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BACTERIORHODOPSIN IN LIPOSOMES

II. EXPERIMENTAL EVIDENCE IN SUPPORT OF A THEORETICAL MODEL

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Summary

In the preceding article equations describing relevant ion flows in illuminated suspensions of bacteriorhodopsin liposomes have been derived. Here these equations are subjected to experimental tests. Changes in permeability characteristics of the liposomal membrane are brought about by addition of specific ionophores and change of medium composition. Using light-driven proton uptake and electrochemical potential differences for protons across the membrane as observation parameters, ridig attempts to falsify the derived equations are unsuccessful.

Agreement between equations and experimental results is established on the point of: (i) the antagonistic effect of valinomycin and nigericin on the two components of the proton-motive force, (ii) the time dependence of the changes in transmembrane electrical and chemical potential differences after the onset of illumination.

In three independent experimental systems evidence was obtained for the correctness of the postulated dependence of the turnover rate of the photochemical cycle on back pressure by the transmembrane electrochemical potential difference for protons.

Abbreviations: ACMA, 9-amino-6-chloro-2-methoxyacridine; BRh, bacteriorhodopsin; S_{13}, 5-chloro-3-*tert*-butyl-2'-chloro-4'-nitrosalicylanilide; SF 6847, 3,5-di-*tert*-4-hydroxybenzilidene malonitrile.

Introduction

Bacteriorhodopsin is probably the most extensively characterized electrogenic proton pump. Its structure has been largely resolved with the determination of most of its amino acid sequence [1,2] and three dimensional structure [3]. Its spectral characteristics have been extensively studied (e.g. see Ref. 4) and have resulted in the proposal of the photochemical cycle of bacteriorhodopsin [5], playing an essential role in the light-driven translocation of protons. Its function has been clearly established both by measurement of light-dependent proton movements into or out of reconstituted vesicles, consisting of purified lipids and bacteriorhodopsin only [6—8] and by electrical measurements on reconstituted bacteriorhodopsin membranes [9—12].

Many reports describing properties of reconstituted bacteriorhodopsin vesicles have reached the literature: different reconstitution procedures have been reported [6,13,14], varying effects of ionophores have been observed [8,13,15,16] and the kinetics of light-driven proton movements have been studied [17—19]. However, a general description, allowing a quantitative evaluation of ion translocation in bacteriorhodopsin vesicles is still lacking.

The application of bacteriorhodopsin in reconstitution of biological energy conversions [13] was an important reason to develop such a description. In the preceding article two other reasons have already been exposed. The pure and well-defined system of bacteriorhodopsin liposomes is a good test case for the applicability of the theory of (linear) irreversible thermodynamics to biological systems. Moreover, bacteriorhodopsin is one of the few light-driven proton pumps lacking respiratory chain segments, so that it probably is one of the best examples of relatively direct interaction of photons and protons. A description of the bioenergetics of such an energy-conserving system lacked precedence.

In the preceding article such a description has been given. Equations relating ion transport and the flux through the photochemical cycle of bacteriorhodopsin to the relevant thermodynamic forces have been presented. In this article the results of some necessary experimental tests of the developed theory will be disclosed.

The results agree with predictions, if the relations in which bacteriorhodopsin is regarded as a voltage source (i.e. sensing thermodynamic back pressure from the proton-motive force) are applied. We conclude that the clear picture of the thermodynamic entity of bacteriorhodopsin emerging from the theoretical description is operationally correct.

Materials and Methods

Materials. Bacteriorhodopsin was purified from *Halobacterium halobium* as described in [20]. The resulting purple membranes contain less than 0.3 mol ethanol-extractable lipoic acid/mol bacteriorhodopsin (not shown). Soy bean phospholipids and egg phosphatidylcholine were isolated as described (Refs. 21 and 22, respectively). Cardiolipin was purchased from Sigma Chemical Company. [^{14}C]Aminomethane (50 μCi · ml^{-1}, 2.23 mM), potassium [^{14}C]-thiocyanate (250 μCi · ml^{-1}, 6.10 mM) and [G-^{3}H]H_2O (5 Ci · l^{-1}) were purchased from Amersham Radiochemical Centre, England. [U-^{14}C]Sucrose

(50 mCi · l^{-1}, 12 μM) was obtained from New England Nuclear. 5-Chloro-3-*tert*-butyl-2'-chloro-4'-nitrosalicylanilide (S_{13}) and 3,5-di-*tert*-butyl-4-hydroxybenzylidene malononitrile (SF 6847), were gifts from Dr. P. Hamm, Monsanto Company, St. Louis (U.S.A.) and Dr. Y. Nishizawa, Sumitomo Chemical Industry, Osaka (Japan), respectively. Nigericin and valinomycin were gifts from Dr. W.C. Pettinga, Eli Lilly and Comp., Indianapolis (U.S.A.). All other reagents were of analytical grade. Twice distilled water was used.

General methods. Reconstitution was carried out as described in [23] unless stated otherwise.

pH measurements were carried out in a 3.7 ml thermostatted (25°C) incubation vessel equipped with a magnetic stirrer. The pH of the suspension was continuously measured using an Ingold glass pH electrode connected to an amplifier (Vibron Electrometer 33 B-2). Usually the vessel contained 0.3—1.0 mg bacteriorhodopsin. Illumination was carried out with a 120 V, 500 W Leitz slide projector equipped with two heat filters. The pH changes were calibrated by the addtiion of 0.01 N oxalic acid.

9-Amino-6-chloro-2-methoxyacridine (ACMA) fluorescence changes were measured in the instrument described by Fiolet et al. [24]. Excitation and emission wavelength were 410 nm and 490 nm, respectively; a number 3482 Corning filter (transmission 550—3400 nm) shielded the suspension from the white actinic light. Simultaneous measurement of proton uptake with a pH electrode in this instrument gave data comparable to those obtained in the proton uptake measurements described above.

Bacteriorhodopsin filter experiments were carried out essentially as described by Blok et al. [23]. A Millipore VSWP 025 00 filter was covered with 0.25 ml of hexadecane containing 150 mg of soy bean phospholipids/ml. Then the filter was wiped to remove excess lipids. Bacteriorhodopsin liposomes (soy bean phospholipids 20 mg · ml^{-1}, bacteriorhodopsin 1.0 mg · ml^{-1}, sonication 300 s) in 150 mM KCl were diluted with an equal volume of 75 mM KCl, 50 mM $CaCl_2$. 0.25 ml of the resulting suspension was applied to one side of the lipidated Millipore filter, which had already been clamped in between the two compartments of the Teflon measuring vessel. After a 1 h incubation both compartments of the vessel were filled with 150 mM KCl, and the calomel electrodes were immersed into the different compartments. As external voltage source of 10 V battery (Mallory; 8X PX 625) was used. The electric circuit (see Fig. 10) was analogous to that described by Mueller et al. [25]. The external resistance (R_u) was kept below 1 GΩ. Experiments with any one filter were terminated when either the filter's resistance or the photopotential at zero countervoltage began to change significantly.

Light intensities were evaluated using a Photometer/Radiometer (Model 450, E.G. and G.).

Flow dialysis. Membrane potential and transmembrane pH difference in bacteriorhodopsin liposomes were calculated from the uptake of methylamine (CH_3NH_2) and SCN^-, respectively, using the relations:

$$\Delta\psi = 59.2 \text{ mV} \cdot \log_{10} K_{SCN^-}$$

and

$$\Delta\mu_H (\equiv \Delta pH) = 59.2 \text{ mV} \cdot \log_{10} K_{MeNH_2}$$

with

$$K_x = \frac{[X]_{in}}{[X]_{out}}$$

The accumulation ratio K_x was calculated from the variations in $[X]_{out}$. The latter was measured by means of the flow dialysis procedure developed by Colowick and Womack [26]: by means of a peristaltic pump (LKB 12 000 varioperpex) flow medium is sucked through the well-stirred 0.6 ml lower compartment of a perspex vessel into different test tubes at a flow rate of 7 ml · min^{-1}. The tube between the flow dialysis vessel and the peristaltic pump has an inner diameter of only 0.3 mm thus reducing dead space. The tube between the flow medium bottle and the vessel has a much larger diameter to decrease the minimal inlet resistance. This inlet resistance can be regulated by means of a tube clip, thus controlling the pressure in the lower compartment of the vessel. Upper and lower compartment are separated by a single dialysis membrane cut from dialysis tubing (Arthur H. Thomas Co., Philadelphia, wall thickness 2 μm, pretreated by boiling 1 h in 1 mM Na_2H_2EDTA, and by washing with distilled water). Both the upper and the lower compartment are well-stirred by a couple of tiny stirring bars and a large stirring disc, respectively, both slaves to a magnetic stirrer, on which the vessel rests. In most experiments the pH of the reaction suspension in the upper compartment was recorded by means of a combined pH glass and reference electrode (Ingold type Lot 403 M3). All experiments took place at (and with flow medium preincubated at) room temperature (about 25°C). In experiments with energization by light a Leitz Wetzlar projector housing a 500 W lamp with two heat filters was used. The usual volume of the suspension in the upper compartment was 0.75 ml. Sampling was started at the moment label was added: either about 20 μl of stock $KS^{14}CN$ or about 70 μl of stock $^{14}CH_3NH_2$. In most runs also 9 μl of stock ^3H_2O was added to check for faults in stirring and volume changes. No significant deviations from ideality for ^3H_2O dialysis have been observed. No medium pH changes larger than 0.1 unit were met. From each 7 ml sample 1.0 ml was assayed for radioactivity in the presence of 6.0 ml of a Triton/toluene/PPO (diphenyloxazole)/dimethyl-POPOP (2,2'-*p*-phenylenebis[4-methyl-5-phenyloxazole], Kodak Eastman) mix (1 l/2 l/16.7 g/375 mg) in a Mark I, Unillux II, or Isocap 300 (Nuclear Chicago) liquid scintillation counter.

In control experiments without energization the semilogarithmic plot of tracer concentration versus time yielded straight lines, thus supporting the assumption [26] that the dialysis of the label is a unimolecular process. Theoretical considerations lead to the conclusion that in such plots (as well as in bilinear plots) the lines obtained before energization and after deenergization will be parallel but will not coincide. This effect came out clearly in our flow dialysis experiments. In contrast it is not observed in publications of others [27,28]. In a plot of the dialysis of label, as a function of the total amount of label already dialyzed, lines before energization are expected to extrapolate to lines after deenergization. This prediction was verified (see also Fig. 3). The points lying on the resulting single line (the dashed line in Fig. 3) now indicate the reference label concentration at each point, i.e. the concentration the label

would have, if there would be no accumulation. If the ratio between reference and actual dialysis of substance X at each time point is defined as S_x, then K_x can be calculated from

$$K_x = \frac{S_x - 1}{f_{ih}} + 1$$

Here f_{ih} represents the volume fraction of the suspension occupied by the internal volume of the liposomes.

The internal volume of the bacteriorhodopsin liposomes was equated to the total membrane-enclosed volume in the preparation. It was routinely determined from the retention of K^+ on passage through a Sephadex G-50 column. The observed internal volumes all appoached 0.8 μl/mg phospholipid. Substitution of K^+ as marker by minute amounts of labelled sucrose, or larger amounts of glucose, yielded essentially the same value for this parameter.

Binding of SCN^- by the bacteriorhodopsin liposomes appears not to influence the measurement of membrane potential. Addition of unlabelled potassium thiocyanate in order to increase the average thiocyanate concentration from 0.16 to 0.26 or even 2.5 mM did not decrease the observed accumulation ratio, whereas it did slow down the kinetics (not shown). The addition of bacteriorhodopsin liposomes in the dark decreased the rate of dialysis of $S^{14}CN^-$ and $^{14}CH_3NH_2$ and in proportion to the dilution factor.

Results

1. The effect of ionophores on the steady-state proton uptake

Bacteriorhodopsin reconstituted in liposomes is probably the best defined 'biological' system for the study of the effect of ionophores on 'energized biomembranes'. Bacteriorhodopsin liposomes prepared under conditions analogous to our standard conditions take up protons, if they are illuminated [6]. After some minutes further net uptake of protons stops. The number of protons taken up/unit of bacteriorhodopsin will be related to the transmembrane pH difference ($\Delta\mu_H$) via the buffer capacity of the inner phase of the liposomes:

$$\int_0^\infty \frac{-J_H}{\beta}\,dt = (\Delta\mu_H)^{**} \tag{40}$$

Here β represents the buffer capacity in mol $\cdot$ $V^{-1} \cdot g^{-1}$ bacteriorhodopsin (pH expressed in volt) and J_H the rate of proton uptake. The symbol ** refers to the steady state attained: that of zero net proton uptake. For most of our present purposes it will suffice to recognize that $\int_0^\infty J_H\,dt$ increases monotonically with $(\Delta\mu_H)^{**}$ and can thus be used as a qualitative measure of this parameter.

Fig. 1 shows a record of such an experiment; by use of a pH electrode net proton uptake into bacteriorhodopsin liposomes is measured. Concentrating on $J_H = 0$, steady state (i.e. **), conditions we may conclude in view of Eqn. 23 †

† Numbers of equations refer to equations in either this or the preceding article; symbols are defined in this paper, but more rigidly so in the preceding one.

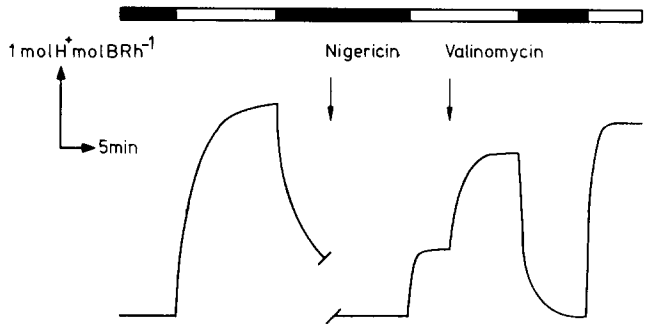

Fig. 1. The antagonistic effect of valinomycin and nigericin on the steady-state proton uptake. Reconstitution conditons: soy bean phospholipids 20 mg · ml^{-1}, bacteriorhodopsin 1 mg · ml^{-1}, sonication 165 s, pH 6.4. Additions: nigericin 10 ng, valinomycin 100 ng, as indicated. The white parts in the bar represent the periods of illumination.

that $\Delta\mu_H > 0$, so that $\alpha > 0.5$. This means that effectively more than half of the bacteriorhodopsin pumps are oriented in such a way that protons are transported inward. Protonophores are expected to increase specifically L_H^1, the electric proton conductance of the membrane [29]. Eqn. 23 then predicts that $(\Delta\mu_H)^{**}$ can be diminished by protonophore, so that the remaining $(\Delta\mu_H)^{**}$ depends in a hyperbolic fashion on the amount of protonophore added (see Table I). In view of the possible dependence of the intraliposomal buffer capacity on pH and the yet unknown relation between efficiency and concentration of protonophore Table I can only qualitatively support this prediction. This table does, however, verify the more specific prediction that the efficiency of protonophore is independent of the action of valinomycin, whenever electroneutral proton diffusion processes are negligible. These experiments were necessary, because Hwang and Stoeckenius [15] observed a valinomycin-induced increase in the efficiency of a protonophore in abolishing steady-state proton uptake. Not even the occurrence of important electroneutral proton movement can explain their observations (valinomycin + protonophore should

TABLE I

EFFECT OF VALINOMYCIN ON THE EFFICIENCY OF THE PROTONOPHORE S_{13}

Reconstitution conditions: soy bean phospholipids 20 mg · ml^{-1}, bacteriorhodopsin (BRh) 2 mg · ml^{-1}, sonication 375 s, pH 5.8. For the pH measurements the sample was diluted six-fold. The concentration of valinomycin was 2 μM. J_{Hi}, the initial rate of proton uptake; n.d., not determined.

[S_{13}] (μM)	Extent of proton uptake (μmol · g^{-1} BRh)		J_{Hi} (μmol · g^{-1} BRh · min^{-1})	
	+ Valinomycin	— Valinomycin	+ Valinomycin	— Valinomycin
0	195	165	1005	278
0.1	186	145	n.d.	n.d.
1	167	130	n.d.	n.d.
10	101	95	585	161
100	25	25	164	32

then give a higher proton uptake than protonophore alone, see Eqn. 23). Probably effects of the addition of valinomycin other than that of an increase in potassium permeability of the membrane are involved, as in the experiments of these authors [15] the initial rate of proton uptake decreases on addition of valinomycin. This is in contrast to other both experimental (see Ref. 13 and Table I) and theoretical (Ref. 30, see below) findings.

To further test our equations the parameters L_e (non-proton electric permeability coefficient) and L_n (electroneutral proton permeability coefficient) were varied using the specific ionophores valinomycin (K^+) and nigericin (K^+/H^+ exchange), respectively. Antagonistic effects of these ionophores on proton uptake and the 'energized state of the membrane' [31] have been observed in chloroplasts [32], submitochondrial [33] and bacterial [34] particles. Qualitative explanations in terms of changes in ion distribution [35] and electrochemical potential differences [27,28,36] have been given. In none of these experimental conditions the influence of endogenous ion transport systems can be excluded. The purity of the system used here allows for a quantitative approach. The observation that nigericin decreases $(\Delta\mu_H)^{**}$ is easily understood: it is predicted by Eqn. 28, as this K^+/H^+ antiporter increases L_{KOH}. The question may now arise whether nigericin simultaneously decreases the proton-motive force ($\Delta\tilde{\mu}_H$) itself. The observation that $(\Delta\mu_H)^{**}$ can be restored by the addition of valinomycin makes such a decrease unlikely. Clear insight is obtained from Eqns. 28—34 as valinomycin specifically increases L_K^l: valinomycin is expected to antagonize the action of nigericin. The antagonistic effect approaches completeness at low valinomycin and nigericin concentrations. Thus Eqn. 32 is supported by the experiment shown in Fig. 1. At the low nigericin concentration used much higher concentrations of valinomycin decrease L_{KOH}/L_K^l so much that almost the original $(\Delta\mu_H)^{**}$ is reached (cf. Eqn. 32). Using the same mathematical approximations (Eqn. 31) Eqn. 34 was derived, which now predicts that on addition of this amount of nigericin the proton-motive force $(\Delta\tilde{\mu}_H)^{**}$ remains approximately constant. Clearly a rise in $(\Delta\psi)^{**}$ (see Eqn. 33) must compensate for the loss in $(\Delta\mu_H)^{**}$. In measurements of the 'energized state' [31] using ACMA fluorescence quenching the antagonistic effect of valinomycin and nigericin turns out to disappear with increasing concentration of these ionophores. This is shown in Fig. 2, with $\log_{10}(Q/(1-Q))$ as an indication of $\Delta\tilde{\mu}_H$ [37]. Here Q represents the fraction of ACMA fluorescence that is quenched by energization of the liposomes.

As the quantitative implications of fluorescence quenching measurements to probe $\Delta\mu_H$ are subject to much doubt (e.g. Ref. 24), the uptake of the weak base aminomethane was monitored and used as a quantitative measure of $\Delta\mu_H$. To be sure not to disturb the bacteriorhodopsin liposomes in any manner, the aminomethane uptake was measured using low concentrations of labelled aminomethane in a flow dialysis set up [26]. In Fig. 3 results are presented in a manner that makes the reference level coincide with a straight interpolation (dashed line in Fig. 3) between the parts of the curve that represent unenergized conditions. This way the flow dialysis data can be converted to a plot of $\Delta\mu_H$ versus time by the formulae presented under Materials and Methods. Both ACMA fluorescence quenching and aminomethane uptake reflect the predictions of the effect of different ionophores on $(\Delta\mu_H)^{**}$ as

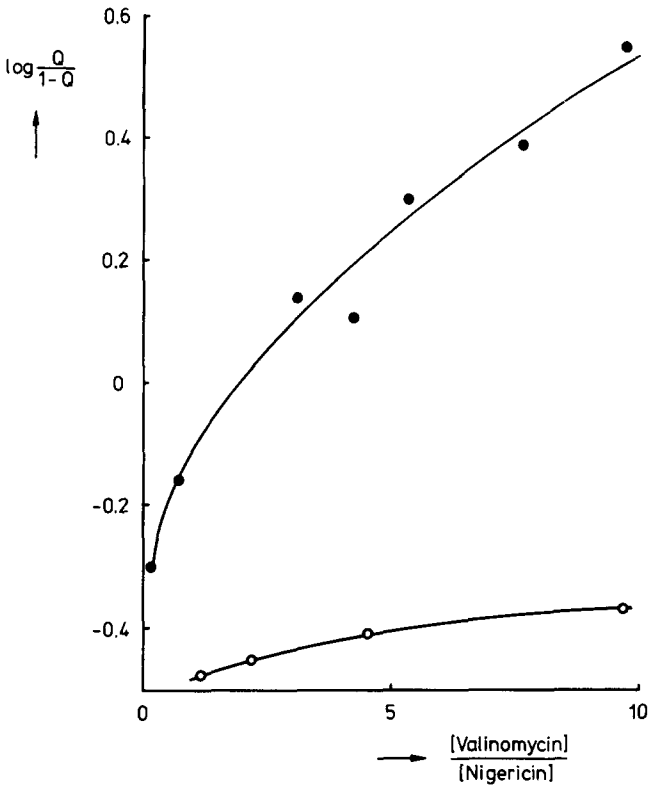

Fig. 2. The antagonistic effect of valinomycin and nigericin on ACMA fluorescence depends on the absolute nigericin concentration. Reconstitution conditions: soy bean phospholipids 20 mg · ml^{-1}, bacteriorhodopsin 1 mg · ml^{-1}, sonication 165 s, pH 6.4. Experimental conditions: 3.5 times diluted bacteriorhodopsin liposomes; ACMA 5 μM; nigericin: •———•, 5.4 ng · ml^{-1}; ○———○, 34 ng · ml^{-1}; valinomycin was added in the mol/mol ratio relative to nigericin indicated on the abscissa. $1 - Q$, the fraction of fluorescence remaining as compared to deenergized conditions. In the absence of added ionophores $\log_{10}(Q/(1 - Q))$ amounted to 0.95.

formulated in section 2.3. of the preceding article.

As a quantitative measure of $\Delta\psi$, the uptake of the lipid soluble anion of the strong acid thiocyanic acid was used. The uptake of $S^{14}CN^-$ was measured in flow dialysis experiments, which were duplicates of those measuring aminomethane uptake. In bacteriorhodopsin liposomes consisting of bacteriorhodopsin and soy bean phospholipids no SCN^- uptake could be measured. Since on addition of nigericin the ΔpH decreased by about 50 mV and was increased again by about 45 mV on addition of valinomycin, a significant uptake of SCN^- was predicted by the Eqns. 32 and 33. Clearly the absence of SCN^- uptake either disproves the proposed model, or SCN^- does not respond to $\Delta\psi$ under these conditions. Recent experiments with chromatophores [38] can be explained, if it is assumed that the electric charge of the vesicle membrane has a significant influence on the permeability of the membrane for thiocyanate. Also in the system studied here the negative charge of the soy bean phospholipids [21] may have prevented SCN^- permeation across the liposomal membrane. To check this possibility the experiments were repeated with bacterio-

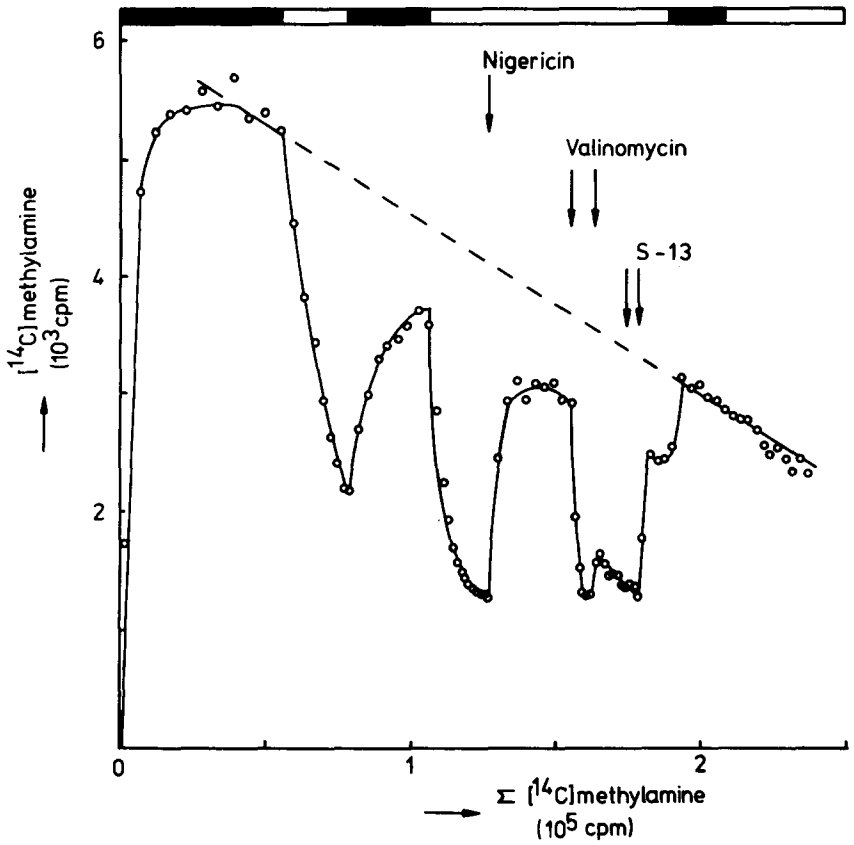

Fig. 3. Aminomethane uptake in bacteriorhodopsin liposomes is also subject to the antagonistic effect of valinomycin and nigericin. Reconstitution conditions: soy bean phospholipids 133 mg · ml^{-1}, bacteriorhodopsin 5 mg · ml^{-1}, sonication 675 s, pH 5.6. Additions: nigericin 0.13 μg · ml^{-1}, valinomycin 4 μg · ml^{-1} and 20 μg · ml^{-1}, S_{13} 2 μM and 62 μM. Aminomethane uptake was assayed by flow dialysis.

rhodopsin liposomes of such a composition that at pH 7 the net charge of the liposomal membrane would be positive rather than negative: 120 mg · ml^{-1} egg phosphatidylcholine, 2.4 mg · ml^{-1} stearylamine and 6 mg · ml^{-1} bacteriorhodopsin. As can be seen in Fig. 4 this indeed led to uptake of SCN^- under the expected condition, i.e. after addition of nigericin.

Also shown in this figure is that indeed $(\Delta\tilde{\mu}_H)^{**}$ remains approximately constant throughout the series of additions of nigericin and valinomycin, until it is lowered by protonophore and darkness. The $(\Delta\psi)^{**}$ measured may seem rather low in comparison to the maximum of $\Delta\mu_H$ reached, but this is due to the use of a very limited amount of nigericin. If more nigericin is added (see Fig. 5), a higher membrane potential can be reached. In that case, however, the assumption that electric proton permeability (caused by the presence of both H^+/K^+ exchange and a permeability for K^+ or Cl^-) is small as compared to the true electric proton permeability (cf. Eqn. 31) appears not to be valid anymore: on addition of nigericin the $\Delta\tilde{\mu}_H$ decreases. This is in full agreement with

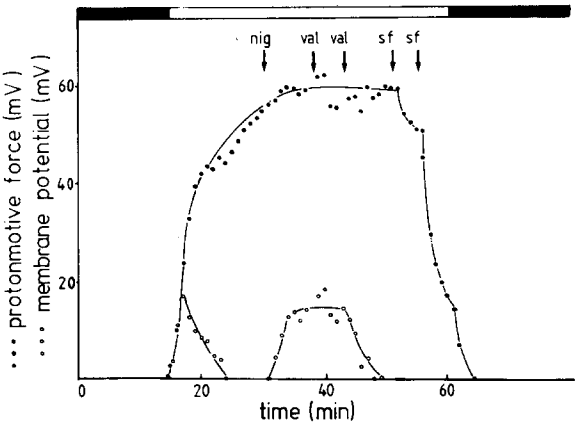

Fig. 4. Membrane potential (○———○) and total proton-motive force (●———●) in illuminated bacteriorhodopsin liposomes; the influence of nigericin, valinomycin, and a protonophore. Reconstitution conditions: egg phosphatidylcholine 115 mg · ml^{-1}, stearylamine 2.3 mg · ml^{-1}, bacteriorhodopsin 5.6 mg · ml^{-1}, sonication 675 s, pH 6.2. Additions: nigericin 0.13 μg · ml^{-1}, valinomycin 0.66 μg · ml^{-1} and 6.6 μg · ml^{-1}, SF6847 3 μM and 60 μM. The membrane potential and transmembrane pH difference were monitored via aminomethane and thiocyanate uptake in parallel flow dialysis experiments.

Eqn. 30, which takes the place of Eqn. 34 in cases where Eqn. 31 is no longer valid. The reported K^+ carrier activity of nigericin at higher concentrations of the ionophore [39] could be a second reason why the proton-motive force diminishes then L_K^1 and L_{KOH} will be increased at the same time.

2. The development in time of $\Delta\psi$, $\Delta\mu_H$ *and* $\Delta\tilde{\mu}_H$

In section 2.4. of the preceding article the actual values of physical parameters such as the membrane capacitance and the membrane conductance of bacteriorhodopsin liposomes prepared in the usual way were estimated. From the outcome it was concluded that an initial $\Delta\psi$ should be visible. The size of this initial membrane potential relative to the maximum $(\Delta\mu_H)^{**}$ is predicted to depend critically on the permeability coefficient for protons relative to that for the K^+ and Cl^- (cf. Eqn. 35). Moreover, the initial electric potential is expected to decay relatively quickly (cf. Fig. 4 of the preceding article). Although an initial membrane potential was already observed in Fig. 4, the time resolution of the flow dialysis set up used by us was brought closer to its maximum by more rapid sampling. This led to the results of Fig. 6. In the experiment represented by Fig. 5 also the $\Delta\mu_H$ was monitored so that it can be concluded from this figure that at the point of time where $\Delta\psi$ has already passed its maximum value and slowly decreases, $\Delta\tilde{\mu}_H$ is still increasing. This is in strict accordance to the predictions of the model description (see section 2.4. of the preceding article).

3. The effect of medium composition on $\Delta\psi$, $\Delta\mu_H$ *and* $\Delta\tilde{\mu}_H$

According to Eqn. 35 at low $\Delta\mu_H$ the magnitude of the initial $\Delta\psi$ relative to the maximum $(\Delta\mu_H)^{**}$ depends on the permeability of the membrane for ions other than protons relative to the proton permeability. As the membrane of the

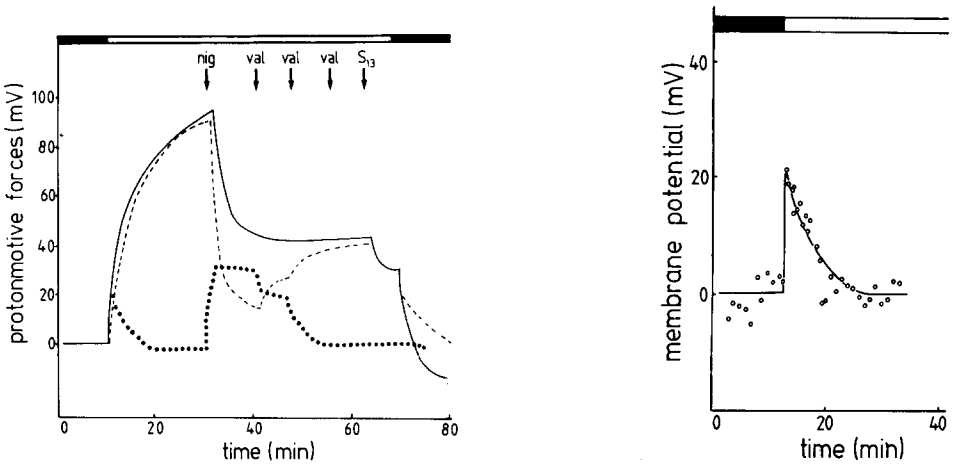

Fig. 5. Membrane potential (•———•) transmembrane pH difference (- - - - -) and proton-motive force (———) in illuminated bacteriorhodopsin liposomes; the influence of nigericin, valinomycin, and protonophore at high nigericin concentrations. Reconstitution conditions: egg phosphatidylcholine 120 mg · ml^{-1}, stearylamine 2.4 mg · ml^{-1}, sonication 90 s, pH restored to 6.4, bacteriorhodopsin 6 mg · ml^{-1}, sonication 675 s, pH restored to 6.4. Additions: nigericin 1.0 μg · ml^{-1}, valinomycin 4.8 μg · ml^{-1}, 29 μg · ml^{-1} (added twice) and S_{13} 60 μM. Further as described in the legend to Fig. 4.

Fig. 6. Initial membrane potential after the onset of illumination of bacteriorhodopsin liposomes. Conditions as described in Fig. 5, except that 10-s samples were taken instead of 60-s samples.

bacteriorhodopsin liposomes used in the experiments of Figs. 4—6 was positively charged, Cl^- rather than K^+ was expected to be responsible for the largest part of the non-proton electric permeability. According to Eqn. 35 replacement of Cl^- by an impermeant ion such as citrate is expected to have the following effect on the initial $\Delta\psi$: since L_e will then significantly decrease, $(\Delta\psi)^*$ is expected to be much larger at the same values for $(\Delta\mu_H)^*$ (* refers to the steady state of electroneutral total flow). Comparison of Fig. 7 to Fig. 5 shows that even after correction for a difference in maximum $\Delta\tilde{\mu}_H$ this is the case (compare for instance t = 30 min in Fig. 7 to t = 12 in Fig. 5).

According to Eqn. 23—25 the antagonistic effects of nigericin and valinomycin should occur in the case of low permeability of the bulk ions (L_e is low) also, although the effect of the addition of nigericin on $\Delta\psi$ at a low L_e is expected to be small. On the contrary the effect of nigericin on $(\Delta\mu_H)^*$ is expected to be quite drastic. Also here experiment and theory match.

Fig. 7 shows that in potassium citrate medium it takes less than half a minute for the membrane potential to reach half its maximum value. Substitution of citrate by chloride is expected to decrease the electric resistance of the liposomal membrane (R_m): the $t_{1/2}$ of the establishment of the membrane potential in 150 mM KCl will be even shorter than half a minute. This is in agreement with the results presented in Fig. 6 and provides experimental support for the value of 1 s for the $t_{1/2}$ in such a system, which was estimated in the preceding article (cf. Eqn. 10). Thus the validity of the steady-state condition of electroneutral flow already a few seconds after the onset of illumination is documented.

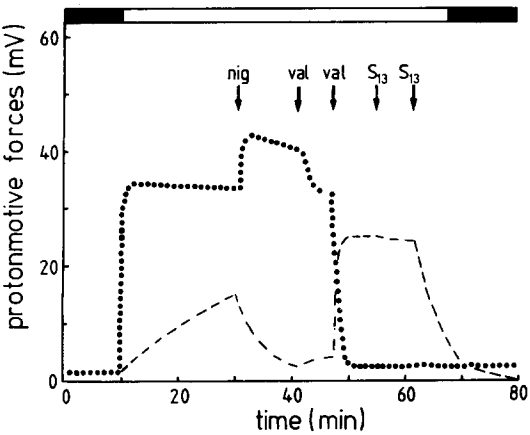

Fig. 7. Membrane potential (•———•) and transmembrane pH difference (- - - - -) in illuminated bacteriorhodopsin liposomes in a medium with an impermeant anion. Sonication conditions: egg phosphatidylcholine 120 mg · ml^{-1}, stearylamine 2.4 mg · ml^{-1}, bacteriorhodopsin 6 mg · ml^{-1}, potassium citrate 75 mM, pH 6.0. Additions: nigericin 0.48 μg · ml^{-1}, valinomycin 2.4 μg · ml^{-1} and 28.8 μg · ml^{-1}, S_{13} 3 μM and 60 μM. Further as described in the legend to Fig. 4.

Although the internal buffer capacity does not explicitly appear in the derived equations, its effect on the rate of decay of the initial membrane potential can easily by discussed in terms of the presented model. The effect of an increase in buffer capacity on the build up of $\Delta\mu_H$ in the state that preceeds the steady state of electroneutral flow is a straightforward effect of slowing down the rise of $\Delta\mu_H$. The $\Delta\psi$ is not affected. Even when the steady state of net electroneutral flow has been reached, there is no effect of the buffer capacity on the rate of proton uptake (cf. Eqn. 18): still the effect of buffer capacity is limited to the trivial buffering away of the normal number of protons entering the internal phase. As $(\Delta\mu_H)^*$ increases, Eqn. 15 must be applied. This equation expresses a back pressure of the $\Delta\mu_H$ on the J_H^*. If two preparations differ only in internal buffer capacity, then for the same number of protons having been pumped inwards, the one with the higher buffer capacity will have the lower $\Delta\mu_H$. Initially no difference in $\Delta\psi$ will be detected between the two preparations, so that the $\Delta\tilde{\mu}_H$ will be higher in the preparation with the lower buffer capacity. This higher $\Delta\tilde{\mu}_H$ will, however, then exert a higher back pressure on the proton pump. Meanwhile also the back leakage of protons will increase. Whilst for $\Delta\mu_H$ dissipation only proton movement is determining, in $\Delta\psi$ dissipation the movement of any ion is effective: the lower $(\Delta\tilde{\mu}_H)^*$ in highly buffered systems will lead to a higher ratio of $\Delta\psi$ to $\Delta\mu_H$ during the electroneutral steady-state phase. At the end of this phase, however, the differences between the two systems will fade away. In the steady state of zero net proton flow no effect of buffering capacity on $(\Delta\mu_H)^{**}$, $(\Delta\psi)^{**}$ and $(\Delta\tilde{\mu}_H)^{**}$ is expected (cf. Eqns. 23—25). Except for the last prediction (the appropriate steady state has not yet been reached) the other predictions can be checked to fit in (see Fig. 7).

4. The effect of light intensity and the back pressure of $\Delta\tilde{\mu}_H$

In the postulated model the effect of varying the light intensity is proposed to be fully embodied by a proportionality of L_ν with light intensity. Consequently the flow through the photochemical cycle is predicted to be proportional to the light intensity. As both the amount of bacteriorhodopsin and the duration of a photochemical cycle are limited, saturation behaviour is expected to occur at high light intensities (cf. Ref. 40). Therefore in its proportional form Eqn. 8 can only apply to light intensities below the half-saturating intensity. From the turnover time of the photochemical cycle (8 ms, see Ref. 40) it can be estimated that at half-saturation 1 einstein ($21 \cdot 10^4$ J) is absorbed every 16 ms. The absorbed power then equals $1.3 \cdot 10^7$ W/mol bacteriorhodopsin. Using the absorption coefficient of bacteriorhodopsin at 570 nm ($6.3 \cdot 10^3$ $m^2 \cdot mol^{-1}$, see Ref. 41) it can be estimated that incident 570 nm light intensity must be $2.1 \cdot 10^3$ $W \cdot m^{-2}$ for half-maximal saturation. The essential conclusion from the above calculation is that for light intensities below 15 $kW \cdot m^{-2}$ (white light), L_ν can indeed be expected to be proportional to light intensity.

Many experiments at such light intensities seem to contradict the above conclusion: ATP synthesis [40], the initial velocity of proton uptake (J_{Hi}) and the steady-state proton uptake [15] show saturation behaviour with respect to light intensity at lower light intensities. The latter two parameters can be examined in Fig. 8. In double-reciprocal plots straight lines (correlation coefficient 0.998 and 0.999, respectively) are found, indicating apparent K_m values for light of 0.50 $kW \cdot m^{-2}$ and 0.16 $kW \cdot m^{-2}$, respectively. The model tested in this paper can solve the dilemma. Eqns. 18 and 23 do predict these dependencies of J^*_{Hi}, and (if buffer capacity does not depend too much on $\Delta\mu_H$)

$$\int_0^\infty \frac{J_H}{\beta}\, dt,$$

on light intensity, if L_ν is taken proportional to the latter. The physical background of this saturation effect is the postulated inhibiting effect of $\Delta\tilde{\mu}_H$ on

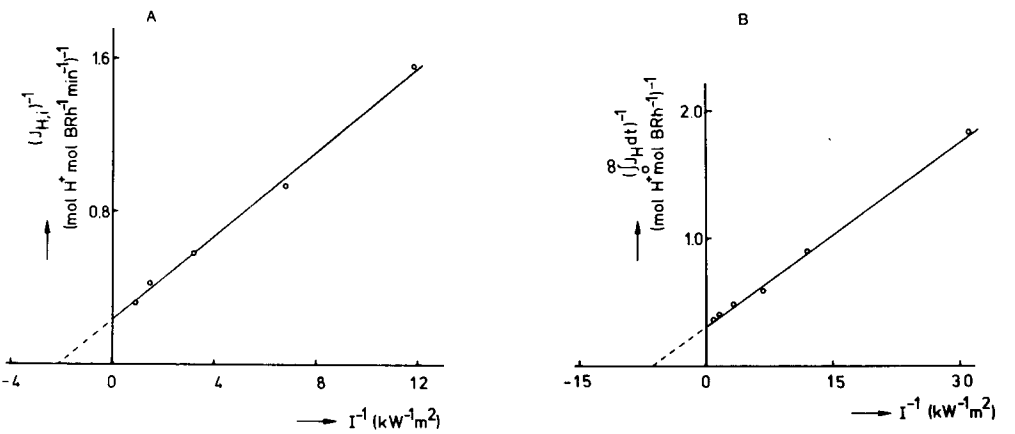

Fig. 8. The dependence of (A) the initial rate of proton uptake and (B) the steady-state proton uptake on light intensity. Reconstitution conditions: soy bean phosphatidylcholine 20 $mg \cdot ml^{-1}$, bacteriorhodopsin 1.0 $mg \cdot ml^{-1}$, sonication 150 s, pH 6.5. Light intensity was varied by changing the input voltage of a 500 W Tungsten filament lamp (Philips, Eindhoven). J_{Hi} = initial rate of protein uptake. I = light intensity. $\int_0^\infty J_H\, dt$ = the total proton uptake in the $J_H = 0$ steady state.

the flux through the photochemical cycle. For, the corresponding description in which there is no back pressure of $\Delta\tilde{\mu}_H$, yields Eqns. 18′ and 23′, which are falsified by the results presented in Fig. 8. Apart from the observation that the apparent K_m for light is lower than the calculated K_m, also the difference between the K_m observed in the initial rate experiment and the K_m found in the steady-state proton uptake measurement pleads against a hyperbolic dependence of L_ν on the light intensity at the light intensities used (cf. Eqns. 18′ and 23′). The model with $\Delta\tilde{\mu}_H$ back pressure predicts such a K_m difference. From Eqn. 18:

$$K_m((J_{Hi})^*) = \frac{L_H^1 + L_e}{n^2} \tag{41}$$

and from Eqn. 23:

$$K_m((\Delta\mu_H)^{**}) = \frac{L_H^1 + \dfrac{L_n \cdot L_e}{L_n + L_e}}{n^2} \tag{42}$$

Here n represents the number of protons pumped/absorbed photon.

In order to become independent of the theoretical considerations presented above, the inhibition by $\Delta\tilde{\mu}_H$ of the flux through the photochemical cycle was assayed in a more direct manner. Taking the kinetics of the transient presence of the 640 nm intermediate as a measure of J_ν (the flow through the photochemical cycle), it can be shown (see Fig. 9) that J_ν^{**} increases on addition of a high concentration of either protonophore, or valinomycin plus nigericin: these (combinations of) ionophores increase the rate of appearance and possibly the rate of decay of the 640 nm intermediate after an 8 μs flash. The action of the ionophores occurs via the transmembrane difference in electrochemical activity of protons rather than via an effect on the average pH: their effect is independent of the orientation of bacteriorhodopsin. As is shown in a more exact form in section 2.5. of the preceding article, these observations argue against a model without $\Delta\tilde{\mu}_H$ back pressure (Eqn. 38′), and can very well be explained by the model with $\Delta\tilde{\mu}_H$ back pressure (Eqn. 38). In this way back pressure has been demonstrated for medium pH values ranging from pH 5 to pH 7 for liposomes with either orientation. The back pressure effect in Fig. 9 may seem limited in size. One should, however, keep in mind that the steady-state proton-motive force generated by the repeated flashes will only be small (estimation less than 30 mV).

A serious competitor of the model presented was the one obeying exactly the same equations, except for the effect of light intensity. In that alternative model light intensity was embodied in A_ν (the thermodynamic force exerted by light) rather than L_ν (the proportionality constant between flow through the photochemical cycle and its driving force). A_ν, the analogue of ΔG_p in the ATPase reactions, was postulated to depend logarithmically on the concentration of photons and thus on light intensity. The most direct way to choose between this model and the one presented in section 1 of the preceding article and used until now is to measure the dependence of A_ν on light intensity.

A technically convenient system to do this is the bacteriorhodopsin-on-Millipore-filter system described in Ref. 23. Although physically the system is a

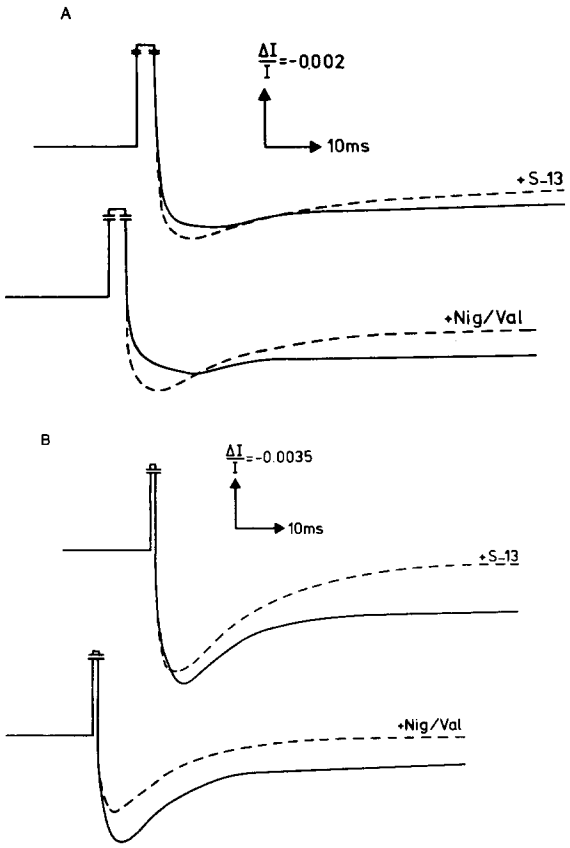

Fig. 9. The rise and fall of the 660 nm absorbance in bacteriorhodopsin liposomes in the presence and absence of uncoupling agents. The experiments were carried out as described under Materials and Methods. S_{13} (10 μM), valinomycin (1 μM) and nigericin (1 μM) were added as indicated. (A) Reconstituted vesicles that show proton uptake upon illumination. Reconstitution conditions: soy bean phospholipids 10 mg · ml^{-1}, bacteriorhodopsin 10 mg · ml^{-1}, sonication 450 s, pH 6.0. (B) Reconstituted vesicles that show proton extrusion upon illumination (cardiolipid 0.6 mg · ml^{-1}, bacteriorhodopsin 1.5 mg · ml^{-1}, sonication 30 s, pH 6.5, 18°C) were prepared according to Happe et al. [14]. The absorbance was measured as described elsewhere [63] except that the temperature was 18°C. I, 660 nm light intensity; ΔI, change in 660 nm light density.

little complicated, by means of the electrical analogue shown in Fig. 10 relations between photoeffect and countervoltage can be predicted for: (i) the ideal current source model, i.e. no feedback pressure effect of $\Delta\tilde{\mu}_H$:

$$\Delta V_m = L_\nu\,(1-2\alpha)A_\nu\, f\,(R_a, R_s, R_h \text{ and } R_p) \tag{43}$$

and (ii) the voltage source model with back pressure:

$$\Delta V_m = g(L_\nu, R_p, R_s, R_h \text{ and } R_a)\cdot[(1-2\alpha)A_\nu(1+\frac{R_p}{R_s}) - V_{md}] \tag{44}$$

Here ΔV_m represents the photopotential (the increase in measured potential caused by switching on the light); V_{md} the dark potential (countervoltage in

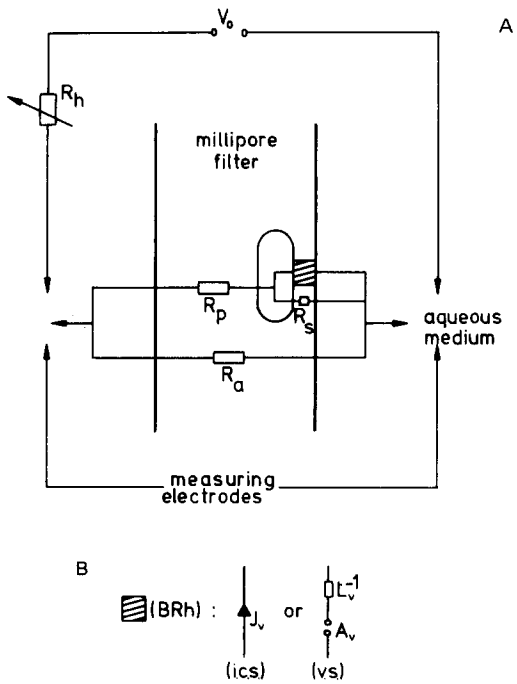

Fig. 10. (A) The electrical analogue for the bacteriorhodopsin filter system. (B) The electrical analogue for bacteriorhodopsin seen either as an ideal current source or as a voltage source with $V_{chem} = A_\nu$ and L_ν^{-1} as internal resistance. V_0, external voltage; BRh, bacteriorhodopsin; R_h, externally applied resistance; R_a, resistance across the filter less $[(1/R_p + 1/R_s)^{-1}]$; R_s, resistance between aqueous compartment and the bacteriorhodopsin-impregnated side of the filter; R_p, resistance between the aqueous compartment and the other side of the filter; ics, ideal current source; vs, voltage source; J_ν, the flow through the photochemical cycle; A_ν, thermodynamic force exerted by the photon; $L_\nu{}^{-1}$, internal resistance of bacteriorhodopsin. In this figure the stoicheiometric number n (the number of protons pumped/photon absorbed) is taken to equal 1.

the dark); R_a, R_s, R_h, R_p are defined by Fig. 10 and f and g depend only on the variables following between brackets. In the calculations it has been assumed that light acts as a 'switch' in the bacteriorhodopsin molecule: in the dark, the resistance of the source is supposed to be infinitely high. If this assumption is replaced by the assumption that A_ν becomes equal to zero in the dark, whereas the internal resistance of the voltage source remains the same, no dependence of the photoeffect on the countervoltage is expected. Fig. 11 shows representative experimental results obtained with the filter system. The important conclusions are:

(a) The photoeffect depends on the countervoltage (see also Refs. 9 and 10) and thus the ideal current source model is inappropriate (Fig. 11a).

(b) The slope of the curves depends on light intensity and therefore the effect of light intensity must at least partly be reflected by L_ν (Fig. 11b).

(c) Within the limits of experimental error the curves intersect the V_{md} axis at the same point and therefore A_ν must be independent of light intensity (Fig. 11b).

Although especially the interpretation of the results obtained in the filter

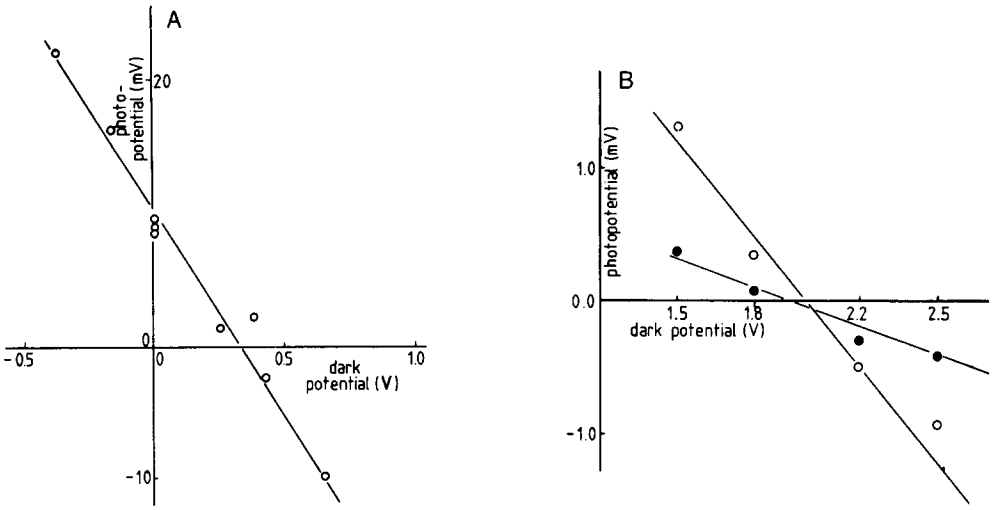

Fig. 11. Light-induced increase in electric potential across a bacteriorhodopsin-impregnated Millipore filter. The experiment was carried out as described under Materials and Methods. In experiment (A) a relatively low external resistance (10 MΩ, i.e. one-fifth of the resistance of the filter) was used. The filter in (B) had been wiped less thoroughly, which resulted in a resistance of 0.10 GΩ, equal to the applied external resistance. (A) The relation between photopotential and dark potential. The straight line is the least-squares best fit (with a correlation coefficient 0.99). (B) The effect of light intensity on this relation. Light intensities: ○, 3 W · m^{-2}; ●, 20 W · m^{-2}.

system by use of an electric analogue is not as straightforward as it may seem ($\Delta\mu_H$ may play an important role in values for $\Delta\tilde{\mu}_H$), the combination of its results with those obtained in the three other types of experiments (i.e. the light dependence of the rate of initial proton uptake, the light dependence of the steady-state total proton uptake and the effect of ionophores on the rapid flash kinetics of the 640 nm intermediate in the photochemical cycle) can be conceived as a promising initial support for the proposed model.

Discussion

In this article a description of the energetics of bacteriorhodopsin liposomes in terms of irreversible thermodynamics was developed, tested and not falsified. During the experimental stage preceding this paper the developed theory even led to predictions (initial $\Delta\psi$) that had not yet been experimentally established. When carried out the essential experiments yielded the predicted results, but not until the lipid composition of the liposomal membrane was changed. The former characteristics of the theory prove that it is a useful one [42], whereas the changing of the lipid composition rather than the rejection of the theory may show that in our hands it already functioned as a paradigm [43].

It may be useful to summarize the assumptions made:

(i) Linear proportional relations, between flows and forces, and thus a fixed stoicheiometry between J_H^{ν} and J_{ν}.

(ii) Homogeneity, i.e. the assumption that a preparation consisting of a large number of bacteriorhodopsin liposomes, which will no doubt differ from each

other in the values of many of the important parameters, can be described as one large average liposome.

(iii) An electric capacity of the liposomes low relative to the buffer capacity of the inner space.

(iv) L_v proportional to light intensity, A_v independent of light intensity.

(v) Thermodynamic back pressure of $\Delta\tilde{\mu}_H$ on J_v.

Of these assumptions only the first two have not been verified directly in this article. To the very first point three things are relevant. Firstly, the postulated linearity is to be regarded as a first-order approximation in an otherwise too complex system. Secondly, all data can be qualitatively fully explained within the frameword of this first-order approximation. Finally, though in some complicated systems relevant deviations from linearity have been found (e.g. Ref. 44), other, simpler systems obey linear (e.g. Refs. 45—47), or even proportional [48,49] and Onsager-symmetrical [46,50] relations between flows and forces. As to the second assumption one justification is that in the experiments reported in this paper the average number of bacteriorhodopsin molecules/liposome sufficiently exceeds one to ensure that relative differences in number of proton pumps/liposome are within small limits. Both on theoretical and experimental level work is to be carried out concerning the relevance of this problem.

Thus the basis of the developed theory is quite firm. In addition the theory is a useful one. The purposes it has already served, or may still serve are summarized below:

(1) The question of how the effect of light intensity should be considered. Conclusion: in L_v (the number of active pumps), but not in A_v (the force of each pump).

(2) The question whether the bacteriorhodopsin proton pump feels the $\Delta\tilde{\mu}_H$ it builds up, as a back pressure. Conclusion: it does.

(3) The simple explanation of the antagonistic effect of low concentrations of valinomycin and nigericin on ΔpH in bacteriorhodopsin liposomes, and the prediction that under these conditions $\Delta\tilde{\mu}_H$ is not altered by these ionophores. This prediction was shown to be in accordance with experimental results.

(4) The prediction of an initial $\Delta\psi$ in illuminated bacteriorhodopsin liposomes. Conclusion: it exists.

(5) The explanation of the small effect of valinomycin on the steady-state proton uptake in bacteriorhodopsin liposomes.

(6) The definition of experimental conditions to be used, when the permeability of the membrane to ions or their acid-base conjugated neutral forms are to be measured.

After this discussion of the characteristics of the theory, some extra attention should be paid to part of the results presented in this article. One such result is the small, but reproducible increase in steady-state proton uptake on addition of valinomycin (see Table I). In bacteriorhodopsin liposomes prepared in the cholate dialysis procedure [21] this increase is even higher [64]. Racker and Hinkle [8] observed a 'variable' effect of valinomycin on the steady-state proton uptake, but did not interpret these observations. In terms of the description presented above, interpretation is straightforward: Eqn. 23 in which L_e contains L_K^1, valinomycin's catalytic activity, predicts that valinomycin does

not have any effect on the steady-state proton uptake, unless L_n differs from zero. Therefore, the stimulation of steady-state proton uptake seems to indicate a significant permeability of the liposomal membrane to either HCl or KOH (or Cl^-/OH^- or K^+/H^+ exchange). Reports of passive HCl permeability through lipid bilayers have reached the literature [51—53], but also K^+/H^+ exchange activity has been postulated [54,55]; to distinguish between the two possibilities ion substitution studies are to be performed.

Another point of interest is the liability of the light-driven proton pump bacteriorhodopsin to $\Delta\tilde{\mu}_H$ back pressure. Although photosynthetic electron transport could be shown to be inhibited by a proton-motive force (for review see Ref. 56), the back pressure effect could not be located in either of the two photosystems itself. This is in line with expectations, as the chlorophyll-containing photosystems do not function as proton pumps; they rather polarize the membrane [56]. It is not until the electron carriers participate in the reaction that real proton transport from bulk phase to bulk phase occurs. Thus proton-motive force back pressure on the photoreaction in chloroplasts may not seem remarkable, being merely analogous to respiratory control in mitochondria [57]. The purity of bacteriorhodopsin isolated by the usual procedure [58] ensures us that the back pressure effect is embodied in the single polypeptide itself. Its molecular basis will lie in dependence of interconversion rates of the intermediates in the photocycle on the activities of protons on either side of the membrane. Especially those interconversions involving proton uptake or release are interesting in this respect.

Theoretically back pressure of $\Delta\tilde{\mu}_H$ on J_ν could be brought about by two effects: $\Delta\tilde{\mu}_H$ may increase the duration of the photochemical cycle, thus decrease the amount of 570 nm intermediate. Then at higher $\Delta\tilde{\mu}_H$ fewer bacteriorhodopsin would be able to absorb a photon and undergo a proton-translocating photochemical cycle. Alternatively the photochemical cycle of bacteriorhodopsin may be effectively reversible (just like the mitochondrial ATPase), the direction into which it moves being governed by statistics so that the average direction is dictated by the balance between the proton-motive force and that part of the photo energy available to the photochemical cycle. Our experiments with the bacteriorhodopsin-filter-system indicate that the $\Delta\tilde{\mu}_H$ effect is not exerted via the activity of bacteriorhodopsin (L_ν), but rather by a balance between $\Delta\tilde{\mu}_H$ and A_ν. Also the results obtained with the rapid-kinetics experiments reported in this and Hellingwerf et al. [63] exclude an effect via the 570 nm intermediate concentration only.

The remaining model of bacteriorhodopsin is that of a normal battery (through which current can flow in both directions) switched on by the absorption of a photon. The effect of $\Delta\tilde{\mu}_H$ on the photochemical cycling rate is equivalent to that of an external voltage applied to a battery with an internal resistance.

$$J = \frac{V_{chem} - V_{out}}{R_i} \tag{45}$$

parallels

$$J_H^\nu = \frac{A_\nu - \Delta\tilde{\mu}_H}{1/L_\nu} \tag{46}$$

Experiments with bacteriorhodopsin liposomes analogous to the present bacteriorhodopsin filter experiments are to be carried out to check the obtained results.

A second reason to repeat the experiments reported in Fig. 11 with bacteriorhodopsin liposomes is the quantitation of A_ν. From Fig. 11a it can already be concluded that

$$A_\nu < 0.42 \text{ V} \tag{47}$$

For simplicity of this discussion, n is taken to equal 1 [17], although the value of this stoicheiometric number is still under discussion in the literature. Similarly α is taken to equal zero. The extra compartments presumably present in the filter system do, however, not allow for the definition of more than an upper limit for A_ν. Bacteriorhodopsin liposomes may lack such extra compartments. A lower limit for A_ν was obtained in the $\Delta\tilde{\mu}_H$ measurements in this article: 0.09 V (using higher bacteriorhodopsin to lipid ratios we could recently increase this value to 0.18 V). In bacteriorhodopsin liposomes with bacterial orientation a proton-motive force of 0.13 V was measured [65]. Literature data also allow an estimate of this lower limit; 0.15 V [59,60], and a downward shift of the upper limit to 0.28 V [9].

An important conclusion can already be drawn: less than 20% of the energy of an absorbed photon can be converted into proton-motive energy. Although this fraction would be higher, if the stoicheiometry would be higher than the one proton/photon reported by Lozier et al. [17], it is comparable in size to maximum efficiencies reported in other photoenergy-conserving systems [61]. That these maximal efficiencies are so low can be easily explained, if one takes into account the following considerations: for an organism in evolution it is not of importance whether it uses the energy of a photon efficiently. The energy content of the majority of the photons emitted by the sun will always be much higher than the energy necessary to build up an adequate proton-motive force. Far more important is the frequency with which photons of the wavelength to be absorbed appear: if this frequency is high, lower pigment concentrations are needed to absorb the same number of photons and to accomplish the same number of photochemical cycles. The solar spectral irradiance, when calculated in einstein $\cdot$ m^{-2} $\cdot$ nm^{-1}, has a maximum around 590 nm, with values 90% of the maximum at 450 and 780 nm. Thus for primitive biological photoconverters with the simplest stoicheiometry of one proton/photon an absorption maximum of 590 nm (efficiency below 20%, relative irradiance 100%) rather than one around 3000 nm (efficiency approximately five times higher, relative irradiance 9%) will be most favorable. That not all photonenergy (2.18 V) occurs in the A_ν term may serve two related purposes. Firstly it will be useful to do away with the 80% energy dissipation in controlled manner, i.e. not via a high-energy proton. Secondarly a lower value of A_ν will subject the proton pump to a back pressure control by the proton-motive force; thus the proton-motive force and the linked [62] phosphorylation potential will be controlled.

We conclude that a model based on linear irreversible thermodynamics has served the purposes of explanation of earlier data, prediction of yet unknown phenomena, and a framework for clear theoretical and experimental questions.

Acknowledgements

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References

1 Bridgen, J. and Walker, I.D. (1976) Biochemistry 15, 792–798
2 Ovchinnikov, Y.A., Abdulaev, N.G., Feigina, M.Y., Kiselev, A.V. and Lobanov, N.A. (1977) FEBS Lett. 84, 1–4
3 Henderson, R. and Unwin, P.N.T. (1975) Nature 257, 28–32
4 Henderson, R. (1977) Annu. Rev. Biophys. Bioeng. 6, 87–109
5 Stoeckenius, W. and Lozier, R.H. (1974) J. Supramol. Struct. 2, 76–79
6 Racker, E. (1973) Biochem. Biophys. Res. Commun. 55, 224–230
7 Kayushin, L.P. and Skulachev, V.P. (1974) FEBS Lett. 39, 39–42
8 Racker, E. and Hinkle, P.C. (1974) J. Membrane Biol. 17, 181–188
9 Drachev, L.A., Jasaitis, A.A., Kaulen, A.D., Kondrashin, A.A., Liberman, E.A., Nemecek, I.B., Ostroumov, S.A., Semenov, A.Yu. and Skulachev, V.P. (1974) Nature 249, 321–324
10 Shieh, P. and Packer, L. (1976) Biochem. Biophys. Res. Commun. 71, 603–609
11 Herrmann, T.R. and Rayfield, G.W. (1976) Biochim. Biophys. Acta 443, 623–628
12 Dancsházy, Z. and Karvaly, B. (1976) FEBS Lett. 72, 136–138
13 Racker, E. and Stoeckenius, W. (1974) J. Biol. Chem. 249, 662–663
14 Happe, M., Teather, R.M., Overath, P., Knobling, A. and Oesterhelt, D. (1977) Biochim. Biophys. Acta 465, 415–420
15 Hwang, S.-B. and Stoeckenius, W. (1977) J. Membrane Biol. 33, 325–350
16 Happe, M. and Overath, P. (1976) Biochem. Biophys. Res. Commun. 72, 1504–1511
17 Lozier, R.H., Niederberger, W., Bogomolni, R.A., Hwang, S.-B. and Stoeckenius, W. (1976) Biochim. Biophys. Acta 440, 545–556
18 Eisenbach, M., Bakker, E.P., Korenstein, R. and Caplan, S.R. (1976) FEBS Lett. 71, 228–232
19 Bakker, E.P., Eisenbach, M., Garty, H., Pasternak, C. and Caplan, S.R. (1977) in Proc. of the ICN-UNCL Symposium on Molecular Aspects of Membrane Transport, in the press
20 Oesterhelt, D. and Stoeckenius, W. (1974) Methods Enzymol. 31, 667–678
21 Miller, C. and Racker, E. (1976) J. Membrane Biol. 26, 319–333
22 Pangborn, M.C. (1951) J. Biol. Chem. 188, 471–476
23 Blok, M.C., Hellingwerf, K.J. and Van Dam, K. (1977) FEBS Lett. 76, 45–50
24 Fiolet, J.W.T., Bakker, E.P. and Van Dam, K. (1974) Biochim. Biophys. Acta 368, 432–445
25 Mueller, P., Rudin, D.O., TiTien, H. and Wescott, W.C. (1964) in Recent Progress in Surface Science (Danielli, J.F., Pankhurst, K.G.A. and Riddiford, A.C., eds.), Vol. 1, pp. 379–393, Academic Press, New York
26 Colowick, S.P. and Womack, F.C. (1969) J. Biol. Chem. 244, 774–777
27 Ramos, S., Schuldiner, S. and Kaback, H.R. (1976) Proc. Natl. Acad. Sci. U.S. 73, 1892–1896
28 Ramos, S. and Kaback, H.R. (1977) Biochemistry 16, 848–854
29 Skulachev, V.P. (1971) Curr. Top. Bioenerg. 4, 127–190
30 Montal, M. (1976) Annu. Rev. Biophys. Bioeng. 5, 119–175
31 Kraayenhof, R. (1970) FEBS Lett. 6, 161–165
32 Fiolet, J.W.T. (1975) A Glimpse of the Chloroplast Energized State, pp. 64–70, Ph.D. thesis, University of Amsterdam, Mondeel, Amsterdam
33 Rottenberg, H. and Lee, C.-P. (1975) Biochemistry 14, 2675–2680
34 Singh, A.P. and Bragg, P.D. (1976) Eur. J. Biochem. 67, 177–186
35 Singh, A.P. and Bragg, P.D. (1977) Biochim. Biophys. Acta 464, 562–570
36 Ramos, S. and Kaback, H.R. (1977) Biochemistry 16, 854–859
37 Schuldiner, S., Rottenberg, H. and Avron, M. (1972) Eur. J. Biochem. 25, 64–70
38 Gromet-Elhanan, Z. and Leiser, M. (1978) in The Proton and Calcium Pumps (Azzone, G.F., Avron, M., Metcalfe, J.C., Quagliariello, E. and Siliprandi, N., eds.), pp. 81–91, Elsevier, Amsterdam

39 Toro, M., Gómez-Lojero, C., Montal, M. and Estrada-O, S. (1976) J. Bioenerg. 8, 19—26
40 Oesterhelt, D., Hartmann, R., Fischer, U., Michel, H. and Schreckenbach, Th. (1975) in Proc. of the 10th FEBS Meeting (Desnuelle, P. and Michelson, A.M., eds.), Vol. 40, Enzymes: Electron Transport Systems, pp. 239—251, North-Holland Amsterdam
41 Oesterhelt, D. and Hess, B. (1973) Eur. J. Biochem. 37, 316—326
42 Popper, K.R. (1969) Conjectures and Refutations, Routledge and Kegan Paul, London
43 Kuhn, Th.S. (1970) The Structure of Scientific Revolutions, 2nd edn., The University of Chicago Press, Chicago
44 Michel, B.E. (1977) Plant Physiol. 60, 259—264
45 Padan, E. and Rottenberg, H. (1973) Eur. J. Biochem. 40, 431—437
46 Rottenberg, H. (1973) Biophys. J. 13, 503—511
47 Van der Meer, R., Akerboom, T.P.M., Groen, A.K. and Tager, J.M. (1978) Eur. J. Biochem. 84, 421—428
48 Nicholls, D.G. (1977) Eur. J. Biochem. 77, 349—356
49 Nicholls, D.G. (1974) Eur. J. Biochem. 50, 305—315
50 Dunlop, P.J. and Gosting, L.J. (1959) J. Phys. Chem. 63, 86—93
51 Hauser, H., Oldani, D. and Phillips, M.C. (1973) Biochemistry 12, 4507—4517
52 Nicholls, P. and Miller, N. (1974) Biochim. Biophys. Acta 356, 184—198
53 Robertson, R.N. and Thompson, T.E. (1977) FEBS Lett. 76, 16—19
54 Azzone, G.F., Massari, S. and Pozzan, T. (1976) Biochim. Biophys. Acta 423, 27—41
55 Pozzan, T. and Azzone, G.F. (1976) FEBS Lett. 71, 62—66
56 Hauska, G. and Trebst, A. (1977) in Current Topics in Bioenergetics (Sanadi, D.R., ed.), Vol. 6, pp. 151—220, Academic Press, New York
57 Lardy, H.A. and Wellman, H. (1952) J. Biol. Chem. 195, 215—224
58 Oesterhelt, D. and Stoeckenius, W. (1971) Nat. New Biol. 233, 149—152
59 Bakker, E.P., Rottenberg, H. and Caplan, S.R. (1976) Biochim. Biophys. Acta 440, 557—572
60 Michel, H. and Oesterhelt, D. (1976) FEBS Lett. 65, 175—178
61 Lien, S. and San Pietro, A. (1975) in NSF/RANN Report, An Inquiry into Biophotolysis of Water to Produce Hydrogen, pp. 1—8, Indiana University, Bloomington
62 Danon, A. and Stoeckenius, W. (1974) Proc. Natl. Acad. Sci. U.S. 71, 1234—1238
63 Hellingwerf, K.J., Schuurmans, J.J. and Westerhoff, H.V. (1978) FEBS Lett. 92, 181—186
64 Hellingwerf, K.J., Scholte, B.J. and van Dam, K. (1978) Biochim. Biophys. Acta 513, 66—77
65 Hellingwerf, K.J., Tegelaers, F.P.W., Westerhoff, H.V., Arents, J.C. and van Dam, K. (1978) in Energetics and Structure of Halophilic Microorganisms (Caplan, S.R. and Ginzburg, M., eds.), pp. 283—290, Elsevier/North-Holland Biomedical Press, Amsterdam