Linear Relations between Proton Current and pH Gradient in Bacteriorhodopsin Liposomes†

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ABSTRACT: The dependence of proton movement across the membrane of bacteriorhodopsin liposomes on the pH gradient was investigated. Under the appropriate experimental conditions, endogenous proton (or hydroxyl) leakage, proton movement catalyzed by protonophore or nigericin, and light-driven proton translocation depend linearly on the pH gradient. This justifies the use of linear proton flux vs. protonmotive force relations in a recent mosaic thermodynamic description of ion translocation in bacteriorhodopsin liposomes [Westerhoff, H. V., Scholte, B. J., & Hellingwerf, K. J. (1979) *Biochim. Biophys. Acta 547*, 544–560]. Since bacteriorhodopsin liposomes are a model system for all biological energy transducing systems in which proton pumps are involved, these findings also explain linear relations between proton flux and protonmotive force observed in and postulated for those systems. In cases where the membrane potential is not clamped at a low value, an initial phase of rapid proton movement occurs, followed by a phase of slower proton movement. The rate of proton movement during the slow phase is again linear with the pH gradient. Such a linear relation is not observed for the fast phase. Since the rapid proton movement phase is also observed in liposomes without bacteriorhodopsin, it is not due (only) to dissociation of scalar protons from bacteriorhodopsin. We suggest that during the initial phase of proton movement, the proton flux is not yet electrically compensated by the fluxes of other ions.

Reconstituted bacteriorhodopsin has been used as a model system for proton pumps (Stoeckenius et al., 1979). It has been useful in founding the concept (Mitchell, 1961) that the protonmotive force can act as a high energy intermediate in biological energy transduction. At the same time, it is the most straightforward example of a proton pump that is not chemiosmotic (Mitchell, 1966) in the strict sense (Mitchell, 1977). The elucidation of the mechanism of this proton pump will bring with it new concepts (Honig et al., 1979) of proton pump mechanisms.

Bacteriorhodopsin reconstituted into (single-bilayer) liposomes has also been used to develop (Westerhoff et al., 1979) a novel method for the quantitative description of the coupling of ion fluxes, both to one another and to the (photo-) chemical reactions that ultimately drive them. The same method has been applied to other systems of biological energy transduction (Westerhoff & Van Dam, 1979, 1981), such as mitochondrial oxidative phosphorylation (Van Dam & Westerhoff, 1977) and gluconeogenesis (Van Dam et al., 1978). However, the correctness of these quantitative descriptions has up to now only been checked qualitatively, which is insufficient for a quantitative theory. Before the theory can be fully applied to bioenergetic systems in general, both its basic postulates and its experimental predictions must be verified.

The central postulate (Westerhoff & Van Dam, 1979) implies proportional relations between rates of translocation reactions (J_i) and their driving forces (ΔG_i). From underlying statistical mechanics, it can be shown that such a proportionality should always hold near equilibrium (Onsager, 1931). The range of the near-equilibrium domain is, however, not specified for the general case. For ordinary chemical reactions the domain has been stated to be relatively small, i.e., $\Delta G \ll RT$ (Katchalsky & Curran, 1967; Erecinska & Wilson, 1979). For other special cases, such as free diffusion (Dunlop & Gosting, 1959) and simple enzyme-catalyzed reactions with often trivial boundary conditions (Rottenberg, 1973; Van der Meer et al., 1980), this near-equilibrium proportionality domain appears to be larger. However, no theoretical foundation for the assumption of a proportional relation between proton current and the pH gradient [as postulated in Westerhoff et al. (1979)] exists [see, however, Heinz (1978), Läuger (1979), and Del Castillo et al. (1979)]. For mitochondria (Nicholls, 1974b; Sorgato & Ferguson, 1979), the relation between protonmotive force and proton current was concluded to be linear within but a limited region of the relevant proton current.

These considerations warrant a straightforward demonstration of proportional relations in a model system for proton-mediated energy transduction: bacteriorhodopsin liposomes. Mechanistic assumptions, such as a constant H^+/O stoichiometry in the mitochondrial experiments (Nicholls, 1974b; Sorgato & Ferguson, 1979), should not be involved.

This paper proves that in bacteriorhodopsin liposomes proton leakage current, either mediated by ionophores or passive, is linear with the pH gradient driving it. In addition, it will be demonstrated that the active (light-driven) proton current is linear with the pH gradient. Thus the use of linear flow force relations in quantitative descriptions of biological energy transduction finds support in the model system of bacteriorhodopsin liposomes.

Materials and Methods

Measurement of the pH Gradient. If the buffer capacity of the intravesicular space is known, the pH gradient at any time can be calculated from the total number of protons taken up (Mitchell & Moyle, 1967a). Both to eliminate effects of heterogeneity of the liposome preparation and to simplify the calculation of the internal pH, the bacteriorhodopsin liposomes were loaded with a buffer mixture with a buffer capacity that is independent of pH in the relevant pH range (Hellingwerf,

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1979; Kell & Morris, 1980). The pH gradient is then given by

$$\delta(\Delta pH) = (-1/\beta_{H,in})\vec{\delta H}^+/V_{in} - \delta pH_{out} \quad (1)$$

where $\beta_{H,in} = \vec{d}H^+/(V_{in}dpH_{in})$ represents the intravesicular buffer capacity.[1]

The components of such a buffer mixture must be impermeant. Therefore acids which are charged in both the acidic and the conjugated base form, and which are harmless to the membrane and bacteriorhodopsin, were chosen. Between pH 6.0 and pH 4.0, the buffer capacity of the buffer mixture (buffer 12) finally selected is essentially constant. All experiments were carried out in such a manner that the part suspected of nonlinearity had an inner buffer capacity that varied less than 5%.

Less than 1%/h of the internal buffer capacity leaked out of the bacteriorhodopsin liposomes both in the dark and in the light. At pH 6.2, the internal buffer capacity of bacteriorhodopsin liposomes containing 150 mM KCl was less than 0.01 μmol of H^+ (pH unit)$^{-1}$ (mg of lipid)$^{-1}$, which is approximately equal to 0.02 M H^+/pH unit. With bacteriorhodopsin liposomes containing the pH-independent buffer mixture (buffer 11 or buffer 12), the difference between the observed inner buffer capacity and the inner buffer capacity calculated from the inner volume and the buffer capacity of buffer 11 (0.12 M H^+/pH unit) was insignificant. Also reflecting the higher internal buffer capacity, the extent of proton uptake is about 10 times higher when the bacteriorhodopsin liposomes contain buffer 11 than when they contain a KCl solution only (Hellingwerf, 1979). These high extents of proton uptake indicate that buffer 11 does not damage bacteriorhodopsin or the liposomal membrane.

By centrifugation through Sephadex columns (Penefsky, 1977), the liposomes were transferred to a medium containing the nonpermeant sulfate anion. Sodium sulfate and potassium sulfate were mixed so that their concentrations equalled their concentrations in buffer 11. Thus medium 12 was defined. The difference in tonicity of this external medium and buffer 11 was corrected by the addition of 112 mM glucose to buffer 11. The resulting buffer mixture (cf. Materials) was named "buffer 12". The amount of glucose that had to be added was determined by measuring the swelling and shrinkage (light scattering) of nonsonicated liposomes in which buffer 11 had been included as a function of the concentration of externally added medium 12.

With buffer 12 as the internal medium and medium 12 (+ traces of buffer 12) as the external medium, the relation between proton flow and pH gradient can be measured through the use of eq 1.

Instrumentation. Proton movement into or out of the (bacteriorhodopsin) liposomes was measured through its effect on the external pH, using the pH meter described by Hellingwerf et al. (1979) (pH meter 1). The reference electrode was connected to the vessel via a salt bridge containing saturated KCl or Na_2SO_4. The hydrostatic pressure was adjusted so that less than 3 mM/h of the salt leaked into the vessel. Alternatively "pH meter 2" was used. "pH meter 2" consists of a cylindrical (i.d. 0.75 cm) Perspex vessel, painted black and equipped with a thermostat (25 °C), and a magnetic stirrer. An Ingold 10 403 3059 combined pH and reference electrode sticks into the vessel from a side in such a manner that its planar surface forms a continuum with that side. In all experiments that were liable to electrode artifacts, control injections of (different types of) acids or bases were carried out in the absence of vesicular material. If such an injection caused a pH overshoot, or a slow relaxation, the reference compartment of the electrode was rinsed and refilled with 3 M KCl + saturated AgCl. Approximately 1 mM/h potassium leaked into the vessel. Illumination was carried out by using a 150-W, 20-V xenon lamp (Osram), two heat filters, one UV filter (U.G.1), a flexible light guide, and a shutter. Light intensities were measured by using an EG&G photometer (Radiometer, Model 450) with a Multiprobe detector, Model 550-2; 100% light intensities were 0.65 and 0.17 kW/m^2 for pH meters 1 and 2, respectively (white light). The spectra of the two lamps differ only slightly.

Analyses were carried out by means of hand-drawn tangents (as a control) and a Hewlett-Packard 2100s computer. In the latter case, traces were digitalized by using a Tektronix 4954 screen. Rates were then calculated by using a subroutine, written by Jan Wilms, based on the 25-point first derivative method (quadratic convolution) of Savitzky & Golay (1964). The computer analysis had the disadvantage that no tangents were drawn to the initial part of the decay curve; the subroutine uses an interpolation method to calculate the tangent. Unless otherwise indicated, the figures shown have been calculated by the computer. In such plots, only one of every five points is indicated by a symbol. Unless otherwise indicated, straight lines are linear least-squares fits using all the points. We wish to stress that (by evaluating a fixed number of points per period of time rather than per amount of change in the pH gradient) the computer program systematically overestimates the correlation coefficients. Therefore the correlation coefficients given should only be used to objectively compare the different curves with each other. For some experiments, we give the hand-drawn analysis of the curves (as indicated in the legend). Such an analysis should be considered less accurate and the calculated correlation coefficient as unbiased.

Materials. "Buffer 12" consists of 100 mM citrate, 60 mM tartrate, 100 mM phosphate, 125 mM pyrophosphate, 75 mM β-glycerol phosphate, 100 mM oxalate, 50 mM malate, and 112 mM glucose. The cations were sodium and potassium so that buffer 12 contains 640 mM potassium and 715 mM sodium. "Medium 12" consists of 320 mM K_2SO_4 plus 357 mM Na_2SO_4. Bacteriorhodopsin liposomes were prepared as described (Hellingwerf et al., 1978a) except that the medium was buffer 12, pH 6.0; sonication was 60 times (15 s followed by 45-s silence) at room temperature, usually at concentrations of 10 g/L lipid (unless otherwise indicated) and 1.0 g/L bacteriorhodopsin in a volume of 2 mL. For preparation of French press liposomes, 4 mL of such a suspension of bacteriorhodopsin liposomes was stored for 2 days at −70 °C. After thawing at room temperature, the suspension was passed 4 times through a French pressure cell press (American Instrument Co.). Just before each experiment, 0.1 mL of a preparation was applied to a 4-mL Sephadex G-50 Coarse column in a 5-mL disposable syringe, equilibrated in water, and washed with "medium 12". Centrifugation for 1.0 min at 2000 rpm ($670g_{max}$) in a bench centrifuge then caused elution of about 0.2 mL. This volume was put into the pH meter and supplemented to 1.0 mL (pH meter 2) or 3.8 mL

[1] Symbols and abbreviations used [see also Westerhoff et al. (1979)]: μ_x, chemical potential of substance x; $\tilde{\mu}_x$, electrochemical potential of substance x; pX, −log (activity of substance x); $\Delta\mu_x$, $\mu_{x,in} - \mu_{x,out}$; $\delta\mu_x$, change in time of μ_x; dx, infinitesimal amount of x crossing the membrane; β, buffer capacity; J_x, outward flux of substance x; $J_{H'}$, proton flux pumped by bacteriorhodopsin; L_x^1, permeability coefficient of the membrane for ion x; L_{KOH}, permeability coefficient for H^+/K^+ exchange; L_p, activity coefficient of bacteriorhodopsin (proportional to light intensity); S13, 3-*tert*-butyl-5,2′-dichloro-4′-nitrosalicylanilide.

(pH meter 1) with "medium 12" (final pH 6.4 ± 0.2). Internal volumes of the bacteriorhodopsin liposomes, deduced from K^+ exclusion by a Sephadex G-50 column at 1*g* (Hellingwerf et al., 1979), were about 0.75 mL/g of phospholipid.

L-α-Phosphatidylcholine (type V-e from egg yolk, 100 g/L in chloroform/methanol, 9:1) was purchased from Sigma Chemical Co. Soybean phospholipids were isolated as described by Kagawa & Racker (1971). 3-*tert*-Butyl-5,2′-dichloro-4′-nitrosalicylanilide (S13) was a gift from Dr. P. Hamm, Monsanto Co. (St. Louis, MO). Nigericin and valinomycin were gifts from Dr. W. C. Pettinga, Eli Lilly (Indianapolis, IN). Chlorpromazine was purchased from Serva (Heidelberg).

Results

(*1*) *Passive Proton Leakage.* We set out to determine the relation between proton leakage and the pH gradient in the region of the latter parameter that is relevant to energy transduction in bacteriorhodopsin liposomes. Thus it sufficed to consider the proton reappearance in the medium when the light is switched off after an illumination period long enough to reach the steady state of proton uptake. Proton flux and pH gradient can be determined from the slope and deviation from the dark value of the pH_{ex} vs. time plot provided by the recorder (cf. Materials and Methods). In the subsequent figures, the pH gradient decay curves will generally be plotted as proton efflux rate vs. the remaining pH gradient.

The passive efflux of protons is expected to be driven not only by the pH gradient but also by the electric potential across the membrane. Evidently this component of the protonmotive force cannot be measured in our experimental setup. It may, however, be clamped at a value close to zero; if the potassium buffer capacity is defined analogously to the H^+ buffer capacity:

$$\beta_{K_{in}} = \frac{\overrightarrow{d[K^+]}}{dpK_{in}} \qquad (2)$$

it is equal to 1.47 M/p*K* unit, which is more than 10 times the pH buffer capacity. Even though the potassium buffer capacity decreases as K^+ leaves the inner liposomal space, the change in chemical potential for internal K^+ is less than 5 mV if the internal pH changes by 60 mV (1 pH unit). Since the potassium gradient is always small, it can be used to clamp the membrane potential to low values. For this purpose, valinomycin, the K^+ ionophore, should be added in excess.

In initial experiments, in which the bacteriorhodopsin liposomes were preincubated with the valinomycin, the latter requirement appeared to be in conflict with the objective of analyzing the relation between proton leakage rate and pH gradient in the relevant region of the pH gradient; at the high concentrations needed, valinomycin reduced the steady-state pH gradient by more than 50% as a result of its inhibitory activity on the photocycle of bacteriorhodopsin (Rott & Avi-Dor, 1977). For analysis of whether linearity exists in ΔpH ranges that are comparable to the usual steady-state pH gradients, a whole efflux trace from an uncorrupted steady state should be analyzed. In order to make this possible, valinomycin should be added during the steady state of proton uptake rather than during preincubation. Illumination should then be terminated as quickly as possible.

Figure 1 shows the result of such an analysis of proton efflux traces at different valinomycin concentrations. At the low, but also at the higher, valinomycin concentrations, the membrane potential appears to limit the rate of proton efflux; an increase in valinomycin concentrations increases the proton

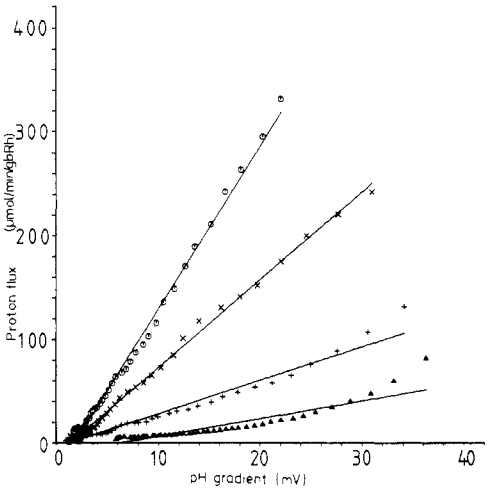

FIGURE 1: Proton efflux as a function of pH gradient after illumination has been terminated. Bacteriorhodopsin liposomes (0.1 mL) (10 g/L soybean phosphatidylcholine and 1.0 g/L bacteriorhodopsin, "buffer 12"; 45 × 15 s sonication) were incubated in pH meter 1 and supplemented with 3.6 mL of "medium 12". After the steady state of proton uptake had been induced by illumination, the appropriate amount of valinomycin was added, and after another 40 s, illumination was terminated. The four runs were carried out in sequence with a single preparation. (▲) 0.2, (+) 1.0, (×) 5.0, and (⊙) 10 μg of valinomycin. Straight lines drawn are linear least-squares fits with correlation coefficients 0.926, 0.986, 0.999, and 0.997, respectively.

flux at a given pH difference. This was to be expected, because the efflux of protons will cause a diffusion potential which is positive on the outside (Junge, 1975). Unless it is efficiently dissipated by potassium movement mediated by valinomycin, this diffusion potential will have an inhibitory effect on the proton efflux. At the highest valinomycin concentrations used, proton flux no longer increases linearly with valinomycin concentration, which would indicate that an almost saturating amount of valinomycin is present ($L_K^I \gg L_H^I$). Then the curves are nearly linear. We conclude that under conditions where the membrane potential is kept low, the relation between pH gradient and passive proton efflux tends to be linear.

A critical analysis of what may be expected for proton efflux curves under conditions where no excess valinomycin is present yields a problem. In terms of the formalism of "mosaic" irreversible thermodynamics developed by Westerhoff et al. (1979), the proton and potassium flows during the efflux phase are given by

$$J_H = L_H^I \Delta\tilde{\mu}_H + L_{KOH}(\Delta\mu_H - \Delta\mu_K) \qquad (3)$$

$$J_K = L_K^I \Delta\tilde{\mu}_K + L_{KOH}(\Delta\mu_K - \Delta\mu_H) \qquad (4)$$

Note that $\Delta\mu_X = -RT(\ln 10)\Delta pX = -59(\Delta pX)$ mV at room temperature. Just before the light is switched off, $\Delta\tilde{\mu}_K$ will equal zero, and both $\Delta\mu_K$ and the membrane potential will be very close to zero (Hellingwerf et al., 1979). As was mentioned above, $\Delta\mu_K$ is nearly constant, and therefore only the membrane potential will change the moment the light is switched off (cf. Heinz et al., 1981). This change will be caused by the efflux of the electrically charged protons. It is not until the membrane potential has reached its maximum that the potassium ion flux induced by it quantitatively compensates for the proton efflux. However, once this is so, the so-called steady state of electroneutral total ion flow has been reached, so that the proton flow can be rewritten as (Westerhoff et al., 1979)

$$J_H^{*e} = \frac{L_H^I L_K^I}{L_H^I + L_K^I}\Delta\mu_H + L_{KOH}(\Delta\mu_H - \Delta\mu_K) \qquad (5)$$

According to this equation, the relation between proton efflux

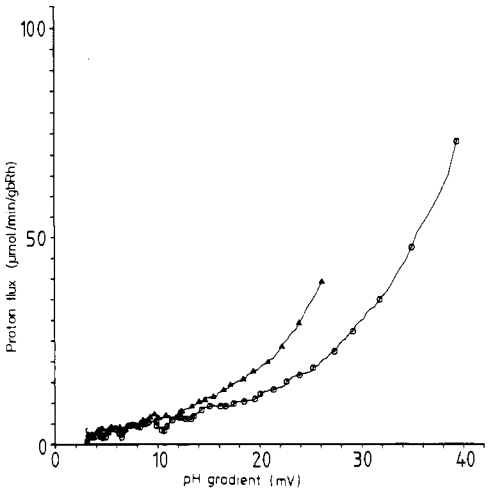

FIGURE 2: The relation between proton efflux and pH gradient depends on the course of the experiment. Bacteriorhodopsin liposomes as in Figure 1 were first subjected to 10 × 15 s preillumination to bring the bacteriorhodopsin into the light-adapted form. A 0.2-μg sample of valinomycin was added. Then illumination with 100% light intensity was carried out until the steady state of proton uptake had been reached. After a period of darkness long enough for a decay of the pH gradient (O) by more than 98%, illumination with 15% light intensity was initiated (neutral density filter, Oriel Corp., Stanford, CT). After the steady state had been reached, the illumination was terminated (▲).

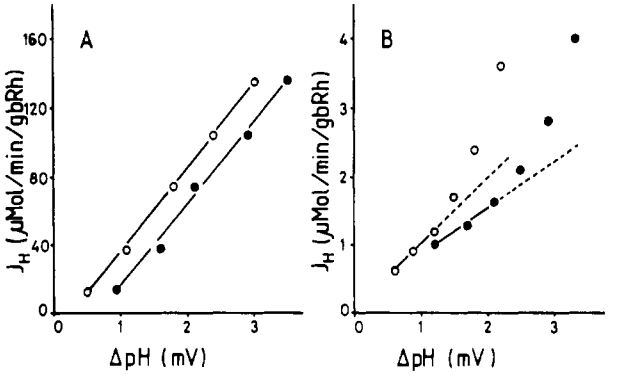

FIGURE 3: Preincubated valinomycin does linearize the relation between passive proton efflux and pH gradient. The conditions are as in Figure 1, except that pH meter 2 was used. Valinomycin preincubated with the bacteriorhodopsin liposomes. (B) Valinomycin (0.2 μg), light intensity reduced by closing the diaphragm until the steady-state proton uptake was similar to the case with 40 μg of valinomycin [(A), 100% light intensity]. Analysis by hand of the efflux traces. Open and closed symbols are duplo analyses (by hand) using different drift extrapolations. Correlation coefficients of straight lines (not shown) obtained through linear least-squares analysis of all the points were 0.97 (B) and 0.999 (A).

and the pH gradient should be linear, even if valinomycin would not be present in excess. This is in contradiction with the experiment of Figure 1.

Therefore, it seems that either eq 3 or eq 4 or the assumption that the steady state of electroneutral flow has been reached is incorrect (cf. Stoeckenius et al., 1979). A completely different explanation would be that as the illumination is terminated, also protons that are not involved in the pumping process (scalar protons) dissociate from bacteriorhodopsin [Eisenbach et al., 1976, 1978; Garty et al., 1977; Eisenbach & Caplan, 1979; see, however, Stoeckenius et al. (1979)]. The first of these four possibilities seems unlikely in view of the fact that at high valinomycin linearity is observed (cf. Figure 1).

So that the third and fourth possibilities could be tested, the experiment of Figure 2 was carried out. In one preparation, two efflux traces were made, one beginning at a pH gradient created by full illumination and the other beginning at a (lower) pH gradient caused by illumination with reduced light intensity. If the steady-state condition of electroneutral total ion flow would apply continuously, the proton flow would only be determined by the pH gradient. In that case, the two plots of proton efflux vs. pH gradient should be superimposable. If, on the other hand, the electroneutrality steady-state condition would not continuously apply, one would expect higher proton fluxes for the initial parts of the plots. In the full illumination plot, this would occur at high pH gradients and, in the low illumination plot, at the lower pH gradients, i.e., where the off-reaction begins. Figure 2 shows that the two plots are not superimposable. Thus it appears to take considerable time for the total flow to become electroneutral; the third possibility is confirmed.

The experiment of Figure 2 also bears upon the fourth possibility. If the dissociation of the scalar protons would be induced by the relaxation of a light conformation of bacteriorhodopsin to a dark conformation (Eisenbach et al., 1978), then the reduction of light intensity by 85% would be expected to reduce the rapid phase of medium acidification much more than is apparent from Figure 2 (see also Figure 3). The light intensities used here are far below those saturating bacteriorhodopsin (Hellingwerf et al., 1979). We suggest that the linearization of the relation between proton flow and pH gradient by valinomycin (Figure 1) is due to more rapid attainment of the steady-state condition of electroneutral total flow.

The control experiment of Figure 3 shows that a limitation in the rate of insertion of valinomycin into the liposomal membrane does not by itself account for the linearizing effect of this ionophore on the flux vs. pH gradient curves. In this experiment, the bacteriorhodopsin liposomes were preincubated with a high concentration of valinomycin. In a parallel experiment, bacteriorhodopsin liposomes were incubated with very little valinomycin at a reduced light intensity so that the steady-state pH gradient was similar in magnitude. Even in this region of low pH gradients, valinomycin linearizes the relation between proton efflux and pH gradient under conditions that exclude a time-dependent integration artifact.

The origin of the deviation from linearity in the high flux part of the proton flux vs. pH gradient curves suggests several methods to obtain full linearization. The use of ions other than potassium to dissipate the membrane potential appeared to be ineffective, however. In order to keep the membrane potential constant during the total duration of the pH gradient decay, their concentration would have to be in the order of 0.5 M (see above). Already at much lower concentrations, thiocyanate inhibited light-driven proton uptake. Neither chloride nor nitrate was as effective as potassium and valinomycin in their effect on the initial rate of proton uptake. We tried to increase the activity of valinomycin or decrease the permeability of the liposomal membrane to protons (or hydroxyl ions) by manipulation of the net charge of the liposomal membrane. Table I shows the effect of adding either dicetyl phosphate or stearylamine to the egg phosphatidylcholine used to prepare the bacteriorhodopsin liposomes. For comparison, the results of the experiment of Figure 1 are also summarized in Table I. The negatively charged lipid does not produce the desired increased activity of valinomycin. The positively charged stearylamine has the effect of making valinomycin ineffective.

Another approach used was to prepare the bacteriorhodopsin liposomes in a different manner. We chose the so-called French press method, which has been shown to yield larger

Table I: Effect of Varying Lipid Composition on the Relation between pH Gradient and Passive Proton Efflux[a]

lipid	valinomycin [mg/(g of lipid)]	correlation coeff	slope (s^{-1} V^{-1})	H^+ uptake (H^+/BRh)
egg PC	0.2	0.926	0.7	20
	1.0	0.986	1.4	18
	5.0	0.999	3.7	15
	10.0	0.997	6.8	10
+5% DCP	6.5	0.973	1.6	20
	13.0	0.997	4.5	13
+2% stearylamine	0.2	0.942	0.8	32
	1.0	0.958	0.7	29
	4.0	0.992	0.8	24
	10.0	0.966	0.8	21
+2% stearylamine (French press liposomes)	0.2	0.941	0.2	38
	4.0	0.982	1.3	24
	10.0	0.993	2.2	16

[a] Bacteriorhodopsin liposomes as in Figure 1 except that, where indicated, 0.2 g/L stearylamine or 0.5 g/L dicetyl phosphatate (DCP) was present in addition to the 10 g/L egg phosphatidylcholine (egg PC). For the French press liposomes, an inner volume of 1.2 mL/g of phospholipid was measured and used in the calculation.

monobilayer vesicles than the sonication technique, not only with pure lipid vesicles (Barenholzt et al., 1979) but also with bacteriorhodopsin and pure lipids (P. W. Van Dijck, personal communication). Table I shows that with this preparation the positive charge of the lipids does not eliminate the activity of valinomycin; the ionophore increases both the slope and the degree of linearity. Indeed, at the highest concentration of valinomycin, the relation in French pressure cell liposomes seems to be more linear.

As yet, no situation has been presented in which valinomycin is present in such an excess that we can be certain that the proton efflux is not affected by the rise of a membrane potential. The concentration of valinomycin added during the steady state of proton uptake just before the light was switched off could, however, not be increased further without running the risk that insertion of valinomycin into the membranes would become rate limiting. In order to be able to use high valinomycin concentrations after all, the way by which the pH gradient was generated was altered; after preincubation of bacteriorhodopsin liposomes, the external pH was suddenly changed by adding acid or base (cf. Kell & Morris, 1980). Alternatively the liposomes themselves were injected into a pH meter preincubated with medium 12 at the external pH desired. Although the former method is more liable to artifacts caused by relaxation phenomena of the pH electrode itself (see Materials and Methods), the results of the two methods were similar. Figure 4 shows that at valinomycin concentrations of 6.5 mg/g of lipid and higher the relation between proton efflux and pH gradient is indeed linear, even though the rate of proton efflux still increases with the valinomycin concentration.

From this section we conclude that if rapid attainment of the steady state of electroneutral net flux is made possible, passive proton flux via endogenous leakage pathways depends linearly on the pH gradient.

(2) *Protonophore-Stimulated Proton Efflux.* Protonophores increase the proton permeability of the liposomal membranes, thereby increasing the L_H^1 coefficient, which is the slope in the plots of passive proton efflux vs. pH gradient. In view of the reported interaction of negatively charged protonophores with the valinomycin–K^+ complex (Yamaguchi & Anraku, 1978; Anraku, 1979), we used the positively charged protonophore chlorpromazine (Kraayenhof, 1971). Figure 5 shows that chlorpromazine does indeed increase the slope in the efflux vs. ΔpH plots. This increase is limited, as could be expected from the inefficiency of this cationic protonophore (Kraayenhof, 1971). Within the experimental error, the proton flux

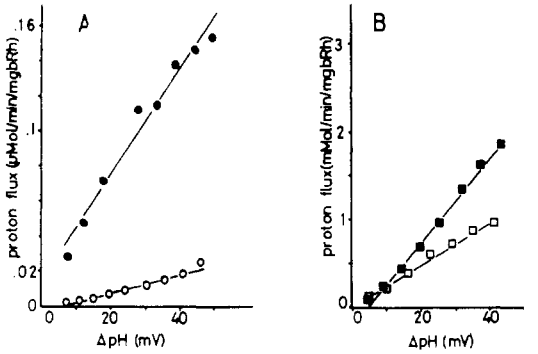

FIGURE 4: With excess valinomycin, the relation between passive proton efflux and the pH gradient is linear in the relevant range of the pH gradient. Bacteriorhodopsin liposomes were prepared as described (Figure 3). After a 15-min preincubation in the presence of the indicated amount of valinomycin in a total volume of 0.2 mL, the bacteriorhodopsin liposomes (1 mg of lipid) were added to pH meter 2, which had been preincubated with 0.8 mL of medium 12 brought to pH 3.94 with sulfuric acid. The observed alkalinization was interpreted analogously to the passive proton leakage in illumination experiments. By adding NaOH instead of bacteriorhodopsin liposomes to the pH meter, it was checked that less than 5% of the apparent proton movement could be due to artifactual pH electrode response. (○) 0.2, (●) 6.5, (□) 20, and (■) 40 μg of valinomycin. Correlation coefficients (all points) are 0.979, 0.985, 0.996, and 0.997, respectively. The analysis was performed by hand.

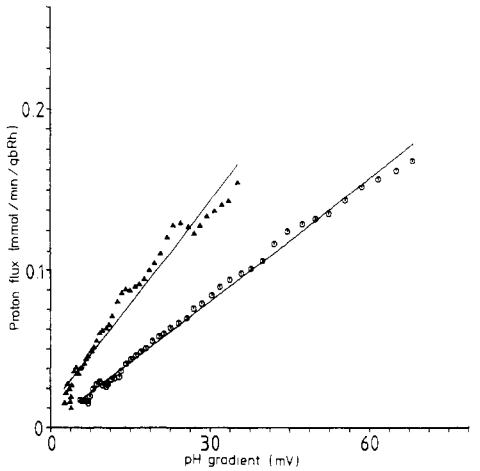

FIGURE 5: Chlorpromazine increases the proton permeability of the membrane retaining linearity. Bacteriorhodopsin liposomes as in Figure 3, except that 0.2 g/L stearylamine was included in the lipid mixture. A 6.5-μg sample of valinomycin and no (⊙) or 100 μM (▲) chloropromazine were present. Correlation coefficients are 0.997 and 0.980, respectively.

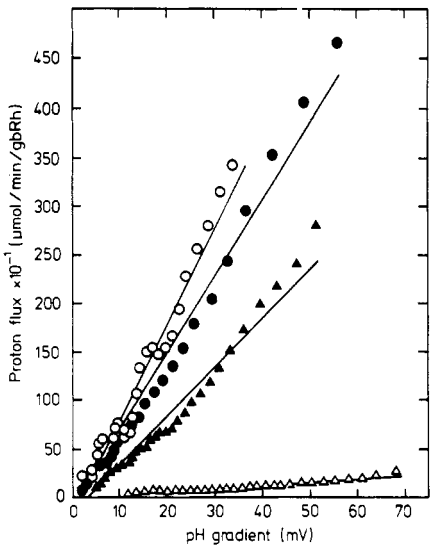

FIGURE 6: Stimulation of the passive proton efflux by an anionic protonophore. A preparation (0.1 mL) of bacteriorhodopsin liposomes (30 g/L egg phosphatidylcholine, 0.6 g/L stearylamine, 3 g/L bacteriorhodopsin, and buffer 12) was centrifuged through a Sephadex column containing medium 12 and supplemented with medium 12 to a volume of 3.0 mL. One milliliter was incubated in pH meter 2. Valinomycin (6.5 mg/g of lipid) and S13 were added after the steady state of proton uptake had been reached. Valinomycin was always added more than 20 s before the light was switched off. (△) No, (▲) 30 nM, (●) 50 nM, and (○) 100 nM S13. Correlation coefficients of the linear least-squares fits shown are 0.983, 0.987, 0.992, and 0.986, respectively.

depends linearly on the pH gradient.

In the same type of preparation, we looked at the effect of the anionic protonophore S13 on the rate of passive proton efflux. As is shown in Figure 6, this rate is greatly stimulated by this protonophore. The interpretation of this stimulatory effect is, however, not straightforward: it can be due to either an increase in the proton permeability coefficient [S13 is known to be much more efficient than chlorpromazine (Kraayenhof, 1971)] or activation of the valinomycin by a direct interaction between the two ionophores. Also, in the present system of bacteriorhodopsin liposomes, we have evidence for such an interaction (Arents et al., 1981).

We conclude that protonophore-mediated proton leakage also depends linearly on the pH gradient.

(3) Nigericin-Catalyzed Proton Movement. As the degree of proportionality between pH gradient and proton efflux is expected to depend on the mechanism of proton movement across the membrane, it is relevant to examine whether the relation is also proportional or if proton permeation occurs via an entirely different mechanism. Such a different mechanism is the proton/potassium ion exchange catalyzed by nigericin (Ovchinnikov et al., 1974). The above-noted problem of absence of electroneutrality is expected to be irrelevant in nigericin-catalyzed proton permeation since nigericin (at the amount per milligram of lipid used here; cf. Toro et al., 1976) only permeates in either of its uncharged forms. Figure 7 shows that especially at the higher nigericin concentrations the relation between pH gradient and proton flux is linear. The fact that in the absence of nigericin and at low concentrations of this ionophore nonlinearity is observed reflects the endogenous H^+ leakage through the membrane (see above).

(4) Linear Relation between the pH Gradient and the Active Proton Flow. Up to now the process that is essential for energy transduction in bacteriorhodopsin liposomes has not been considered in this paper. The earlier papers (Westerhoff et al., 1979; Hellingwerf et al., 1979) also postulated a linear

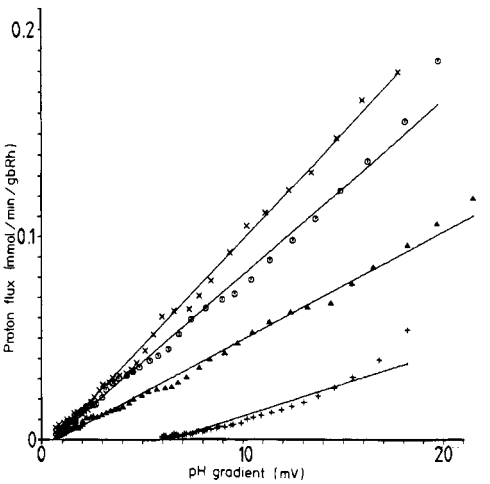

FIGURE 7: Nigericin both increases and linearizes passive proton efflux. A total of 0.1 mL of bacteriorhodopsin liposomes (soybean phospholipids (10 g/L), bacteriorhodopsin (1.0 g/L), and "buffer 12") was treated as described in the legend to Figure 3. Nigericin [(+) 0, (▲) 0.33, (⊙) 0.66, and (×) 1.0 ng] was added in the preincubation period (▲), or during the steady state of proton uptake, about 12 s before the light was switched off. Correlation coefficients were 0.963, 0.996, 0.997, and 0.998, respectively. The experiment was carried out with one sample of bacteriorhodopsin liposomes in one continuous incubation.

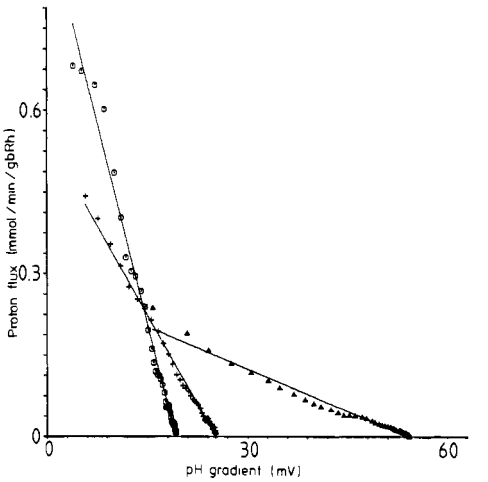

FIGURE 8: The rate of light-driven proton influx is linear with the pH gradient. Valinomycin was present as indicated (15-min preincubation) [(▲) 0, (+) 0.05, and (⊙) 13 μg], as further indicated in Figure 3, except that soybean phospholipids were used. Correlation coefficients 0.989, 0.998 and 0.994, respectively.

relation between the protonmotive force and the light-driven proton flow. This linearity can be checked by looking at the rate of proton disappearance from the medium as a function of the pH gradient that is built up during illumination. The picture is complicated by the occurrence of proton back-leakage. However, we already have checked (cf. *Passive Proton Leakage*) that eq 3 correctly describes the proton back-leakage. If eq 6 (Westerhoff et al., 1979) is correct,

$$J_H^{\nu} = -L_{\nu}(A_{\nu} - \Delta\tilde{\mu}_H) \qquad (6)$$

then the dependence of the rate of proton uptake on the protonmotive force should be given by

$$J_H = -L_{\nu}A_{\nu} + (L_H^{\,l} + L_{\nu})\Delta\tilde{\mu}_H + L_{KOH}(\Delta\mu_H - \Delta\mu_K) \qquad (7)$$

We will now show that the linear relation between proton uptake and pH gradient predicted by this equation is indeed observed. Thus, not only eq 7 but also eq 6 will be verified.

Figure 8 shows the relation between pH gradient and proton uptake rate for three concentrations of valinomycin. The linear

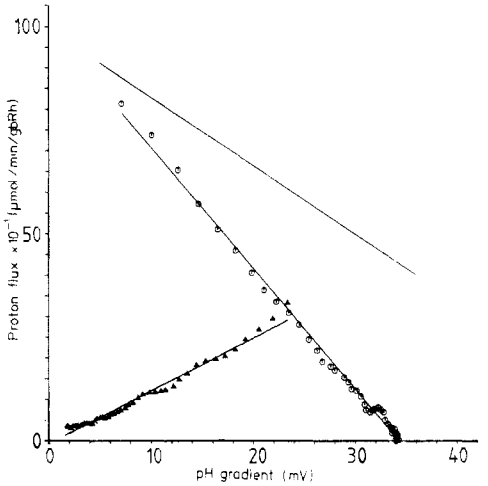

FIGURE 9: The rate of the light-driven proton pump as a function of ΔpH. Correlation coefficients of the linear least-squares lines drawn through "on" (⊙) and "off" (▲) reactions are 0.998 and 0.990, respectively. The third line is the sum of the two other lines and therefore the light-driven proton flux. Preincubation with 13 μg of valinomycin.

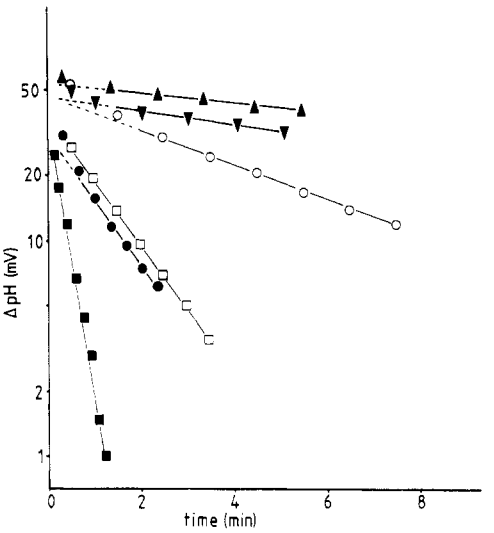

FIGURE 10: A nonexponential passive proton efflux occurs when valinomycin is not present in excess. Analysis was performed by hand. Bacteriorhodopsin liposomes containing 5% (w/w) dicetyl phosphate were prepared, and their passive proton efflux was analyzed as described in Table I. (○) 6.5 and (□) 13 μg of valinomycin per mg of lipid. Liposomes without bacteriorhodopsin containing 5% dicetyl phosphate were exposed to a sudden pH change as described in Figure 4. In the calculations an inner volume of 0.5 mL/g of phospholipid was used. (▲) 0, (▼) 0.2, (●) 6.5, and (■) 13 μg of valinomycin (preincubated).

approximation of the relation between proton uptake and pH gradient is quite good. We conclude that the relation between pH gradient and illumination-induced proton influx is linear.

In order to study more specifically the proton pumping alone, Figure 9 is presented; for a light-on, light-off trace, the proton back-leakage reaction has been subtracted from the "on-phase" plot. The result is a plot of the rate of proton pumping itself as a function of the pH gradient (the line without points in Figure 9).

(*5*) *Interpretation of ΔpH vs. Time Plots.* In view of the observed linearity between proton leakage and pH gradient, a conclusion can be drawn concerning analyses of proton efflux vs. time plots. For a pH-independent internal buffer capacity and relatively large (or constant) outer buffer capacity, eq 8

$$\Delta pH(t) = \Delta pH(0) \exp\left[-(L_H^1 + L_{KOH})\left(\frac{1}{\beta_{in}} + \frac{1}{\beta_{out}}\right)t\right] \quad (8)$$

can be derived. The conclusion is that the pH gradient should follow a monoexponential decay (cf. Kell & Morris, 1980). Figure 10 shows the plot of the protons that still have to leave the bacteriorhodopsin liposomes as a function of time. In the case of high valinomycin, the decay is indeed monoexponential. However, if less valinomycin is present, there is an initial more rapid phase preceding the monoexponential decay. This is related to our earlier contention that it takes some time for the steady state of electroneutral total flow to be reached; the first protons still move without maximal hindrance by the developing electric membrane potential.

That the rapid initial phase in the semiexponential plot is not due to scalar protons coming from bacteriorhodopsin itself is also shown in Figure 10. In this case, liposomes without bacteriorhodopsin were subjected to a pulse of acid.

Discussion

This paper has shown that for four different types of proton translocation across the membrane of bacteriorhodopsin liposomes, the rate of that translocation depends linearly on the pH gradient. In three of these, the pH gradient acted as the driving force for proton movement, i.e., the passive noncatalyzed proton diffusion (cf. Kell & Morris, 1980), the protonophore-mediated proton diffusion, and the nigericin-catalyzed H^+/K^+ exchange. As to the fourth type, the light-induced proton flux, the pH gradient acts as an opposing force, exerting back-pressure (Hellingwerf et al., 1978b) against the proton pump that is driven by the effective thermodynamic energy of the proton (A_p).

The ΔpH values indicated in the figures of this paper never exceed 1 pH unit (60 mV). This might suggest that these experiments are irrelevant to bioenergetics, in which the protonmotive force may well reach values 4 times as high. Since our preparations of bacteriorhodopsin liposomes consist of two-thirds of liposomes devoid of bacteriorhodopsin (P. Staugaard, personal communication; P. W. Van Dijck and K. Van Dam, unpublished results; P. W. Van Dijck et al., unpublished results) and because the inner volumes we measured seem to be a little high for bacteriorhodopsin liposomes (E. Bakker, personal communication), the pH gradient in the bacteriorhodopsin liposomes probably exceeded 2 units. Moreover, because we used the pH gradient that is created by steady illumination, our results are relevant for bacteriorhodopsin liposomes. In any case, the range of the pH gradient for which linearity has now been shown to hold significantly exceeds *RT* [26 mV, 0.4 pH unit (see above)]. This extensively linear dependence of proton translocation rates on the pH gradient may seem surprising in view of the fact that the well-known linear flow-force relations of irreversible thermodynamics have only been proven for systems much closer to equilibrium than *RT*. However, it has been shown that for enzyme-catalyzed reactions obeying certain boundary conditions, linearity follows from the well-known enzyme kinetic equations for 80% of the total velocity range (Rottenberg, 1973; Van der Meer et al., 1980). Recently these theoretical analyses have been extended to theoretical proton pumps with similar results [H. V. Westerhoff et al., unpublished results; see also Heinz (1978)].

In other systems, indications for similar relations have been obtained earlier. In mitochondria (Nicholls, 1974b), but also in submitochondrial particles (Kell et al., 1978, Sorgato & Ferguson, 1979), the relation between oxidation rate and

protonmotive force was partly linear in titrations with inhibitors of respiration. With the help of the nontrivial assumption of constant H^+/O stoichiometries, this was taken as an assay method for linear relations between passive or protonophore-mediated proton back-leakage and the protonmotive force (Nicholls, 1974a). In these systems, the nature of the proton leakage pathway is unclear. It can reside in futile cycles of translocators [P_i, adenine nucleotides, and Ca (Nicholls & Crompton, 1980)], in specific regulated proton conductance pathways (Nicholls, 1974a), or just in passive leakage across the membrane. The relatively pure system of bacteriorhodopsin liposomes shows that the latter pathway is relevant and has the same ohmic characteristic that the mitochondrial proton leakage has at not too high membrane potentials.

The mitochondrial respiration-linked proton pumps have also been shown to be linearly inhibited by the protonmotive force (Padan & Rottenberg, 1973; Azzone et al., 1978; Van Dam et al., 1980). Thus the phenomenon observed here for the light-driven proton pump is certainly not unique. It may, however, seem surprising that a light-driven proton pump can indeed be described in terms of mosaic irreversible thermodynamics since Hill (1977) pointed out that the description of a light-driven chemical reaction was impossible within his theoretical irreversible thermodynamic framework.

The fact that in our present experiments the observed relations between passive proton efflux and pH gradient, if linear, are rarely proportional, is not a reflection of the linear, nonproportional relations derived by Rottenberg (1973) and Van der Meer et al. (1980); it appears that the drift of the pH electrode has a significant effect on the abscissa intersection point, though not on the degree of linearity (cf. Figure 3).

Although a linear relation between membrane potential and proton flux has not yet been established, the linear relations shown in this paper can be taken as partial support for the linear relations between proton flow and protonmotive force that were basic postulates in the quantitative description of bacteriorhodopsin liposomal processes developed (Westerhoff et al., 1979) and qualitatively tested (Hellingwerf et al., 1979) earlier. In papers submitted simultaneously with this paper [e.g., Arents et al. (1981) and Westerhoff et al. (1981a,b)], it is shown that this mosaic, nonequilibrium thermodynamic description can indeed be used to quantitatively describe the effect of variations in ion permeabilities and light intensity on the two energetically important output parameters of light-energized bacteriorhodopsin liposomes: the initial rate of proton uptake and the steady-state pH gradient.

The success of this theoretical method of analysis of bioenergetic experiments is relevant not only to the study of bacteriorhodopsin as bioenergy transducer but also to a, as yet unknown, number of other bioenergy transducing systems, as all of these can be described analogously on a theoretical level (Westerhoff & Van Dam, 1979).

Some interesting details of the experiments presented in this paper should not be left undiscussed. The first is relevant to the conclusion we drew earlier after analysis of some experiments by use of the mechanistic thermodynamic description (Hellingwerf et al., 1979). Bacteriorhodopsin is inhibited in its proton pumping activity by the protonmotive force [see also Hellingwerf et al. (1978b)]. In the present paper, we have direct proof for this conclusion (Figure 9).

The second point is the appearance of the phase during which the proton movement is electrically not yet fully compensated. This is unusual in bioenergetic measurements with the low time resolution we use. For uncompensated proton movement across the inner mitochondrial membrane, it has been calculated (Mitchell, 1966) that the membrane potential would already equal 0.25 V after translocation of 1 nmol of H^+/mg of protein, i.e., some 0.02 s after the oxidation proton pump has been started. However, electric relaxation phenomena can be expected to be slower in the present system of bacteriorhodopsin liposomes; the ratio between electric capacity and buffer capacity in this system is lower than that in mitochondria (Mitchell, 1966; Mitchell & Moyle, 1967b).

Also the observation that upon addition of excess valinomycin the electrogenic fast phase and the slow phase merge is in accordance with expectations; the slower phase is less inhibited by a membrane potential (compensating K^+ movement can occur), so that it becomes kinetically indistinguishable from the rapid phase.

The third point is closely related to the second. If, after the steady-state pH gradient has been reached by illumination of bacteriorhodopsin liposomes and illumination has been terminated, the number of protons still present inside the liposomes is plotted as a function of time (cf. Figure 10), a monoexponential decay is obtained only if excess valinomycin is present. Similarly Eisenbach et al. (1976, 1978) showed that in subbacterial particles as well as in bacteriorhodopsin liposomes both light-on and light-off proton movements were not monophasic. These authors interpret the slow phase in the same way as we do. The interpretations of the initial, more rapid phase differ quite drastically. Eisenbach et al. (1978) conclude that the rapid phase is caused by nonvectorial Bohr protons dissociating from the bacteriorhodopsin when illumination is stopped. They reject the interpretation of the fast phase in terms of the proton flux being more rapid because it is not yet inhibited by a membrane potential, on the following grounds. First, they calculate that in their subbacterial particles only 1% of the proton movement could not be compensated by movement of other ions. Their calculations turn out differently for bacteriorhodopsin liposomes with their 20-fold higher surface to volume ratio [radii of about 15 nm (P. Staugaard and P. W. Van Dijck, personal communication) vs. 0.25 μm for the subbacterial particles] and their much larger inner volume to protein ratio (in our case, about 8 mL/g of protein). In that case the fraction of proton movement that may be electrogenic could be as high as 20%. However, the results of such calculations should be considered as rough estimates.

A second reason for Eisenbach et al. (1978) to refute the interpretation of the nonmonophasic behavior given by Stoeckenius et al. (1979) and us (this paper) is that their kinetics were clearly biphasic and could not be fitted with a gradually decreasing rate constant for proton movement. However, the fact that in their experiments the intravesicular buffer capacity may well have been pH dependent does not allow for such a detailed kinetic conclusion (see below). Moreover, when the vesicular membrane is depicted as an ideal capacitance, one can calculate that approximate biphasicity is expected also for the interpretation of initial proton flow as an electrogenic phase.

In principle, experiments with protonophores should allow discrimination between the scalar protons and the electrogenic phase interpretations. Garty et al. (1979) show that in subbacterial particles FCCP increases the rate constant of the slow phase more than that of the rapid phase. This finding is inconsistent with the electrogenic-phase interpretation. However, the same experiment shows that the extent of the rapid phase is highly FCCP sensitive. Both this finding and Figures 2 and 3 of this paper are inconsistent with the scalar-proton interpretation, but readily explained by the elec-

trogenic-phase interpretation. In view of the limitations imposed on kinetic analyses in cases where the pH dependence of the vesicular buffer capacity is unknown (see below), the fact of the low extent and, in relation to the response times of pH electrodes, the high rate of proton movement in the presence of FCCP limits these kinetic analyses even more. Also, because the FCCP experiment was carried out with subbacterial particles, which still contain various other transport systems, this paradoxical contradiction of both interpretations of nonmonophasic proton movement should not be taken as definitive. Interpretation of biphasic pH gradient decay kinetics in terms of scalar vs. vectorial protons should be limited to cases in which the membrane potential has been completely eliminated.

A fourth point is the possible influence that heterogeneity of our preparation may have had on the analyses. Our preparation consists of about two-thirds of liposomes that are devoid of bacteriorhodopsin (P. Staugaard, personal communication; P. W. Van Dijck and K. Van Dam, unpublished results; P. W. Van Dijck et al., unpublished results). Since in the experiments where the pH gradient was induced through illumination, only the liposomes containing bacteriorhodopsin count, the relevant buffer capacity is 3 times smaller than the one we used in our calculations. The effect of this type of heterogeneity is a 3-fold underestimation of the actual pH gradient. On the other hand, since heterogeneity of the volumes and permeability properties would be expected to cause deviations from monophasicity in the proton movement, it would never make our proton current vs. pH gradient seem more linear than they are.

Another aspect of heterogeneity, i.e., that of heterogeneous orientation of bacteriorhodopsin, could, however, provide some trouble for our conclusion that bacteriorhodopsin is inhibited by an opposed pH gradient. It might be contended that the opposed pH gradient would switch on bacteriorhodopsin of inverse orientation rather than inhibit those with the orientation of the majority. Indications for the inhibitory effect of the pH gradients have also been obtained by using flow dialysis to determine the pH gradient (Hellingwerf et al., 1979), a method which only kept track of those liposomes that were acidic inside. Moreover, Hellingwerf et al. (1978c) have shown that there is no significant subpopulation of bacteriorhodopsin liposomes in our type of preparation exhibiting light-driven proton extrusion. Finally, carboxypeptidase analysis according to Gerber et al. (1977) showed that in our type of preparations more than 85% of the bacteriorhodopsin molecules are oriented inside out. We consider it unlikely that the maximal heterogeneity of each liposome containing on average 15% of the bacteriorhodopsin in the inverse orientation that would switch on upon an increase in ΔpH could cause a reduction of the net proton flux by more than 35% (cf. Figure 9). We would expect either that at least part of the correctly oriented bacteriorhodopsin would be switched off or that all bacteriorhodopsins in the correct orientation are gradually inhibited and all bacteriorhodopsins in the inverse orientation are gradually stimulated by an increasing pH gradient. We conclude that the possible effects of this type of heterogeneity are limited to a significant (3-fold) underestimation of the pH gradient in experiments where this gradient is illumination induced.

A fifth point to be stressed is the usefulness of pH-independent buffer mixtures (Hellingwerf, 1979; Kell & Morris, 1980). They not only simplify pH gradient measurements but also allow the rational analysis of proton influx and efflux traces. Here it should be stressed that for a given functional relation between pH gradient and proton efflux, the kinetics of the decay of the protonmotive force still depend on the pH dependence of the buffer capacity on either side of the membrane. Thus, even if the effect of the membrane potential has been eliminated, kinetic analysis of proton movements in cases with pH-dependent buffer capacities should be interpreted with much more caution than is common practice (Mitchell & Moyle, 1967b; Eisenbach et al., 1978).

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