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THE EFFECT OF IONOPHORES AND LIGHT INTENSITY ON THE INITIAL RATE OF PROTON UPTAKE INTO BACTERIORHODOPSIN LIPOSOMES CAN BE QUANTITATIVELY DESCRIBED BY MOSAIC NON-EQUILIBRIUM THERMODYNAMICS

H.V. WESTERHOFF, J.C. ARENTS and K.J. HELLINGWERF *

Laboratory of Biochemistry, B.C.P. Jansen Institute, University of Amsterdam, Plantage Muidergracht 12, 1018 TV Amsterdam (The Netherlands)

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The initial rate of light-induced proton uptake into bacteriorhodopsin liposomes was analyzed for its quantitative dependence on system parameters. All relevant parameters were varied: the proton pump activity of bacteriorhodopsin (by varying the light intensity), the electrophoretic proton permeability of the liposomal membrane (by adding protonophore), the electroneutral proton permeability (by adding nigericin) and the non-proton electric permeability of the membrane (by adding valinomycin). The results are in strict, quantitative agreement with the predictions of a theoretical description of bacteriorhodopsin liposomes based on mosaic non-equilibrium thermodynamics (Westerhoff, H.V., Scholte, B.J. and Hellingwerf, K.J. (1979), Biochim. Biophys. Acta 547, 544–560). Furthermore, the analysis showed that bacteriorhodopsin is inhibited by the membrane potential it develops.

Introduction

Thanks to the concept and experiments of Mitchell [1] and many others, it is now clear that protons play an important role in energy transduction in biological systems [2]. Two important aspects remain unsolved: is the bulk phase-to-bulk phase difference in the proton electrochemical potential the only high-energy intermediate in processes such as oxidative and photosynthetic phosphorylation [3–7]? And: what is the quantitative impact of the chemiosmotic processes on the cellular metabolism? Both questions should be tackled by quantitative analysis.

Early quantitative approaches to membrane-mediated biological energy conversions were only of limited use, because they made use either of equilibrium equations for a non-equilibrium system [8], or of irreversible thermodynamic equations that are devoid of mechanistic parameters [9–11]. The former type of theory has never been developed far enough to include the effect of the main coupling agent, the proton. The latter theory can describe processes in terms of rather abstract parameters, such as the degree of coupling, q, but its potential help in elucidating a mechanistic problem is limited. Quite early, different authors [12,13] set out to try to find the mechanistic meaning of these abstract parameters. The approach that resulted (for a review see Ref. 14) consisted in first describing the elemental processes in terms of the forces that drive them, the flows that result and the mechanistic coefficients that relate the two types of parameters. Using the appropriate steady-state conditions, the large set of elemental equations can be reduced to a much smaller set of equations relating the experimentally determinable output and input flows to the output and input forces [15,16]. In contrast to the

* Present address: Department of Microbiology, Biological Centre, University of Groningen, 9751 NN Haren, The Netherlands.

equations of the above-mentioned type of irreversible thermodynamics [11], the proportionality coefficients in these equations are explicit functions of the 'mechanistic' parameters that define the elemental processes [15,17,18]. After and in addition to other applications to experimental systems (e.g., Ref. 19), this method was recently elaborated for oxidative phosphorylation [17,18,20,21].

Theoretical [22,23] and experimental [24] studies showed that the form of the elemental flow-force relationships may vary depending on the type of process considered and its degree of kinetic reversibility [23,25]. The latter aspect led Westerhoff and Van Dam [25] to rename the method as 'mosaic non-equilibrium thermodynamics'.

In order to allow optimal testing of the practical correctness and power of the mosaic non-equilibrium thermodynamic method, it was also elaborated [26] for the well defined system of purified bacteriorhodopsin (the light-driven proton pump [27]; for reviews see Refs. 28 and 29) reconstituted with pure lipids. A first semi-quantitative look at the quality of the predictions of the theory for the latter system [30] gave confirmatory results as well as interesting conclusions about bacteriorhodopsin itself. This paper shows that the theory quantitatively predicts the (combined) effects of light intensity and ionophores on the initial rate of light-induced proton uptake into bacteriorhodopsin liposomes.

Materials and Methods

Bacteriorhodopsin liposomes were prepared as described [30] by sonication for 60 periods of 15 s (each time followed by 45 s silence, at room temperature). Light adaptation was induced by a series of 10 periods of 15 s illumination followed by 15 s darkness. In control experiments it was checked that after such a pretreatment the initial rate of proton uptake could be measured reproducibly. The initial rate of proton uptake is expressed either in μmol/min per g bacteriorhodopsin or in mol H^+/mol bacteriorhodopsin per s.

Proton uptake into the bacteriorhodopsin vesicles was assayed either with the pH meter described by Hellingwerf et al. [30] (pH meter 1), or in a 1 ml vessel, equipped with a magnetic stirrer (pH meter 2). In the latter case, illumination was carried out with a 150 W, 20 V xenon lamp (Osram), equipped with two heat filters and using a flexible light guide. Light intensity was varied with neutral density filters (Oriel Corporation, Stanford, CT). Light intensities were at 100%: 0.65 and 0.17 kW/m^2 for pH meters 1 and 2, respectively. The number of protons corresponding to a measured pH shift was determined by adding known amounts of oxalic acid, essentially before ionophores had been added. Routinely, ethanol concentrations were kept below 1% (v/v). At least up to this concentration ethanol by itself had no significant effect on the initial rate of proton uptake.

L-α-Phosphatidylcholine (type V-e from egg yolk, or from soybean, 100 g/l in chloroform/methanol, 9 : 1, v/v) was purchased from Sigma Chemical Co. Nigericin and valinomycin were gifts from Dr. W.C. Pettinga, Eli Lilly and Co., Indianapolis; chlorpromazine was purchased from Serva, Heidelberg. All other reagents were of analytical grade. Twice-distilled water was used.

Results

Although the quantitative description of ion movement in bacteriorhodopsin liposomes should in principle be capable of describing the ion fluxes under all conditions, two conditions are easily accessible to experimental analysis. These are the conditions in which the light-driven pH gradient has come to a steady-state value and the condition in which the pH gradient has not yet been built up (the condition occurring just after the light has been switched on). Equations describing the steady-state pH gradient as a function of light intensity and the activity of different ionophores have been developed and tested [31]. The present article will concentrate on the latter condition: the effects are analyzed of light intensity and ionophores on the initial rate of proton uptake occurring during the first 30 s [26] after the light has been switched on. Generally, during this period the proton flux was essentially monophasic and almost constant. When valinomycin was added, proton influx became more rapid, remained monophasic, but decreased already significantly during the first 30 s. In those cases tangents were drawn to the initial parts of the proton influx trace. Again, for as many as 0.5 protons per bacterio-

rhodopsin molecule, no significant difference between tangent and actual curve was visible. This apparent monophasicity of proton movement is in contrast to observations by Caplan and coworkers (see, for example, Ref. 32), who interpreted their observation of biphasicity as being the result of the presence of scalar Bohr protons in addition to the vectorial protons [33] (for a review see Ref. 29; cf. Ref. 28). Recently, however, Govindjee et al. [34], using a preparation similar to ours, also found no evidence for the occurrence of such scalar protons (see also Ref. 28). Possibly the occurrence of scalar protons is linked to the degree of purity of the bacteriorhodopsin and lipids used or the degree of trimerization of the protein.

The appropriate equation has already been derived [26]:

$$J_{H,i} = \frac{n(1-2\alpha)L_\nu A_\nu}{1+(n^2L_\nu + L_H^1)/L_e} \quad (1)$$

describing the dependence of the initial rate of proton uptake ($-J_{H,i}$) on light intensity (L_ν), the proton permeability of the membrane (L_H^1), the membrane conductance for cations and anions (L_e), the number of protons pumped per cycle (n) and the size of the fraction of bacteriorhodopsin that pumps its protons to the inside of the vesicle (α). A_ν represents the (constant) energetic impact of the absorbed photon. Only because their exact values are not yet known with certainty (for the value of n, see Refs. 34 and 35; for that of α, see Ref. 36), and because their exact value is irrelevant to the purpose of this article (cf. Eqn. 1), we shall eliminate n and α from the equations by making both equal to 1.

The effect of an increase in electric (non-proton) conductance

Since bacteriorhodopsin pumps protons only [27,37], the onset of illumination does not only cause a pH gradient across the liposomal membrane, but generally also a membrane potential [27]. Similarly to other bioenergetic systems [38], the electric capacity of the liposomal membrane can be estimated to be less than the internal buffer capacity [26]. The result is that the membrane potential increases more rapidly than the pH gradient [30]. It can even be estimated that already after a few seconds a significant membrane potential can develop which can give rise both to proton back-leakage and 'product inhibition' ('back pressure') of the proton pump itself [30,39]. Such initial membrane potentials can be measured [30]. Their relevance can be demonstrated by determining the effect of an increased electric conductance of the membrane on the initial rate of proton uptake. Thus, valinomycin, the K^+-specific ionophore, has been shown to increase the initial rate of proton uptake in systems that contain K^+ [40]. Eqn. 1 states what the exact effect of valinomycin on the initial rate of proton uptake would look like. To stress this we rewrite the equation as follows:

$$\frac{A_\nu}{-J_{H,i}} = \frac{1}{L_e}\left(1+\frac{L_H^1}{L_\nu}\right)+\frac{1}{L_\nu}. \quad (2)$$

According to this description, the inverse of the rate of proton uptake should depend linearly on the inverse of the electric (non-proton) conductance of the liposomal membrane. Fig. 1 shows what the effect of valinomycin on the initial rate of proton uptake looks like experimentally. At first sight the correspondence between theory and experimental practice seems poor: what could have been a linear relationship between 1/initial rate and 1/[valinomycin] lies within a range of valinomycin concentrations where valinomycin inhibits the initial rate of proton uptake (the high concentrations) and a range in which valinomycin has hardly any effect on the initial rate of proton uptake (the lowest concentrations). However, both non-linear parts were expected: at high concentrations valinomycin does not only act as an ionophore, but it also directly inhibits the turnover of bacteriorhodopsin [41], thereby decreasing L_ν. The valinomycin/bacteriorhodopsin ratio at which this inhibition starts (i.e., 0.09, cf. Fig. 1) is similar to the ratio at which valinomycin begins to be inhibitory to the kinetics of the photocycle [41]. We verified this explanation by looking at the effect of such valinomycin/bacteriorhodopsin ratios on the steady state of proton uptake (not shown).

The other concentration range in which the experiment seems to contradict the theoretical prediction is at low valinomycin concentrations. Here the electric conductance of the liposomal membrane induced by the added valinomycin is still of the same order of magnitude as the endogenous

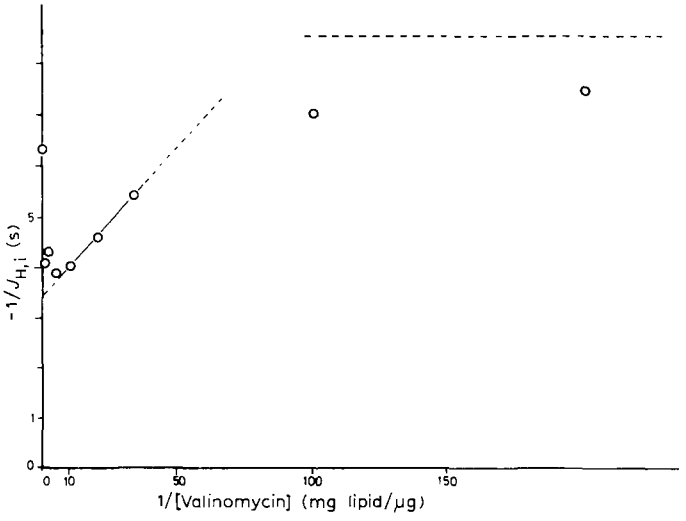

Fig. 1. The effect of valinomycin on the initial rate of proton uptake. Bacteriorhodopsin liposomes (1.0 g/l bacteriorhodopsin, 10 g/l soybean phosphatidylcholine, 150 mM KCl) were prepared as indicated in Materials and Methods. 1.0 ml of this preparation was added to pH meter 2. Its pH was brought from 5.4 to 6.5, the sample was then preincubated until there was no further pH change. Short periods of illumination (with 63% light intensity) were alternated with dark periods long enough to allow for full proton reappearance into the medium. During the dark periods valinomycin was added to reach the concentrations indicated. The horizontal dashed line indicates 1/initial rate of proton uptake at zero valinomycin.

electric conductance of the liposomal membrane: the term L_e in Eqn. 2 is only linearly related, and is not proportional, to the valinomycin concentration:

$$L_e(\text{val}) = L_e(0) + \frac{dL_e}{d[\text{val}]} \times [\text{val}] \,. \qquad (3)$$

That electric conductance is linear with the valinomycin concentration in this system at the concentrations used, i.e., that $\delta L_e/\delta[\text{val}]$ is a constant, has been verified by inspection of its influence on the rate of proton efflux from bacteriorhodopsin liposomes (acid inside) at a certain pH gradient (not shown). If we substitute this expression (Eqn. 3) for L_e in Eqn. 2, we obtain:

$$\frac{A_\nu}{-J_{H,i}} = \frac{1}{L_e(0) + dL_e/d[\text{val}] \times [\text{val}]}\left(1 + \frac{L_H^l}{L_\nu}\right) + \frac{1}{L_\nu} \,. \qquad (4)$$

This equation is in full accordance with the right-hand part of Fig. 1. To illustrate the importance of the already existing electric conductance of the liposomal membrane for the valinomycin effect on the initial rate of proton uptake, it may be of use to confine the discussion to low values of total electric conductance as compared to the light intensity, L_ν, plus the proton conductance, L_H^l. Eqn. 2 can be rewritten as:

$$\left(\frac{-J_{H,i}}{A_\nu}\right) = L_e \Big/ \left(1 + \frac{L_H^l}{L_\nu}\right) \qquad L_e << L_\nu + L_H^l \,. \qquad (5)$$

At low electric conductance, the initial rate of proton uptake should be proportional to L_e and therefore linear to the concentration of valinomycin. Fig. 2A shows plots of the initial rate of proton uptake vs. valinomycin concentration. At low valinomycin concentrations the plots are indeed linear. They are, however, not proportional: at zero valinomycin concentration there is already some electric conductance of the membrane which by this way of plotting can be estimated from the abscissa intersection point. It is equivalent to about 0.05 μg valinomycin per mg lipid.

Fig. 2A is also relevant to the question as to whether part of the proton flux observed may have been due to the rapid association of scalar Bohr protons to bacteriorhodopsin (for a review see Ref. 29; cf. Ref. 28). The rate of disappearance from the medium of the rapid protons (interpreted as scalar Bohr protons by Caplan and coworkers, cf. Ref.

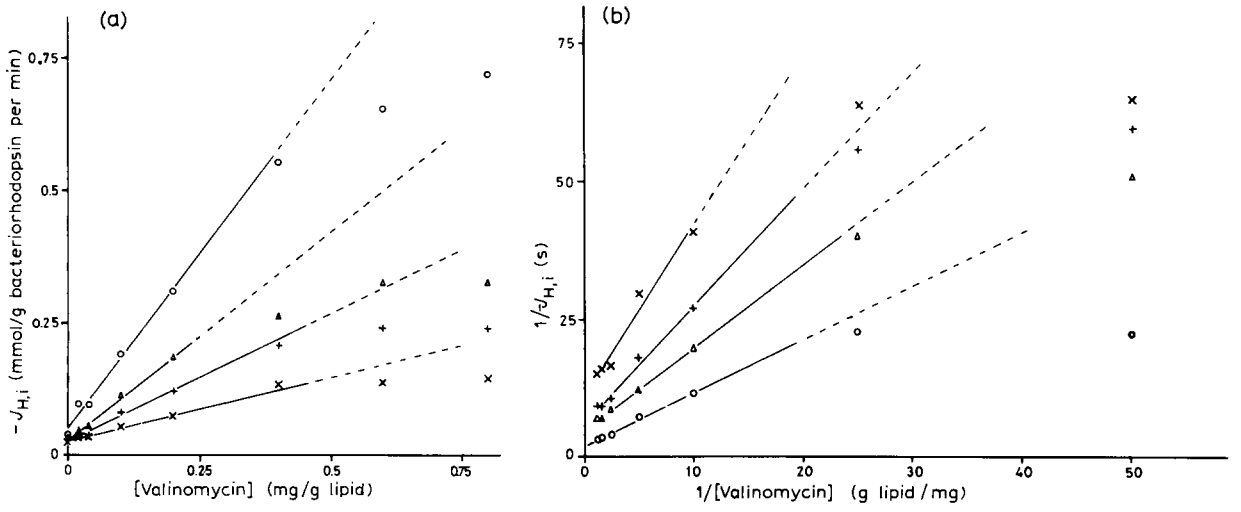

Fig. 2. The effect of valinomycin on the initial rate of proton uptake at different light intensities. 0.1 ml of bacteriorhodopsin liposomes (25 g/l egg phosphatidylcholine, 0.5 g/l stearylamine, 2.5 g/l bacteriorhodopsin, 250 mM K_2SO_4, 1 mM EDTA (potassium salt), pH 5.8), sonicated for 40 periods of 15 (+45) s each, was added to pH meter 1 and supplemented with 3.8 ml of 0.25 M K_2SO_4. Light intensities were (X) 15%, (+) 25%, (△) 50%, (○) 100%. Total ethanol concentration was always below 0.15% (v/v). A and B, normal and double reciprocal plots, respectively, of the same results. A, linear least-squares lines were drawn using the five points with the lowest valinomycin concentration at any light intensity. Correlation coefficients were 0.995, 0.991, 0.998 and 0.992, respectively. B, linear least-square lines were drawn using the five points with the highest valinomycin concentrations. Correlation coefficients were 0.984, 0.994, 0.999 and 0.997, respectively.

29) has been shown not to be influenced by valinomycin [42]. The proton influx measured by us can be stimulated approx. 15-fold by the addition of excess valinomycin (Fig. 2A). (The potential stimulation by agents that dissipate membrane potentials is even larger, in view of the inhibitory effect of valinomycin on bacteriorhodopsin.) This again indicates that the proton 'flux' we measure cannot consist, to a significant extent, of rapidly associating scalar Bohr protons.

The experiment of Fig. 2 was carried out for an additional purpose, i.e., to examine in more detail what variations in light intensity would do to the effect of valinomycin on the initial rate of proton uptake. Eqns. 2 and 5 imply that, independent of light intensity, at low valinomycin concentrations the initial rate of proton uptake should be linear with the valinomycin concentration, whereas at high (but not yet inhibitory) valinomycin concentrations the inverses of the two parameters should be linearly interrelated. Moreover, in the double-reciprocal plot (Fig. 2B), an increase in light intensity (L_p) should decrease the slope, whereas the reverse should occur in the straightforward plot (Fig. 2A). Fig. 2 confirms these theoretical predictions.

It is useful to look at Fig. 2B in some more detail in view of the earlier contention that bacteriorhodopsin is inhibited by the proton-motive force it develops [30,39]. One of the important arguments for this contention was that both the initial rate of proton uptake and the steady-state pH gradient show saturation behaviour with respect to the intensity of the illumination. For the former of these two parameters this is confirmed by the experiment: replotting of the data shows that there is a hyperbolic relationship between initial rate of proton uptake and light intensity (not shown). An important question is whether the observed saturation effects could also be due to saturation of the photochemical cycle itself, independent of a proton-motive force. It was estimated that the light intensity required for half-saturation is much lower than that expected to saturate a photochemical cycle with 8 ms duration [30]. The evidence would, however, be more conclusive if the saturation effect could be shown actually to depend on the presence of the proton-

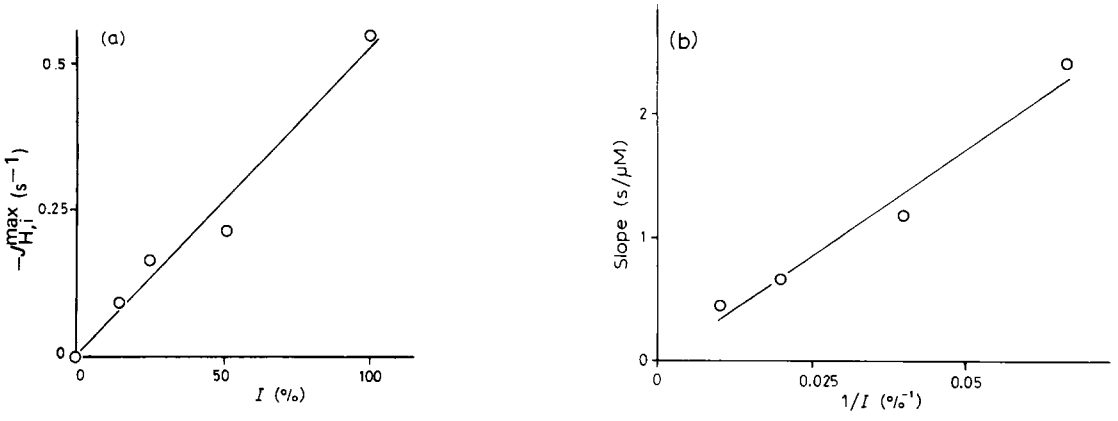

Fig. 3. Detailed analysis of Fig. 2B. (A) The initial rate of proton uptake at infinite valinomycin concentration as a function of light intensity. Extrapolation was carried out in Fig. 2B. (B) The slope in Fig. 2B as a function of light intensity. Correlation coefficients of the linear least-squares lines shown are 0.987 and 0.997, respectively.

motive force. Eqn. 2 and Fig. 2B allow us to extrapolate to a situation where there can be no more back-pressure effect of a proton-motive force on bacteriorhodopsin: the extrapolation to infinite valinomycin concentration (zero $1/L_e$). The membrane potential will then disappear and, since initial rates are measured, the pH gradient will equal zero as well. Considering the points at zero $1/L_e$ in Fig. 2B, we can conclude that in the absence of a proton-motive force an increase in light intensity can still induce an increase in the initial rate of proton uptake. Fig. 3A shows that this increase is even linear. We conclude that at light intensities where the saturation effects are observed, the photochemical cycle itself is still far from saturation and that the observed saturation depends on the presence of the proton-motive force, in this case the membrane potential.

Further analysis of Fig. 2B shows that the slope in this figure is indeed linear with the inverse of the light intensity (Fig. 3B, cf. Eqn. 5). The half-saturating valinomycin concentration should (cf. Eqn. 2) increase linearly with light intensity. The low accuracy with which these K_m values can be determined in Fig. 2B limits the confirmation.

The effect of nigericin on the initial rate of proton uptake

Our mosaic thermodynamic description [26] also took into account the effect of electroneutral proton permeation (either as symport with an anion, or as

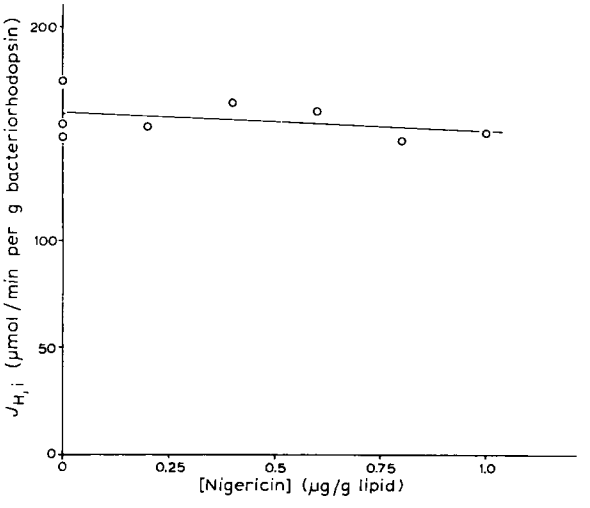

Fig. 4. The effect of nigericin on the initial rate of proton uptake. Exactly as the experiment of Fig. 2, except that 0.10 mg valinomycin/g lipid was continuously present. The amounts of nigericin indicated were present due to addition during the dark periods. At 8 and 16 μg/g lipid, initial rates of proton uptake of 95 and 52 μmol/g bacteriorhodopsin per min were measured. At these high concentrations the extent of proton uptake was, however, too low to consider these measurements as initial-rate measurements. In an experiment with a similar preparation, 0.8 μg/g lipid of nigericin reduced the steady-state proton uptake from 0.17 to 0.107 mmol H^+/g bacteriorhodopsin. The straight line shown is a linear least-squares fit with slope −8 (±9 S.D.) μmol H^+ × g lipid/g bacteriorhodopsin/min per μg nigericin, and correlation coefficient 0.35.

antiport to a cation) on the energetic and flux parameters. These processes could be represented by one parameter, L_n, a conductivity coefficient typically increased by an ionophore like nigericin [43]. In Eqn. 1 this coefficient is absent and therefore the initial rate of proton uptake is expected to be independent of the electroneutral proton conductivity of the liposomal membrane. Nigericin was indeed used to vary L_n. Fig. 4 shows that at concentrations where nigericin already decreases the steady-state pH gradient by more than 40% [30], its effect on the initial rate of proton uptake is still insignificant. However, at much higher nigericin concentrations the initial rate of proton uptake seems to be diminished by the addition of nigericin. Truly initial rates of proton uptake, however, can no longer be measured at these high nigericin concentrations. Even the very low pH gradient that exists shortly after the onset of illumination may then cause a significant back-flow of protons via nigericin, induced by ΔpH. An alternative explanation is the reported property of nigericin at high concentrations to act, by dimerization, as a K^+, and probably also as an H^+, carrier [44], rather than as an H^+/K^+ exchange catalyst.

The effect of increased proton permeability of the liposomal membrane

Since an increase in proton permeability decreases the initial rate of proton uptake to a magnitude that is only detectable with much scattering in the points, we decided to increase first the initial rate of proton permeability by increasing the K^+ permeability of the membrane. To this purpose valinomycin was added in the experiments to be discussed below. Since it has been shown [45] that valinomycin may interact directly with anionic protonophores, we chose a cationic protonophore, chlorpromazine [46], to vary the proton permeability of the membrane. Fig. 5 shows the relationship between the initial rate of proton uptake and the concentration of chlorpromazine. Eqn. 1 rewritten in the form of Eqn. 6:

$$\frac{A_\nu}{-J_{H,i}} = \frac{L_H^l}{L_\nu L_e} + \frac{1}{L_\nu} + \frac{1}{L_e} \qquad (6)$$

tells us that the relationship between the inverse of the initial rate of proton uptake and the chlorpromazine concentration (proton permeability) should be linear. This is confirmed, as can be concluded from the quality of the fit of the linear least-squares lines drawn. We may also check that the slope and the $1/(J_{H,i})$-axis intercept are indeed decreased by an increase in light intensity.

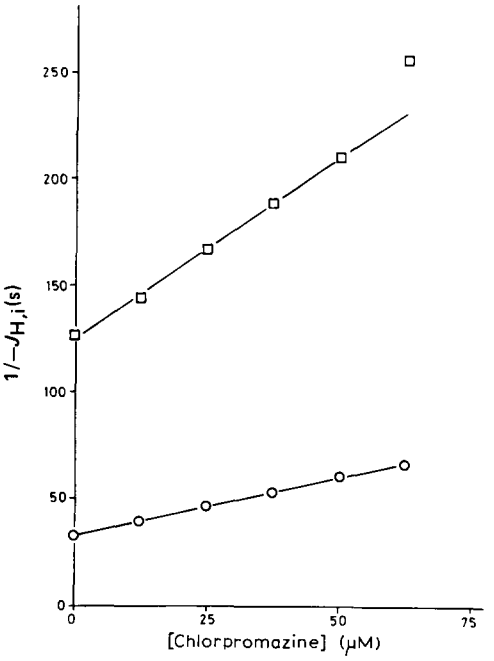

Fig. 5. The effect of the protonophore chlorpromazine on the initial rate of proton uptake. 0.1 ml of the bacteriorhodopsin liposomes of Figs. 2 and 4 was incubated in pH meter 2 and supplemented with 0.70 ml of 250 mM K_2SO_4 and 0.20 mg valinomycin per g lipid. Light intensities were (□) 25% and (○) 100%. Correlation coefficients of the linear least-squares fits shown are 0.999 (0.986 if the point with the highest chlorpromazine would have been included) and 1.000, respectively.

To examine the combined effects of light intensity and proton permeability on the initial rate of proton uptake in more detail, essentially the same experiment was carried out at four different light intensities. As Eqn. 7 shows:

$$\frac{A_\nu}{-J_{H,i}} = \frac{1}{L_\nu}\left(1 + \frac{L_H^l}{L_e}\right) + \frac{1}{L_e} \qquad (7)$$

the inverse of the initial rate of proton uptake should be linear not only with proton permeability (at constant light intensity), but also with the inverse of light intensity (L_ν). This was already confirmed in an earlier article for the case where a protonophore

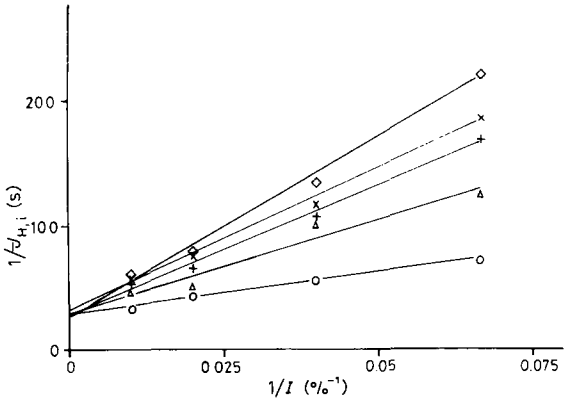

Fig. 6. The effect of light intensity on the initial rate of proton uptake at different concentrations of protonophore. Experiment as in Fig. 2, except that 30 μg of valinomycin per g lipid were continuously present. Chlorpromazine: (○) 0, (△) 15 μM, (+) 30 μM, (X) 45 μM, (◇) 60 μM. Correlation coefficients of the linear least-squares fits are 0.997, 0.975, 0.996, 0.998 and 0.997, respectively.

was absent [30]. Fig. 6 shows that this linearity is essentially still present when protonophore has been added.

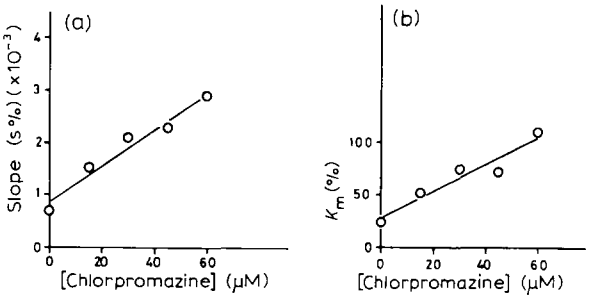

Fig. 7. Detailed analysis of Fig. 6. (A) Its slope vs. protonophore concentration. (B) The half-saturating light intensity as a function of protonophore concentration. Correlation coefficients 0.98 and 0.96, respectively.

Fig. 6 can be analyzed in more detail. Eqn. 7 predicts that the $1/(J_{H,i})$-axis intercept is independent of the proton permeability of the membrane. Fig. 6 confirms this within the limit of experimental error. Eqn. 7 also predicts that the slope in Fig. 6 will increase linearly with proton permeability of the membrane. Moreover, also the half-saturating light intensity 'K_m for light' should bear such a relationship to the proton permeability. Although

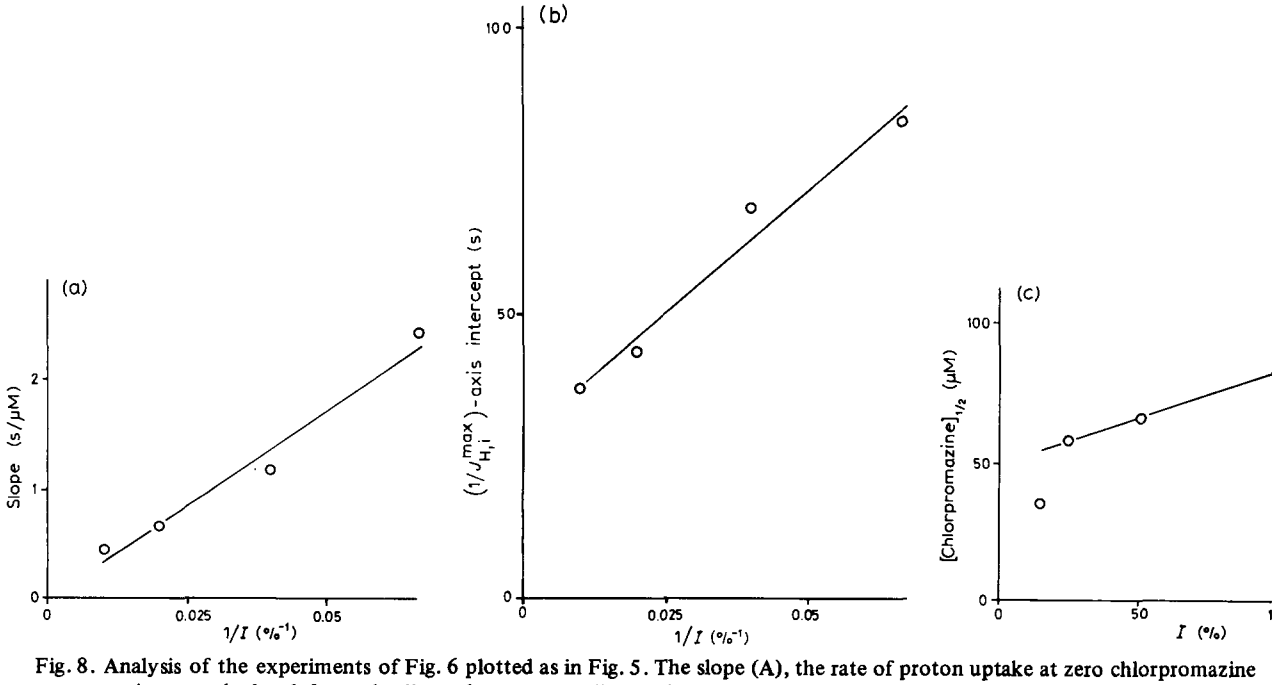

Fig. 8. Analysis of the experiments of Fig. 6 plotted as in Fig. 5. The slope (A), the rate of proton uptake at zero chlorpromazine concentration as calculated from the linear least-squares lines (B), and the concentration of chlorpromazine that reduces the initial rate of proton uptake by 50% (C), as a function of light intensity.

experimental error limits the possibilities for such a fine analysis of Fig. 6, the essence of these statements is confirmed (cf. Fig. 7A and B).

The data of the experiment of Fig. 6 can also be used to make a plot like that in Fig. 5. When such a plot is made, the conclusions are identical to those drawn from Fig. 5, except that they can be elaborated in more detail. The slopes $1/L_v L_e$ of the linear least-squares lines through the points of such a plot should be not only linear, but also even proportional to the inverse of light intensity. The $1/(J_{H,i})$-axis intercept (1/maximum initial rate of proton uptake at any light intensity) should be linear with the inverse of the light intensity. For the dose of chlorpromazine that reduces the initial rate of proton uptake by 50%, linearity with light intensity should hold. That all of this is confirmed within the limits imposed by experimental error is shown by Fig. 8A–C.

Discussion

In this article it has been shown that mosaic non-equilibrium thermodynamics [12–26] quantitatively describe how the initial rate of proton uptake into bacteriorhodopsin liposomes depends on light intensity and on the permeability characteristics of the liposomal membrane. In a parallel paper [31] we show that the same is true for the effect that changes in these parameters have on the steady-state pH gradient. Although the two conditions considered are only two out of a theoretically infinite number of conditions that may be analyzed, they are the most extreme ones, i.e., one of maximum and one of zero net proton flow. We may, therefore, have some confidence that the mosaic non-equilibrium thermodynamic method is equally applicable to the intermediate conditions.

Concomitantly, the theoretical advantage of mosaic non-equilibrium thermodynamics [12–26] over the earlier type of non-equilibrium thermodynamics [9–11] has been shown to have practical value. The effects of changing the characteristics of the energy-transducing systems are no longer hidden in the black box of phenomenological coefficients, but clearly manifest themselves as variations of permeability and activity coefficients, which in turn result in the variations in proton uptake rate actually measured. Non-equilibrium thermodynamics are no longer limited to relationships between input and output flows and forces, but also gives relationships between the parameters characteristic of the biological energy transducer and these input and output flows and forces.

Considering what new insights into the functioning of the energy-transducer bacteriorhodopsin and the ion movements in bacteriorhodopsin liposomes the new theoretical method has brought us, we may begin with the experiments shown in this article. The initial rate of proton uptake saturates with light intensity. This had already been shown by Hwang and Stoeckenius [40]. Our quantitative method of interpretation allows a speculation, followed by the suggestion of an experimental test and then a conclusion concerning the cause of this effect. Rephrasing the above equations, the speculation is that the saturation may arise from the fact that at higher light intensities the membrane potential will be higher, and that this membrane potential inhibits the proton pumping activity of bacteriorhodopsin. The supposition that bacteriorhodopsin is inhibited by the membrane potential it develops leads to the prediction (cf. Eqn. 2) that the K_m for illumination intensity will vary with the degree of dissipation of the membrane potential and thus, for instance, with the amount of added valinomycin. This prediction was confirmed in the experiment of Fig. 2B. A second effect predicted by the back-pressure concept is that (cf. Eqn. 7) the chlorpromazine concentration necessary for 50% reduction of the initial rate of proton uptake increases with light intensity. This point is also confirmed by the experiments (Fig. 8C). An alternative explanation for saturation with light intensity would be that the photochemical cycle itself would be saturated. Since a turn of a cycle takes a certain amount of time, there is a certain 'dead' time in which no photon can be absorbed. To inspect this possibility, we extrapolated the initial rate of proton uptake to infinite valinomycin concentration, i.e., zero membrane potential. This extrapolation was carried out in a double-reciprocal plot, as was suggested by the mosaic thermodynamic theory. When this extrapolated initial rate of proton uptake was plotted as a function of light intensity (cf. Fig. 3A), there was no sign at all of a saturation effect: even if the error

in the determination of the initial rate of proton uptake at 100% light intensity were 10%, the half-saturating light intensity would be over 600%, which is much higher than the light intensity at which saturation behaviour of the initial rate of proton uptake is observed in the absence of excess valinomycin. Thus, the saturation effect is dependent on the membrane potential. This conclusion has been proposed earlier [30,39] after a more qualitative interpretation of some experimental results in terms of the same theoretical description [26] and was confirmed by measurement of the dependence of the kinetics of the photochemical cycle of bacteriorhodopsin on the addition of certain ionophores to bacteriorhodopsin liposomes [30,39]. The mosaic thermodynamic description cannot only be used to assess the effect of added agents that change the permeability characteristics of the system. Likewise, the endogenous permeabilities can be measured. The most obvious example is the endogenous permeability of the liposomal membrane to K^+ and/or Cl^- that can be read from Fig. 2 to be equivalent to 0.06 μg of added valinomycin. We analyzed the experiments of Figs. 2 and 6 with the following results. The endogenous electric permeability for ions other than protons is equivalent to 0.06 mg valinomycin/g lipid. In the presence of 30 μg valinomycin per g lipid the total electric permeability of the liposomal membrane is equivalent to 0.04 μM chlorpromazine. $L_e(0)A_\nu$, $L_H^1(0)$-A_ν and $L_\nu A_\nu$ (at 100% light intensity, pH meter 1) were approximately equal to 0.02, 0.3 and 0.6 s^{-1}, respectively: the bacteriorhodopsin liposomes are relatively leaky towards protons, but much less to other ions (cf. Refs. 28 and 47). The proton pump activity parameter induced by the light intensity used does not vastly exceed the other conductivity parameters. These experiments were, however, directed at proving the correctness of the theoretical relationships rather than finding the values of the permeability parameters. Determination of the parameters given here requires extrapolation in a plot already derived from another plot. They may, therefore, be liable to relatively large experimental error.

The use of the present quantitative method to characterize and compare different preparations of bacteriorhodopsin (e.g., reconstituted with lipid in different manners) appears to be a promising application of the quantitative method developed here (cf. Refs. 30, 31, 48 and 49). Application of the same method to other bioenergy-transducing systems has already given interesting mechanistic information [24].

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References

1 Mitchell, P. (1979) Eur. J. Biochem. 95, 1–20
2 Boyer, P.D., Chance, B., Ernster, L., Mitchell, P., Racker, E. and Slater, E.C. (1977) Annu. Rev. Biochem. 46, 955–1026
3 Padan, E. and Rottenberg, H. (1973) Eur. J. Biochem. 40, 431–437
4 Williams, R.J.P. (1978) Biochim. Biophys. Acta 505, 1–44
5 Van Dam, K., Wiechmann, A.H.C.A., Hellingwerf, K.J., Arents, J.C. and Westerhoff, H.V. (1978) in Membrane Proteins, Proc. 11th FEBS Meet., Copenhagen, 1977 (Nicholls, P., Møller, J.V., Jørgensen, P.L. and Moody, A.J., eds.), vol. 45, pp. 121–132, Pergamon Press, Oxford
6 Azzone, G.F., Pozzan, T., Massari, S. and Pregnolato, L. (1978) Biochim. Biophys. Acta 501, 307–316
7 Kell, D.B. (1979) Biochim. Biophys. Acta 549, 55–99
8 Wilson, D.F., Owen, C.S. and Holian, A. (1977) Arch. Biochem. Biophys. 182, 749–762
9 Onsager, L. (1931) Phys. Rev. 37, 405–426
10 Rottenberg, H., Caplan, S.R. and Essig, A. (1970) in Membranes and Ion Transport (Bittar, E.E., ed.), vol. 1, pp. 165–191, Wiley Interscience, New York
11 Rottenberg, H. (1979) Biochim. Biophys. Acta 549, 225–253
12 Spiegler, K.S. (1958) Trans. Faraday Soc. 54, 1409–1428
13 Kedem, O. and Katchalsky, A. (1961) J. Gen. Physiol. 45, 143–179
14 Katchalsky, A. and Curran, P.F. (1967) Non-Equilibrium Thermodynamics in Biophysics, Harvard University Press, Cambridge, MA

15 Blumenthal, R., Caplan, S.R. and Kedem, O. (1967) Biophys. J. 7, 735–757
16 Oster, G.F., Perelson, A.S. and Katchalsky, A. (1973) Q. Rev. Biophys. 6, 1–134
17 Van Dam, K. and Westerhoff, H.V. (1977) in Structure and Function of Energy-Transducing Membranes (Van Dam, K. and Van Gelder, B.F., eds.), pp. 157–167, Elsevier, Amsterdam
18 Westerhoff, H.V. and Van Dam, K. (1979) Curr. Top. Bioenerg. 9, 1–62
19 Mikulecky, D.C., Huf, E.G. and Thomas, S.R. (1979) Biophys. J. 25, 87–105
20 Lagarde, A.E. (1976) Biochim. Biophys. Acta 426, 198–217
21 Hill, T.L. (1979) Proc. Natl. Acad. Sci. U.S.A. 76, 2236–2238
22 Rottenberg, H. (1973) Biophys. J. 13, 503–511
23 Van der Meer, R., Westerhoff, H.V. and Van Dam, K. (1980) Biochim. Biophys. Acta 591, 488–493
24 Van Dam, K., Westerhoff, H.V., Krab, K., Van der Meer, R. and Arents, J.C. (1980) Biochim. Biophys. Acta 591, 240–250
25 Westerhoff, H.V. and Van Dam, K. (1981) in Membranes and Transport: A Critical Review (A.N. Martonosi, ed.), in the press
26 Westerhoff, H.V., Scholte, B.J. and Hellingwerf, K.J. (1979) Biochim. Biophys. Acta 547, 544–560
27 Kayushin, L.P. and Skulachev, V.P. (1974) FEBS Lett. 39, 39–42
28 Stoeckenius, W., Lozier, R.H. and Bogomolni, R.A. (1979) Biochim. Biophys. Acta 505, 215–278
29 Eisenbach, M. and Caplan, S.R. (1979) Curr. Top. Membranes Transp. 12, 165–248
30 Hellingwerf, K.J., Arents, J.C., Scholte, B.J. and Westerhoff, H.V. (1979) Biochim. Biophys. Acta 547, 561–582
31 Arents, J.C., Hellingwerf, K.J., Van Dam, K. and Westerhoff, H.V. (1981) J. Membrane Biol., in the press
32 Eisenbach, M., Bakker, E.P., Korenstein, R. and Caplan, S.R. (1976) FEBS Lett. 71, 228–232
33 Eisenbach, M., Garty, H., Bakker, E.P., Klemperer, G., Rottenberg, H. and Caplan, S.R. (1978) Biochemistry 17, 4691–4698
34 Govindjee, R., Ebrey, T.G. and Grofts, A.R. (1980) Biophys. J. 30, 231–242
35 Ort, D.R. and Parson, W.W. (1979) Biophys. J. 25, 341–354
36 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, Amsterdam
37 Hellingwerf, K.J. (1979) Structural and Functional Studies on Lipid Vesicles containing Bacteriorhodopsin, Ph.D. Thesis, Veenstra-Visser, Groningen
38 Mitchell, P. (1966) Chemiosmotic Coupling in Oxidative and Photosynthetic Phosphorylation, Glynn Research Ltd., Bodmin, U.K.
39 Hellingwerf, K.J., Schuurmans, J.J. and Westerhoff, H.V. (1978) FEBS Lett. 92, 181–186
40 Hwang, S.-B. and Stoeckenius, W. (1977) J. Membrane Biol. 33, 325–350
41 Rott, R. and Avi-Dor, Y. (1977) FEBS Lett. 81, 267–270
42 Garty, H., Eisenbach, M., Shuldman, R. and Caplan, S.R. (1979) Biochim. Biophys. Acta 545, 365–375
43 Ovchinnikov, Yu.A., Ivanov, V.T. and Shkrob, A.M. (1974) Membrane Active Complexones, Elsevier, Amsterdam
44 Toro, M., Gómez-Lojero, C., Montal, M. and Estrada-O, S. (1976) J. Bioenerg. 8, 19–26
45 Anraku, Y. (1979) in Structure and Function of Biomembranes (Yagi, K., ed.), pp. 193–201, Japan Scientific Societies Press, Tokyo
46 Kraaijenhof, R. (1971) Uncoupling of Energy Conservation in Chloroplast and Mitochondrion, Ph.D. Thesis, Amsterdam, Mondeel-Offsetdrukkerij
47 Nichols, J.W., Hill, M.W., Bangham, A.D. and Deamer, D.W. (1980) Biochim. Biophys. Acta 596, 393–403
48 Westerhoff, H.V., Hellingwerf, K.J., Arents, J.C., Scholte, B.J. and Van Dam, K. (1981) Proc. Natl. Acad. Sci. U.S.A., in the press
49 Arents, J.C., Van Dekken, H., Hellingwerf, K.J. and Westerhoff, H.V. (1981) Biochemistry, in the press