steady-state ΔpH [i.e., $(\Delta F/F)_{max}$] measured from light-induced fluorescence response of trapped pyranine for BR liposomes in K_2SO_4 medium. Note that the highest detergent concentration used in this study is 5 times lower than the one at which solubilization of the membrane begins (M. Paternostre and J.-L. Rigaud, unpublished results). Low amounts of detergent induced an important increase in both initial rates of proton pumping and steady-state ΔpH. As found with valinomycin, the ΔpH levels off [$(\Delta F/F)_{max}$ = 55%] at lower Triton X-100 concentrations than the initial proton pumping rate, due to the parallel increase of the proton back-leakage rate. Higher detergent concentrations increase this leak rate to such an extent that the ΔpH is finally decreased down to near-zero value. Thus, Triton X-100 has the effect of increasing the basic permeability of BR liposomes to protons and most likely to K^+ also, so that it effects a decoupling first of $\Delta\psi$ and second of ΔpH. However, there exists a range of detergent concentration (see Figure 3) in which the initial pumping rate is constant, the proton leak rate increases greatly, and nevertheless the steady-state ΔpH is constant. Again, the retroinhibitory effect of ΔpH can be invoked to explain these features. This confirms that this ΔpH back-pressure has the effect of rendering steady-state ΔpH values independent of proton passive permeability in a certain range.

Effect of Light Intensity upon Proton Uptake. Up to now, we have mainly described the effect of varying the proton and non-proton passive permeability upon proton uptake in BR liposomes. It is important to also investigate what is the effect of varying the rate of proton pumping (or, more precisely, the number of pumping units per liposome) upon formation of the proton gradient.

The simplest way to observe such an effect is to vary the light intensity used for actinic illumination. This allows one to modulate the number of pumping BR molecules without any effect upon passive proton permeability. Light-induced internal pyranine fluorescence responses at different light intensities are shown in Figure 4A for BR liposomes in K_2SO_4 medium in the presence of 0.1 μM valinomycin. The corresponding values of initial apparent pumping rate (V_{ON}) and $(\Delta F/F)_{max}$ are plotted as a function of light intensity in Figure 4B. Initial rates of pumping increase with light intensity, showing limited saturation behavior in the range studied. This saturation effect cannot be due to the increasing presence of a residual $\Delta\psi$ since it has been shown above that the latter was completely abolished at such a valinomycin concentration (note that all experiments described above were performed with the maximum light intensity displayed here). Rather, saturation only reflects the limited number of BR molecules in the sample. On the other hand, $(\Delta F/F)_{max}$ values show a much steeper saturation with light intensity. This leads to a situation where, beyond a certain light intensity value, the steady-state ΔpH values remain constant while the proton pumping initial rate still greatly increases. Similar data were obtained from external pH potentiometric measurements at various light intensities. Namely, the total proton uptake by BR liposomes was found to level off rapidly while the initial uptake rate was still increasing with light intensity (not shown).

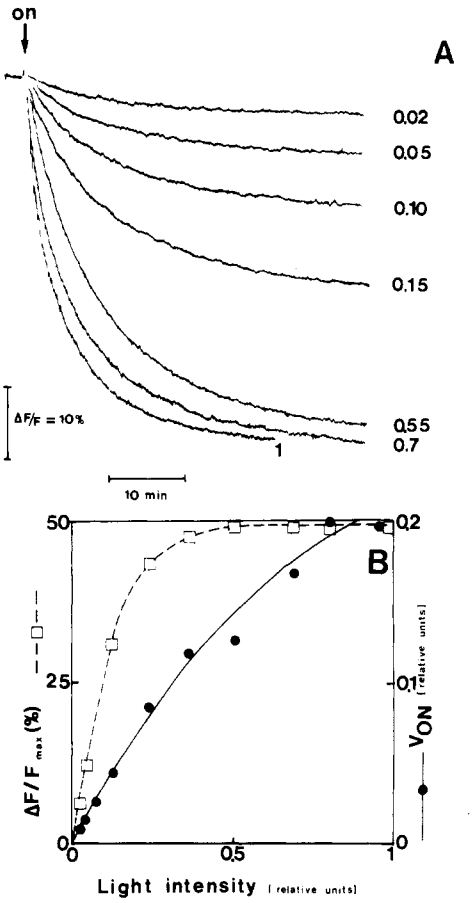

FIGURE 4: (A) Light-induced fluorescence response of pyranine trapped inside BR liposomes in 30 mM K_2SO_4 and 100 mM Pipes–KOH, pH 7.1, in the presence of 0.1 μM valinomycin at 20 °C at different actinic light intensities (relative intensity values are indicated beneath each curve). (B) Effect of actinic light intensity upon initial rates of light-induced pyranine fluorescence response (V_{ON}, closed circles) and upon maximum relative fluorescence decrease [$(\Delta F/F)_{max}$, open squares] for BR liposomes in 30 mM K_2SO_4 and 100 mM Pipes, pH 7.1, in the presence of 0.1 μM valinomycin at 20 °C. Note that V_{ON} values are lower than those of Figures 2 and 3 due to the higher internal buffer capacity used in these experiments (see text for details).

As already suggested (Hellingwerf et al., 1979), these data can be taken as indicative of the back-pressure effect of ΔpH. Due to the high ΔpH values that can be formed with the present BR liposomes, it can be shown unambiguously that ΔpH values can become independent of the initial pumping

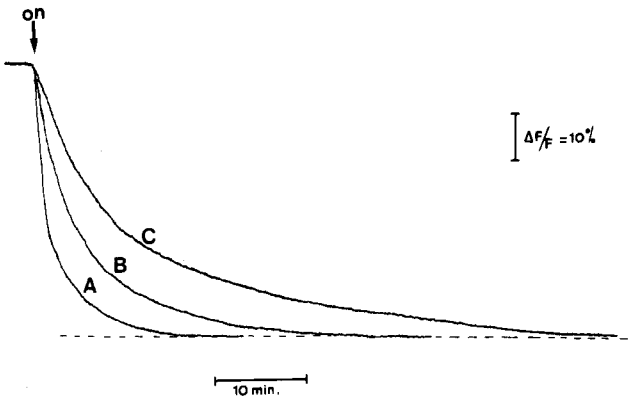

FIGURE 5: Light-induced fluorescence response of pyranine trapped inside BR liposomes in 30 mM K_2SO_4 and 100 mM Pipes–KOH, pH 7.1, reconstituted at lipid to protein ratios (w/w) of 40 (A), 80 (B), and 160 (C) in the presence of 0.1 μM valinomycin at 20 °C.

Table II: Influence of Lipid to Protein Ratio on Steady-State ΔpH in BR Liposomes[a]

lipid/protein (w/w)	ΔpH[b]	proton pumping initial rate (nequiv of H^+/s)	proton back-leakage initial rate (nequiv of H^+/s)	total proton uptake[c] (nequiv of H^+)
40	1.9	101	20	660
80	2.0	50	20.5	700
160	1.9	26	21	650

[a] Liposomes were prepared in 30 mM K_2SO_4 and 100 mM Pipes buffer and contained 200 mol of valinomycin/mol of lipid. [b] Calculated from [^{14}C]methylamine uptake flow dialysis measurements at a lipid concentration of 16 mg/mL. [c] Estimated from external pH potentiometric measurements at a lipid concentration of 4 mg/mL, resuspended in 130 mM K_2SO_4 and 2 mM Pipes, pH 7.1.

rate (i.e., of the number of pumping BR molecules). An explanation is that if the dependence of pumping rate on ΔpH is very steep at high ΔpH values, then increasing the number of pumping units has only very limited effect on the actual pumping rate at these high ΔpH values. Thus the ΔpH remains practically unchanged (see Discussion).

Effect of Lipid to Protein Ratio upon Proton Uptake. Another approach to vary the number of pumping units (and thus initial pumping rate) is to use BR liposomes reconstituted at different lipid to protein ratios. Much caution is necessary in these experiments since we have shown that, at low lipid to protein ratios, the passive proton permeability of the membrane is also increased. We have thus concentrated upon lipid to protein ratios with which proton passive permeability is low and constant (see preceding paper in this issue).

Figure 5 shows light-induced internal pyranine fluorescence responses for BR liposomes reconstituted at lipid to protein weight ratios of 40, 80, and 160 in the presence of 0.1 μM valinomycin. As expected, initial rates of proton pumping increase with protein content (the dependence was found to be linear). On the other hand, the steady-state ΔpH is identical for the three preparations [$(\Delta F/F)_{max}$ = 55%]. The fact that the ΔpH does not vary with lipid–protein ratio is also indicated by parallel [^{14}C]methylamine uptake and external pH potentiometric measurements (Table II).[2] In particular, potentiometric measurements indicate clearly that while the proton pumping initial rate increases linearly with protein content, the total proton uptake remains unchanged. These data also bear out for a back-pressure effect of ΔpH on proton pumping. Again it is shown that the steady-state ΔpH can become independent of the number of pumping BR molecules in liposomes.

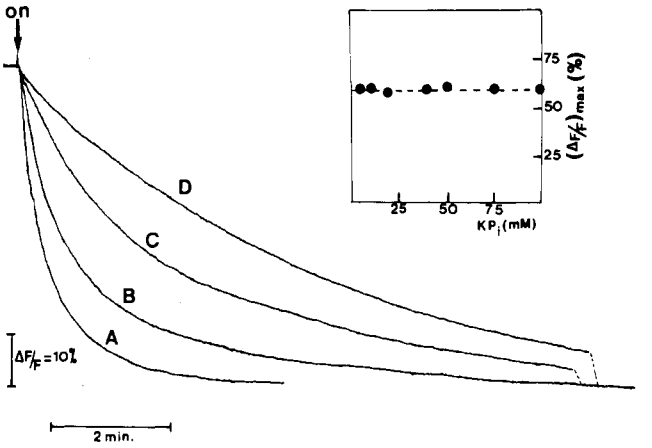

FIGURE 6: Light-induced fluorescence response of pyranine trapped inside BR liposomes in 125 mM K_2SO_4 and 10 mM KH_2PO_4–KOH, pH 7.1 (A); in 115 mM K_2SO_4 and 20 mM KH_2PO_4–KOH, pH 7.1 (B); in 90 mM K_2SO_4 and 50 mM KH_2PO_4–KOH, pH 7.1 (C); and in 45 mM K_2SO_4 and 100 mM KH_2PO_4–KOH, pH 7.1 (D), in the presence of 0.1 μM valinomycin at 20 °C. Inset: Influence of internal buffer upon light-induced maximum relative fluorescence decrease $(\Delta F/F)_{max}$ for BR liposomes in the presence of 0.1 μM valinomycin at 20 °C. Internal buffer capacity was varied through the concentration of KH_2PO_4–KOH, pH 7.1, used during reconstitution from 1 to 100 mM. K_2SO_4 was present at concentrations ranging from 130 to 45 mM to ensure constant osmolarity.

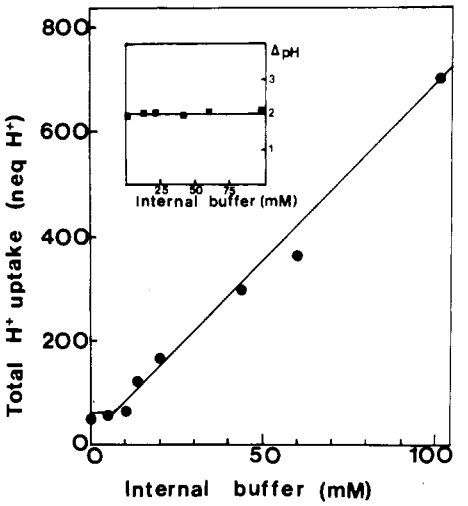

FIGURE 7: Effect of internal buffer concentration upon total light-induced proton uptake in BR liposomes at 20 °C obtained from potentiometric measurements of external pH. BR liposomes were dialyzed against 130 mM K_2SO_4 and 2 mM KH_2PO_4–KOH, pH 7.1, diluted to 4 mg/mL lipid and pretreated with 1 μM valinomycin. Inset: Effect of internal buffer concentration upon steady-state ΔpH for BR liposomes at 20 °C obtained from [^{14}C]methylamine uptake measurements by flow dialysis. BR liposomes (16 mg/mL lipid) were dialyzed against 45 mM K_2SO_4 and 100 mM KH_2PO_4–KOH, pH 7.1, and pretreated with 2 μM valinomycin. Internal buffer capacity was varied as described in the legend of Figure 6.

Effect of Internal Buffering Capacity upon Proton Uptake. We have also tested the influence of internal buffer upon light-induced pumping. For this, BR liposomes were prepared in K_2SO_4 media with different concentrations of phosphate buffer (pH 7.1). Figure 6 shows internal pyranine fluorescence responses for two preparations internally buffered with from 10 to 100 mM phosphate in the presence of 0.1 μM valinomycin. Apparent initial rates of proton pumping appear to decrease with increasing buffering power. In this instance, this only means that, to elicit a given internal pH decrease, more protons are required at high buffer than at low buffer capacity. Steady-state ΔpHs appear, on the other hand, to

be independent of internal buffer capacity (see inset, Figure 6). This is confirmed by parallel [^{14}C]methylamine uptake measurements (see inset, Figure 7). As also shown in Figure 7, the total proton uptake, measured potentiometrically, increases linearly with buffering power, except for the lower ones. This slight nonlinearity is likely to be due to the buffer capacity of the membrane. This can be estimated as equivalent to 5 mM phosphate. It thus appears that the increase of buffering power is compensated by a proportional increase in the extent of proton translocation, leading to a constant steady-state pH gradient. For a simple pump-leak system such buffer-independent ΔpH values are expected. Thus, we have to conclude that the ΔpH back-pressure effect is also by itself independent of internal buffering power.

Discussion

The experiments described in this paper clearly indicate that light-induced proton uptake in BR liposomes is determined by three factors: the number of active pumps, the passive permeability of the membrane, and the back-pressure effects. Their relative importance depends critically upon experimental conditions.

A first set of conditions is met when the passive permeability of the membrane is low. In this case, proton uptake is mostly under the influence of the back-pressure effect of $\Delta\psi$. This situation is best demonstrated in the case of BR liposomes in K_2SO_4 medium where very few protons are pumped in the absence of valinomycin due to this back-pressure effect. The specific involvement of $\Delta\psi$ in this inhibition is proved by the fact that it is overcome by valinomycin, which selectively increases the non-proton conductance of the membrane. Much larger ΔpHs are thus created with initial rates that depend upon valinomycin concentration (note that the back-pressure effect of ΔpH is not supposed to have an effect upon initial pumping rate).

Our results are qualitatively in agreement with those of Westerhoff and collaborators (Hellingwerf et al., 1979; Westerhoff et al., 1981a). However, using small, heterogeneous [see Arents et al. (1981a,b)] BR vesicles prepared by sonication, these authors inferred that a significant $\Delta\psi$ was present only in the early phase of proton pumping since it was rapidly overcome by compensatory ion movements. Thus, back-pressure effects of $\Delta\psi$ could only be observed upon initial rates and not upon steady-state ΔpH values. The fact that, in our case, a strong effect of $\Delta\psi$ back-pressure on steady-state ΔpH was found is certainly to be related to the very low passive permeability of our reconstituted system. Furthermore, it may be remarked that the small effect of valinomycin upon ΔpH found by Hellingwerf et al. (1979) suggests that a significant $\Delta\psi$ must have been remaining in their system at the steady-state, though its measurement may not have been straightforward.

Difficulties in measuring $\Delta\psi$ in reconstituted systems are serious problems. In our case (Rigaud et al., 1983; M. Seigneuret, J.-L. Rigaud, unpublished results), SCN^- uptake measurements by flow dialysis yield $\Delta\psi$ values of the order of −60 mV. This is low considering the high inhibitory effect observed [maximum effects of $\Delta\psi$ on the BR photochemical cycle are obtained at about twice this value; see Helgerson et al. (1985)]. However, as already pointed out (Hellingwerf et al., 1979), quantitative SCN^- uptake measurements can be seriously hindered by experimental artifacts. Possibly the use of electrochromic probes (Ehrenberg et al., 1984) will allow better measurements of $\Delta\psi$.

Interestingly, with BR liposomes in KCl medium, a significant proton uptake is observed even without valinomycin, indicating that compensatory Cl^- movements partially overcome the $\Delta\psi$. This is surprising since in experiments described in the preceding paper (Seigneuret & Rigaud, 1986), Cl^- seemed to be less permeant than K^+ as a charged ion. While further investigation is necessary, we propose two explanations. First, it is possible that Cl^- ions are more accelerated than K^+ ions by membrane potential (due, for example, to ion hydration or surface potential effects). Indeed, it is noteworthy that, in one of the only cases where significant movement of charged Cl^- was inferred in the preceding paper, a significant $\Delta\psi$ must have been present (i.e., with a K^+ gradient and valinomycin). Second, illumination of BR could by changing the conformation of the protein induce ion leakage pathways that show selectivity for Cl^-.

A second set of conditions is met when the non-proton conductance of the membrane is made sufficiently large to allow maximum initial pumping rates.[3] The steady-state ΔpH can then amount to up to 2 pH units and is to a certain extent determined by the proton back-leakage rate. In agreement with the preceding paper (Seigneuret & Rigaud, 1986), such proton permeability occurs through H^+–K^+ exchange and also through HCl diffusion in KCl medium. When the proton leak is made too large, the steady-state ΔpH is decreased as observed in KCl medium/valinomycin and in the presence of Triton X-100 (note that this high effect of detergent suggests that care must be taken of their complete removal to obtain high activity with reconstituted proton pumps). Thus, knowledge of the passive permeability of the membrane allows us to explain many aspects of light-induced proton movements in BR liposomes.

Furthermore, this analysis allows us to point out several facts that cannot be explained on the basis of simple passive permeability. Conditions were found in which the steady-state ΔpH became independent of changes in the rate of proton back-leakage (at constant pumping initial rates). Conditions were also found in which the steady-state ΔpH became independent of changes in the initial rate of proton pumping (at constant rates of proton back-leakage). Such effects are functional manifestations of the back-pressure effect of ΔpH. This back-pressure effect of ΔpH upon proton pumping has already been reported (Hellingwerf et al., 1979; Arents et al., 1981a,b). At the level of the photochemical cycle, much less data exist concerning the effect of ΔpH (Hellingwerf et al., 1979) than on that of $\Delta\psi$ (Quintanilha, 1980; Dancshazy et al., 1983; Helgerson et al., 1983).

The fact that the initial rate of proton pumping and the steady-state ΔpH have different dependence upon actinic light intensity has already been observed previously and furnished one of the early pieces of evidence of the retroinhibitory effect of ΔpH (Hellingwerf et al., 1979). Here, due to the high values of ΔpH obtainable with our system, we have been able

[3] This can be achieved by collapsing the $\Delta\psi$ (and thus its back-pressure effect) by valinomycin or by low amounts of Triton X-100. A drawback of valinomycin is that, as confirmed here, it can also inhibit the proton pumping activity of BR. With small sonicated liposomes, Westerhoff et al. (1981b) found that this inhibition was effective at valinomycin concentrations insufficient to totally collapse the $\Delta\psi$ so that the true uninhibited BR pumping rate could not be observed. This does not appear to be the case in our study since identical maximum initial pumping rates can be observed in three different conditions of media corresponding to three different valinomycin concentrations, namely, in KCl medium (valinomycin, 0.01 μM), in K_2SO_4 medium (valinomycin, 0.025 μM), and in the presence of Triton X-100 (no valinomycin). In both KCl and K_2SO_4 media, this maximum rate remains constant over a wide range of valinomycin concentration before any inhibition is visible. It is thus likely that pumping is hindered neither by $\Delta\psi$ nor by valinomycin within this concentration range.

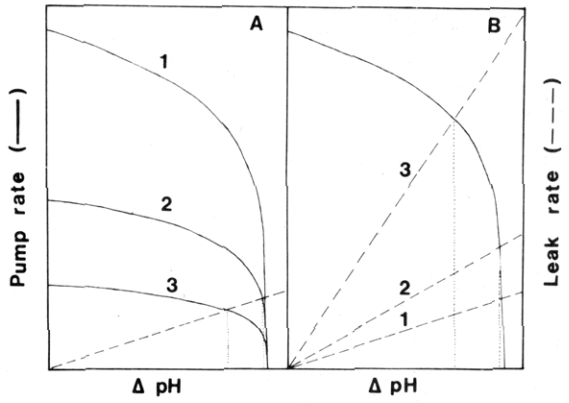

FIGURE 8: Schematic representation of the influence of the back-pressure effect of ΔpH. The proton pumping rate (solid curves) decreases and the proton back-leakage rate (dashed curve) increases with increasing ΔpH. The points where pump and leak rates are identical represent the steady-state ΔpH. (A) Effects of varying pump rate. A decrease by a factor of 2 of the number of pumping units (i.e., of the initial pumping rate) has virtually no effect upon steady-state ΔpH (curves 1 and 2). A decrease by a factor of 4 (curve 3) yields a decrease of the steady-state ΔpH. (B) Effect of varying passive proton permeability. An increase by a factor of 2 of the proton back-leakage rate has no effect upon the steady-state ΔpH (curves 1 and 2) while an increase by a factor of 4 yields a decrease of steady-state ΔpH (curve 3).

to evidence the ultimate consequences of this effect. Namely, the ΔpH was shown to level off with light intensity in spite of the fact that the initial rate of pumping still increased. In the same instance, increasing the number of pumping units by decreasing the lipid to protein ratio was shown to have no effect upon this steady-state ΔpH. Thus all data point to the fact that there is a maximum attainable ΔpH in our BR liposomes (~2 pH units).[4]

It is unlikely that the occurrence of this maximum attainable ΔpH is due to a non-ohmic character of the passive proton permeability of the membrane (i.e., the proton permeability would behave nonlinearly, becoming sufficiently high above a certain value of ΔpH to impede any further increase of the latter). Again, this maximum attainable ΔpH is shown to remain constant when the passive proton permeability is varied within a certain range (with valinomycin through H^+–K^+ exchange or with Triton X-100). This indicates that the proton passive permeability is not the limiting factor in determining the maximum ΔpH value. Furthermore, we have recently found, using the acid–base pulse technique, that the passive proton permeability of the BR liposomes remains ohmic when large (~2 units) ΔpHs acidic inside are generated (unpublished results).

Several theoretical approaches have been used to analyze the characteristics of proton pumping in BR liposomes (Westerhoff et al., 1979; Ho et al., 1979; Ramirez et al., 1983), and our work is consistent with these analyses. However, a qualitative explanation is sufficient for our purpose. The back-pressure effect of ΔpH is basically the fact that the rate of proton pumping by BR decreases progressively as the ΔpH is established. In Figure 8 a possible such dependence of proton pumping rate upon ΔpH is represented. This is by no means intended to represent the exact quantitative situation. The only emphasis is that, in the high ΔpH range, the pump rate (solid line) varies much more steeply with ΔpH than the leak rate (dashed line). Arents et al. (1981) proposed a linear decrease of pump rate with ΔpH, however, their study is limited to a very low (<0.6) ΔpH values. Figure 8A shows that, due to the back-pressure effet, a limited increase of proton passive permeability has practically no effect upon the steady-state ΔpH since it is compensated by an increase of proton pumping rate (decrease of back-pressure effect). A greater increase of permeability can, on the other hand, lead to a lower ΔpH. Similarly, Figure 8B indicates that, to some extent, a decrease of the number of active pumping units (and thus of the initial pumping rate) also has no effect upon the steady-state ΔpH, due to a concomitant decrease of the back-pressure effect. Again, a higher decrease can affect ΔpH. Thus, with this simple scheme, many of our experimental results can be accounted for. Knowledge of the exact dependence of pumping rate upon ΔpH will require further study. Note that, according to Figure 8, low ΔpH have little effect upon the proton pumping rate, in agreement with the work of Quintanilha (1980). There seems to be an important difference betwen the back-pressure effects of ΔpH and $\Delta\psi$ which simply reflects the different kinetics of generation of the two components of the $\Delta\tilde{\mu}_{H^+}$. The $\Delta\psi$ back-pressure affects both initial rates of pumping and ΔpH values while the ΔpH back-pressure does not affect initial rates. A consequence is that the latter back-pressure effect can be delayed by increasing the buffer capacity, allowing more protons to be pumped, as shown above.

Finally, we would like to point out a more technical feature that is derived from this study. Total light-induced proton uptake in BR liposomes is generally expressed as the number of H^+ pumped per mole of BR (Hellingwerf et al., 1978; Lind et al., 1981). This can yield misleading interpretations when different lipid to protein ratios are used since as shown here, the steady-state ΔpH is independent of protein content. Additionally, the number of pumped protons is dependent upon buffering power so that it should be corrected from this factor.

This study suggests that large BR liposomes prepared by reverse-phase evaporation are useful tools for studying in detail the mechanism of light-induced $\Delta\tilde{\mu}_{H^+}$ formation. Due to the low passive permeability, these allowed us to observe large $\Delta\tilde{\mu}_{H^+}$ relevant to physiological conditions. The results obtained here with these liposomes provide a better understanding of how BR regulates its protonmotive force.

ACKNOWLEDGMENTS

We are indebted to A. Bluzat for technical assistance.

Registry No. H^+, 12408-02-5; K, 7440-09-7; Cl^-, 16887-00-6; Triton X-100, 9002-93-1.

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[4] It seems that the internal pH attainable in our experimental conditions is not per se inhibitory for BR. In particular, we have found (unpublished results) that the initial pumping rate of BR liposomes is relatively constant between pH 4 and pH 7.5. This suggests that the back-pressure effect observed here is related to the buildup of a critical ΔpH of about 2 pH units rather than to a critical internal pH. A similar conclusion was drawn by Hellingwerf et al. (1979).

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CORRECTION

Characteristic Ribonucleolytic Activity of Human Angiogenin, by Robert Shapiro, James F. Riordan, and Bert L. Vallee*, Volume 25, Number 12, June 17, 1986, pages 3527–3532.

Page 3530. In column 1, the fourth sentence of the text should read "...after 60 min (lane 4)...increased to 255 min (lane 5)."