Biochimica et Biophysica Acta, 547 (1979) 544—560

BBA 47721

BACTERIORHODOPSIN IN LIPOSOMES

I. A DESCRIPTION USING IRREVERSIBLE THERMODYNAMICS

HANS V. WESTERHOFF, BOB J. SCHOLTE and KLAAS J. HELLINGWERF

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

(Received October 20th, 1978)

Key words: Irreversible thermodynamics; Bacteriorhodopsin; Proton-motive force; Light-driven proton pump; Liposome; Ion permeability; Ionophore

Summary

A comprehensive description of light-induced ion transport in bacteriorhodopsin liposomes is presented. Linear irreversible thermodynamics and the chemiosmotic theory serve as theoretical bases for the formulation of a limited number of fundamental equations. In these equations mechanistic parameters characterize the dependence of ion movement and flux through the photochemical cycle of bacteriorhodopsin on electrochemical potential differences and a so-called light affinity. By making appropriate steady-state assumptions and carrying out mathematical reduction experimentally testable expressions, still containing the mechanistic parameters, are obtained. In the accompanying article rigid trials to falsify these expressions are shown to be unsuccessful.

Glossary

Many of the symbols used refer to the nomenclature used in Ref. 1.

Sub/superscripts:	Referring to:
ν	light driven
H	proton
K	potassium ion
Cl	chloride ion
HCl	hydrochloric acid
KOH	proton/potassium ion exchange
S	entropy
*	steady state of electroneutral net total flow
**	steady state of zero proton flow and *
l	leakage
r	chemical reaction r

Symbol	Definition	Formula	First used in:
J_X	Outward transmembrane flow of substance X		Eqn. 3
J_ν^+	The flow through the photochemical cycle of the bacteriorhodopsin molecules if they would all be oriented in the in vivo orientation		Fig. 2
J_ν^-	Idem in the inverted orientation		Fig. 2
J_ν	The flow through the photochemical cycle	$(1-\alpha)J_\nu^- + \alpha J_\nu^+$	Eqn. 5
$J_H^{\nu+}$	The outward protonflow coupled to J_ν^+		Fig. 2
$J_H^{\nu-}$	The outward protonflow coupled to J_ν^-		Fig. 2
J_H^ν	The outward protonflow coupled to photochemical flow	$J_H^{\nu-} + J_H^{\nu+}$	
J_X^l	The outward leakage of ion X		Fig. 2
J_X^Y	The outward flow of ion X coupled to a flow of ion Y		Fig. 2
A_ν	The thermodynamic force on bacteriorhodopsin exerted by illumination		Fig. 2
μ_X	The chemical potential of substance X, excluding the temperature dependent part		
$\Delta\mu_X$	The gradient of μ_X across the membrane	$\mu_X^{in} - \mu_X^{out}$	Eqn. 13
$\Delta\psi$	The electrical potential across the membrane, $\psi^{out} \equiv 0$		Eqn. 13
$\tilde{\mu}_X$	The electrochemical potential of substance X, excluding the temperature dependent part	$\mu_X + F \cdot \psi$	Fig. 2
$\Delta\tilde{\mu}_X$	The gradient of $\tilde{\mu}_X$ across the membrane	$\tilde{\mu}_X^{in} - \tilde{\mu}_X^{out}$	Fig. 2
L_X	Proportionality constant relating flow to force of an independent reaction involving substance X		Fig. 2
L_X^l	Proportionality constant of electrogenic leakage of ion X		Fig. 2
L_{KOH}	Proportionality constant of H^+/K^+ exchange		Fig. 2
L_{HCl}	Proportionality constant of HCL permeation		Fig. 2
n	Number of protons pumped/turn of the photochemical cycle of bacteriorhodopsin ($n > 0$)		Fig. 2
σ	Local entropy production		Eqn. 1
ϕ	Local dissipation function, i.e. local Gibbs free energy consumption		Eqn. 1
j_S	Local flow of entropy		Eqn. 1
j_i	Local flow of substance i		Eqn. 1
$j_{chem,r}$	Local flow through chemical reaction r		Eqn. 1
A_r	ΔG of chemical reaction r		Eqn. 1

Symbol	Definition	Formula	First used in
Φ_0	Dissipation function in the discontinuous system of bacteriorhodopsin liposomes	$\int_{\text{system}} \phi \, dV$	Eqn. 2
$\overline{J}_S$	The 'average' transmembrane entropy flow	$\dfrac{\int_{\text{membrane}} j_S \operatorname{grad}(-T) \, dV}{\Delta T}$	Eqn. 2
ΔT	Transmembrane temperature difference	$T^{in} - T^{out}$	Eqn. 2
$\delta\tilde{\mu}_X$	Electrochemical potential difference of substance X across the membrane, including the temperature-dependent part	$\Delta\tilde{\mu}_X - \overline{S}_X \Delta T$	Eqn. 2
$\overline{S}_X$	Partial molar entropy of substance X		
$J_{chem,r}$	Total flow through chemical reaction r		Eqn. 2
Φ	The part of Φ_0 not containing the water and heat permeation terms	$\Phi_0 - \overline{J}_S \cdot \Delta T - J_{H_2O} \cdot \Delta\mu_{H_2O}$	Eqn. 3
$1 - \alpha$	The fraction of bacteriorhodopsin molecules in in vivo orientation		Eqn. 5
R_m	Electrical resistance of the liposomal membrane		Eqn. 9
C	Electrical capacity across the liposomal membrane		Eqn. 9
R_i	Internal electrical resistance of bacteriorhodopsin regarded as a voltage source		Eqn. 9
f_e	Fraction of proton flow that is not electrically compensated for	$\dfrac{J_H + J_K - J_{Cl}}{J_H}$	Eqn. 11
L_{eg}	The sum of all electrogenic permeation coefficients	$n^2 L_\nu + L^1_H + L^1_K + L^1_{Cl}$	Eqn. 14
L_e	The sum of all, except proton, electric permeation coefficients	$L^1_K + L^1_{Cl}$	Eqn. 15
L_n	The sum of the electroneutral proton permeation coefficients	$L_{HCl} + L_{KOH}$	Eqn. 15
J^0_{Hi}	The truly initial protonflow ($\Delta\tilde{\mu}_H$ still equal to zero)		Eqn. 16
J_{Hi}	The initial rate of proton uptake after onset of illumination		Eqn. 17
$\Delta\tilde{\mu}^{max}_H$	Steady-state $\Delta\tilde{\mu}_H$ in the absence of added or endogenous neutral proton permeation	$\dfrac{-n(1-2\alpha)L_\nu A_\nu}{n^2 L_\nu + L^1_H}$	Eqn. 34
γ_H	Sum of the parallel proton conductances by straight proton conductance and the permeation via electroneutral H^+ permeation and electrogenic permeation of the coupled ion in series	$L^1_H + \dfrac{L_n L_e}{L_n + L_e}$	Eqn. 39

Introduction

The postulate [2] that proton transport is an essential process in at least some energy-conserving biochemical conversions has not only led to numerous critical experiments in Popper's sense [3], but also to a new paradigma [4] in biochemical sciences. Today bioenergetics is closely related to the study of transport across biomembranes. Oxidative phosphorylation can be considered a central example. The identity of the proton-motive force as high energy intermediate in this process is hardly doubted [5]. Yet on a more refined scale objections exist against the validity of the two phase chemiosmotic hypothesis as formulated by Mitchell ([2], for review see [6]). Insteady of two bulk phases separated by a membrane phase lacking internal electric and concentration capacitance, several authors (e.g. Refs. 7—9) need three phases, in which protons can dwell, to explain their results. To be able to describe these results theory must be refined. It will be clear that equilibrium thermodynamics cannot accurately describe the more complicated chemiosmotic schemes, but the theory of irreversible thermodynamics [10,11] as applied to biological systems [1] may offer certain possibilities. Mitchell [12] developed some equations interrelating thermodynamic flows and forces of different ions across biomembranes; he did, however, not present a full analysis in terms of irreversible thermodynamics of the coupling of ion permeation and chemical reactions. After earlier application of linear irreversible thermodynamics to oxidative phosphorylation [13], Van Dam and Westerhoff [14] using a black box approach proposed a description that bears close relation to mechanistic coefficients such as stoichiometric numbers and proton permeability. Using this terminology they could define the extent to which Mitchell's chemiosmotic theory fails to describe the oxidative phosphorylation, in a thermodynamic sense [8].

The use of linear irreversible thermodynamics has been criticized (e.g. Ref. 15) and therefore it is important to test its predictive value in a system that is biochemically well-defined. Bacteriorhodopsin reconstituted with purified lipids [16,17] offers an attractive test system.

Yet another consideration is reflected in this article: whereas the discription of chemically driven proton pumps in terms of irreversible thermodynamics is relatively trivial [12,14], such a description of a light-driven proton pump as inducer of ion movements lacks precedence in the literature. Questions like: how is an increase in light intensity described in terms of a change in a thermodynamic parameter: the thermodynamic 'force' exerted by light or more directly in a light flow, have to be answered.

In this article linear irreversible thermodynamics, elements of the chemiosmotic hypothesis, and a few evaluated assumptions together with some additional postulates are used to derive relations between experimentally testable parameters. In the accompanying article [18] these relations are shown to survive relevant critical tests. Thus the system can be described in terms of flow-force relations based on fundamental assumptions like full coupling and linearity. Moreover, this strategy of experimental testing of theoretically derived equations turns out to be of great help in setting up those experiments that are relevant for the full definition of light-induced transport in bacterio-

rhodopsin liposomes. Together the two papers will provide a basis for further, more quantitative, critical testing and elaboration of the proposed description.

The model

1. The elements considered

In our description the system of bacteriorhodopsin liposomes is idealized as is shown in Fig. 1. From a thermodynamic point of view the idealized system consists of an aqueous compartment enclosed by a membrane. This membrane is freely permeable to water [19], but much less so to ions [20]. It contains a light-energized proton pump, which may generate an electrochemical potential difference for protons across the membrane [21,22]. This $\Delta\tilde{\mu}_H$ will generally consist of both a membrane potential and a pH gradient. Electrophoretic movement of other ions may result from the former, electroneutral exchange or symport of protons and other ions may be caused by the latter thermodynamic force.

A fundamental equation of irreversible thermodynamics [1,10,11,23] expresses the consumption of Gibbs free energy, a characteristic of irreversible processes, in terms of the products of thermodynamic forces and their conjugate flows:

$$T\sigma = \phi = j_S \text{ grad } (-T) + \sum_i j_i \text{ grad } (-\tilde{\mu}_i) + \sum_r j_{\text{chem},r} A_r \qquad (1)$$

Clearly not all processes that take place within the membrane, can be analyzed in full detail with the present techniques. Since at present interest is confined to the in- and output parameters of the membrane system, the lack of detailed knowledge can be circumvented by integrating Eqn. 1 over the bacterio-

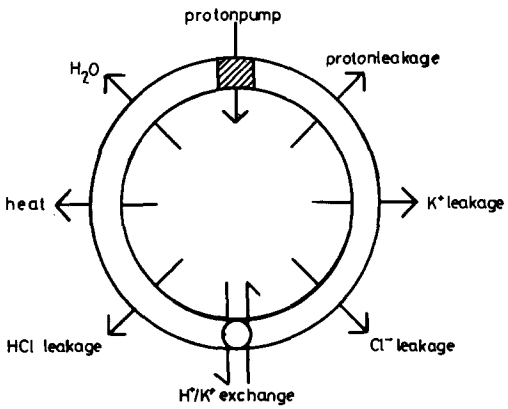

Fig. 1. The idealized bacteriorhodopsin liposome containing a light-driven proton pump in a membrane with some proton, K^+, Cl^-, HCl conductance and allowing some H^+/K^+ exchange. Water and heat conductance of the membrane are very high. The membrane does not exert elastic forces on the liquid. K^+ and Cl^- represent the bulk cation and anion, respectively, of the incubation medium. For non-coupled processes arrows are drawn in correspondence with the sign convention (see legend to Fig. 2).

rhodopsin liposome. The following expression results:

$$\Phi_0 = \overline{J}_S \Delta T + \sum_{i=1}^{n} J_i \Delta\tilde{\mu}_i + \sum_r J_{chem,r} A_r \qquad (2)$$

To obtain this result it has been assumed that chemical reactions that produce any of the described substances, are absent inside the membrane and that within the aqueous phases on both sides of the membrane concentration and electric potential gradients can be neglected. Further simplification is possible in view of the high heat conductance of lipid bilayers [24]. This allows neglection of the coupling of the non-heat fluxes to the temperature gradient (see also Ref. 25). The high permeability of lipid bilayers to water, and the relatively small osmotic effects of the processes to be considered (see below) allow for the same conclusion with respect to the coupling of non-water fluxes to the difference between pressure and osmotic gradient. Elimination of the two essentially non-coupled processes leaves:

$$\Phi = \sum_{i=1}^{n-1} J_i \Delta\tilde{\mu}_i + \sum_r J_{chem,r} A_r \qquad (3)$$

Thus the dissipation of free energy in ideal bacteriorhodopsin liposomes has been split into independent parts. One of these (Eqn. 3) is defined by the sum of the products of transmembrane flow of ions times their electrochemical gradient plus the chemical flows times their chemical affinities.

Fig. 2 enumerates the processes that are taken into consideration, together with the equations they are assumed to obey. In all cases the equations derive from the assumption that fluxes through a process are approximately proportional to the total thermodynamic force across the process. Such an assumption is quite common to the theory of irreversible thermodynamics developed by Onsager [26] and can be proven to hold for near-equilibrium systems [1]. Experimental practice showed that at least some diffusion processes [27,28] obey the linear phenomenological equations. For enzyme-catalyzed reactions in addition to a range of chemical affinities of about RT around equilibrium, in which the flow through the reaction is proportional to the chemical affinity (A_r or ΔG) of the reaction, other ranges of substrate concentration exist where this proportionality is replaced by linearity [29,30].

The so-called light affinity A_ν (see Fig. 2) deserves further comments. At this point it may be taken as a phenomenological parameter linked to the amount of photon energy that can be used to drive the photochemical cycle of bacteriorhodopsin. Since at any one photochemical cycle a bacteriorhodopsin molecule presumably absorbs only one photon, A_ν is expected to be independent of light intensity. If every bacteriorhodopsin molecule undergoes a photochemical cycle driven by a certain thermodynamic force only after a photon has been absorbed, then it seems straightforward to suppose that the total flow through all photochemical cycles is proportional to the number of bacteriorhodopsin molecules that absorb a photon: L_ν will be proportional to both the bacteriorhodopsin concentration and the intensity of illumination.

The third important assumption expressed in Fig. 2 derives from the con-

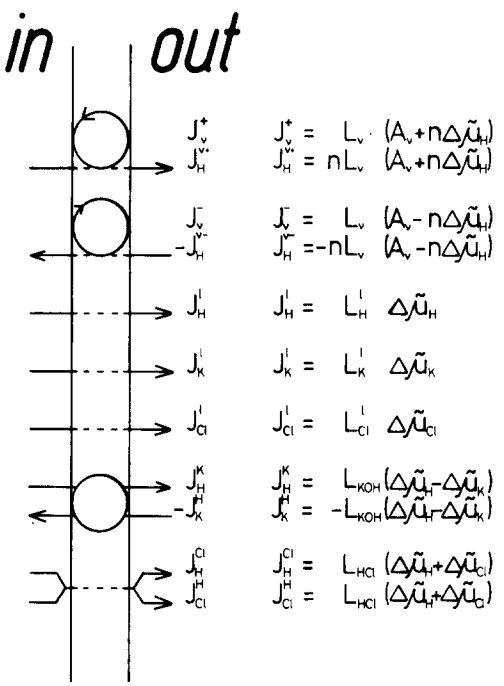

Fig. 2. A schematic representation of the processes and their equations considered in the model. Sign conventions are: $\Delta\tilde{\mu}_X = \tilde{\mu}_X^{in} - \tilde{\mu}_X^{out}$ and $J_X > 0$ if outwards directed. For non-coupled processes the arrows are drawn in correspondence with this sign convention. For further explanation see text.

sideration that, if during a turn of the photochemical cycle n protons are transported across the membrane, the total thermodynamic force does not equal A_v, but rather $A_v - |n||\Delta\tilde{\mu}_H|$. (Due to sign conventions this formula turns out to be: $A_v + n\Delta\tilde{\mu}_H$ for bacteriorhodopsin in in vivo orientation). This idea, which is based on the expression for the ΔG of a four molecular reaction, is equivalent to the idea of Onsager's reciprocity at the single reaction level. As a consequence the flux of pumped protons is expected to be sensitive to the magnitude of the $\Delta\tilde{\mu}_H$ across the vesicle membrane. Although with other proton-translocating enzymes, such as the mitochondrial [31], bacterial [32] and chloroplast [33] ATPases and the mitochondrial respiratory chain [34] this phenomenon, basically originating from the second law of thermodynamics, has been amply observed, it has not yet been proven to occur in bacteriorhodopsin. Recent experiments (Hellingwerf, K.J. et al., unpublished results) do, however, indicate that the flux through the photochemical cycle of bacteriorhodopsin is inhibited by an electrochemical proton gradient. Models of bacteriorhodopsin without back pressure by $\Delta\tilde{\mu}_H$ can as easily be translated into testable equations, as the model presented in Fig. 2. In the accompanying article [18] experiments will be shown to reject the former models in favour of the latter.

2. *Derivation of some testable equations*

2.1. Basal equations and the effect of electroneutral flow. The explicit form

of Eqn. 3 for the ideal bacteriorhodopsin system is:

$$\Phi = J_H \Delta\tilde{\mu}_H + J_K \Delta\tilde{\mu}_K + J_{Cl}\Delta\tilde{\mu}_{Cl} + J_\nu A_\nu \tag{4}$$

with:

$$\begin{aligned} J_\nu &= (1-\alpha) J_\nu^+ + \alpha J_\nu^- \\ J_H &= (1-\alpha) J_H^{\nu+} + \alpha J_H^{\nu-} + J_H^l + J_H^K + J_H^{Cl} \\ J_K &= J_K^l + J_K^H \\ J_{Cl} &= J_{Cl}^l + J_{Cl}^H \end{aligned} \tag{5}$$

Here $(1-\alpha)$ is defined as the fraction of bacteriorhodopsin molecules oriented in the in vivo orientation. Clearly the first three terms of Eqn. 4 correspond with the first term of Eqn. 3; also the last terms of both equations correspond with each other, both measuring energy dissipation in (photo)chemical reaction sequences. One may now postulate the existence of linear and symmetrical phenomenological equations under near-equilibrium conditions [1] leading to:

$$\begin{Bmatrix} J_\nu \\ J_H \\ J_K \\ J_{Cl} \end{Bmatrix} = \begin{Bmatrix} L_{\nu\nu} & L_{\nu H} & L_{\nu K} & L_{\nu Cl} \\ L_{H\nu} & L_{HH} & L_{HK} & L_{HCl} \\ L_{K\nu} & L_{KH} & L_{KK} & L_{KCl} \\ L_{Cl\nu} & L_{ClH} & L_{ClK} & L_{ClCl} \end{Bmatrix} \begin{Bmatrix} A_\nu \\ \Delta\tilde{\mu}_H \\ \Delta\tilde{\mu}_K \\ \Delta\tilde{\mu}_{Cl} \end{Bmatrix} \tag{6}$$

with

$$L_{ij} = L_{ji} \text{ for all i and j} \tag{7}$$

This has the disadvantage of losing insight in the influence of identifiable parameters such as the proton permeability of the membrane (L_H^l) on the different flows. We therefore choose to combine Fig. 2 and Eqn. 5 to yield:

$$\begin{Bmatrix} J_\nu \\ J_H \\ J_K \\ J_{Cl} \end{Bmatrix} = \begin{Bmatrix} L_\nu & n(1-2\alpha)L_\nu & 0 & 0 \\ n(1-2\alpha)L_\nu & n^2 L_\nu + L_H^l + L_{KOH} + L_{HCl} & -L_{KOH} & L_{HCl} \\ 0 & -L_{KOH} & L_K^l + L_{KOH} & 0 \\ 0 & L_{HCl} & 0 & L_{Cl}^l + L_{HCl} \end{Bmatrix} \times \begin{Bmatrix} A_\nu \\ \Delta\tilde{\mu}_H \\ \Delta\tilde{\mu}_K \\ \Delta\tilde{\mu}_{Cl} \end{Bmatrix} \tag{8}$$

After measurement of the concentration gradients and the electric potential across the vesicle membrane at constant light intensity and therefore at constant L_ν and A_ν the magnitudes of the flows can be predicted from the above equations and compared to the experimental results. In the experiments with bacteriorhodopsin liposomes reported up till now not all flows and forces were

measured simultaneously. Although in the accompanying paper [18] measurements of ΔpH and $\Delta\psi$ will be reported, the probe molecule relaxation time may not have been quick enough and permeability constants are not known well enough, to allow careful testing of Eqn. 8. An approach already known to be effective in studying normal enzyme kinetics, will now be of much help: our measurement conditions are often such that some steady-state condition is met. Here we will discuss merely two steady-state conditions, firstly the steady state of electroneutral total flow (marked by one asterisk) and secondly the steady state of zero proton flow (marked by two asterisks; here also the electroneutrality condition is valid). To find out whether a steady-state condition is essentially met, two approaches can be followed: one is to verify the disappearance of the relevant net flow, the other is to estimate by calculation the time it takes for the conjugate force to reach its steady-state value. In the following we will follow the latter strategy to discuss the steady-state of electroneutral flow.

To check whether a steady-state condition is obligatorily met in a system, one must compare the total uptake of the relevant species to the building up of its conjugate forces using an estimated value for the capacity of the system. Discussing the electroneutral flow steady state the relevant capacity is the electrical capacity of the membrane. Assuming a concentric bispherical capacitor with an interior having a relative dielectric constant of about 3 [35], a membrane thickness of 3 nm [36], and a membrane thin relative to the diameter of the vesicle, a membrane electrical capacity of about $1 \cdot 10^{-2}$ F/square meter of external surface area can be calculated. This is in fair agreement with the values reported in literature [37]. Using a surface area/lipid molecule of $70 \cdot 10^{-20}$ m^2 [37], an average lipid molecular weight of 700 $g \cdot mol^{-1}$, and the assumption that bacteriorhodopsin does not contribute much to the surface area, a total electrical capacity of 3 F/g phospholipid is found. At our usual protein to lipid ratio of 0.05 (w/w) the uptake of 100 μmol of protons/g bacteriorhodopsin, which is usually observed after some minutes of illumination, would, if not compensated by the flow of other ions, cause a membrane potential of about 0.16 V. For biomembranes dielectric breakdown voltages of about $30 \cdot 10^6$ $V \cdot m^{-1}$ have been reported [38] which in the present system amounts to 0.1 V.

Bacteriorhodopsin (BRh) liposomes in 150 mM KCl (our usual incubation medium) are examples of preparations exhibiting proton uptake above the 75 μmol $H^+ \cdot g\ BRh^{-1}$ level. This can be accounted for, since electrical resistance of phospholipid bilayers in this medium is probably as low as $10^2\ \Omega \cdot m^2$: in comparable protein-free liposomes Papahadjopoulos and Watkins observed an electric resistance of $10^3\ \Omega \cdot m^2$ [39]; the incorporation of protein and a decrease in the degree of saturation of the hydrocarbon chains generally lower the electric resistance of a phospholipid bilayer [37]. When the bacteriorhodopsin electrogenic proton pump is set into action by switching on the light, a membrane potential will be built up with a $t_{1/2}$ characterized by:

$$t_{1/2} = \left(\frac{1}{R_i} + \frac{1}{R_m}\right)^{-1} C \ln 2 < R_m C \ln 2 \qquad (9)$$

This can be understood after inspection of Fig. 3. This figure shows an electric analogue for bacteriorhodopsin liposomes. It is valid for as long as $\Delta\mu_H$ can be considered insignificant. From the values for the electrical capacity and resis-

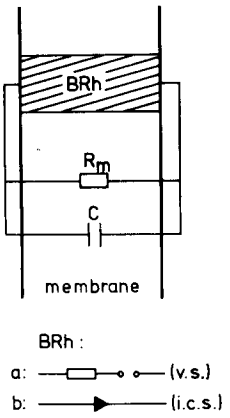

Fig. 3. Electrical analogue of a membrane containing bacteriorhodopsin. The bacteriorhodopsin can be regarded as a voltage source (a, vs), or as an ideal current source (b, ics). BRh, bacteriorhodopsin. R_m, membrane resistance. C, membrane capacitance.

tance of the membrane given above one can conclude that

$$t_{1/2} < 1.1 \text{ s} \tag{10}$$

Using the same electric analogue, the degree to which the pumped proton flow is electrically compensated by ion movement, can be estimated:

$$f_e = \frac{1 + R_m R_i^{-1}}{\exp(t \ln 2/t_{1/2}) + R_m R_i^{-1}} \tag{11}$$

This equation quantitates the degree to which the pumped proton flow is electrogenic, i.e. not electrically compensated by the back leakage of ions. Under these conditions with relatively high electric conductance of the membrane proton flow will be over 95% electrically compensated 4 s after the onset of illumination. If the membrane would have a high electric resistance (R_m) relative to the internal resistance (R_i) of bacteriorhodopsin, then not only the $t_{1/2}$ of the charging of the membrane's capacitance would be higher, but also the degree to which proton flow is electrogenic. Using data given in the accompanying paper [18], estimates of R_i/R_m vary between 2 and 40 so that effectively the former of the two situations is met. This allows us to describe the system after 4 s of illumination as one essentially obeying the steady-state condition of electroneutral total flow.

This rather detailed discussion of the correctness of using the steady-state approximation of electroneutral total flow has been given to show that the total electrical conductance of the membrane is a very important factor in such a discussion: if the electric conductance of the membrane would be two orders of magnitude lower, electroneutrality would not be approached until after 5 min. In the present description of bacteriorhodopsin liposomes we will focus upon the high conductance (i.e. quick electric relaxation) systems, so that already after a few seconds the steady-state condition of electroneutral total flow can be applied.

Electroneutral total flow is expressed as follows:

$$J_H + J_K - J_{Cl} = 0 \tag{12}$$

Using this equation and Eqn. 8, $F\Delta\psi$ can be expressed in the other forces:

$$F\Delta\psi^* = \frac{-nL_\nu(1-2\alpha)A_\nu - (n^2L_\nu + L_H^1)\Delta\mu_H^* - L_K^1\Delta\mu_K^* + L_{Cl}^1\Delta\mu_{Cl}^*}{L_{eg}} \tag{13}$$

with

$$L_{eg} = n^2L_\nu + L_H^1 + L_K^1 + L_{Cl}^1 \tag{14}$$

Insertion of this expression into Eqn. 8 and some rearrangement yields four equations, which are still interdependent. Removing this interdependence one gets the following independent set:

$$\begin{Bmatrix} J_\nu^* \\ J_H^* \\ J_K^* \end{Bmatrix} = 1/L_{eg} \begin{Bmatrix} (L_H^1 + L_e)L_\nu + 4\alpha(1-2\alpha)n^2L_\nu^2 & nL_\nu L_e(1-2\alpha) & -nL_\nu \cdot L_K^1(1-2\alpha) \\ nL_\nu(1-2\alpha)L_e & L_e(n^2L_\nu + L_H^1) + L_nL_{eg} & -L_K^1(n^2L_\nu + L_H^1) - L_{KOH}L_{eg} \\ -nL_\nu L_K^1(1-2\alpha) & -L_K^1(n^2L_\nu + L_H^1) - L_{KOH}L_{eg} & L_K^1(n^2L_\nu + L_H^1 + L_{Cl}^1) + L_{KOH}L_{eg} \end{Bmatrix} \begin{Bmatrix} A_\nu \\ \Delta\mu_H^* + \Delta\mu_{Cl}^* \\ \Delta\mu_K^* + \Delta\mu_{Cl}^* \end{Bmatrix} \tag{15}$$

with: $L_e = L_K^1 + L_{Cl}^1$, and $L_n = L_{HCl} + L_{KOH}$.

2.2. The initial proton flow. A readily accessible experimental variable is the proton uptake occurring soon after the onset of illumination. The true initial proton flow, J_{Hi}^0, will depend on the activity of the bacteriorhodopsin and on the energetic input into the system, but not on any back pressure of $\Delta\tilde{\mu}_H$, as the latter is still equal to zero. Accordingly from Eqn. 8 it is derived that:

$$J_{Hi}^0 = n(1-2\alpha)L_\nu A_\nu \tag{16}$$

In experimental practice the reaction time of the proton uptake measurement is often larger than 10 s, so that not so much J_{Hi}^0, but rather $\frac{1}{30}\int_0^{30} J_{Hi}\,dt$ is evaluated and called initial rate. As was shown in section 2.1. it is estimated that under our usual experimental conditions (see the accompanying paper, Ref. 18) the electrical capacity of the bacteriorhodopsin liposomes is so small as to allow the appearance of a significant electrical potential within 3 s: to describe the results obtained in proton uptake experiments, Eqn. 16 cannot be used as a good approximation. Therefore we should use:

$$J_{Hi} = n(1-2\alpha)L_\nu A_\nu + (n^2L_\nu + L_H^1)F\Delta\psi \tag{17}$$

The experimental check of this equation is hindered by the lack of quickly responding and trustable probes of membrane potential. In view of the discussion in the preceding section an alternative approach is possible: using the electroneutral steady-state condition and hence Eqn. 15 and again neglecting the chemical potential differences for all ions, we obtain:

$$J_{Hi}^* = \frac{n(1-2\alpha)L_\nu A_\nu}{1 + \dfrac{n^2L_\nu + L_H^1}{L_K^1 + L_{Cl}^1}} \tag{18}$$

As protonophores, valinomycin and nigericin are expected to increase L_H^1, L_K^1 and L_{KOH}, respectively, their effect on the observed initial proton uptake is predicted to be negative, positive and nil, respectively.

2.3. The steady-state of zero proton uptake. In proton uptake measurements carried out with bacteriorhodopsin liposomes the velocity of proton disappearance from the medium decreases from a high value, when the light is switched on, to zero in an asymptotic fashion.

The $t_{1/2}$ of this process varies with different system parameters, but lies usually around 2 min. It follows that after some 10 min the approximation of zero net proton flow will be operative; the steady state of zero proton uptake can be considered to be reached. In abstract terms:

$$J_H^{**} = 0 \tag{19}$$

Of course this equation does not necessarily imply that bacteriorhodopsin has stopped its pumping activity; the protons still pumped through the bacteriorhodopsin leave the intraliposomal space via the endogenous proton leak of the membrane, via the endogenous H^+/K^+ exchange mechanism(s), and/or via the endogenous HCl permeation through the membrane. Again in more exact form (cf. Fig. 2):

$$0 = J_H^{**} = (1-\alpha)J_H^{\nu+} + \alpha J_H^{\nu-} + J_H^1 + J_H^K + J_H^{Cl} \tag{20}$$

Steady-state condition (Eqn. 19) allows the expression of $(\Delta\mu_H + \Delta\mu_{Cl})$ in terms of the other thermodynamic forces of Eqn. 15:

$$(\Delta\mu_H + \Delta\mu_{Cl})^{**} = \frac{-n(1-2\alpha)L_\nu L_e A_\nu + [L_{eg}L_{KOH} + L_K^1(n^2L_\nu + L_H^1)](\Delta\mu_K + \Delta\mu_{Cl})^{**}}{L_n L_{eg} + L_e(n^2L_\nu + L_H^1)} \tag{21}$$

Physical considerations now lead to further simplification of this formula. It has already been mentioned that bacteriorhodopsin liposomes show steady-state total proton uptake values of the order of 100 μmol/g bacteriorhodopsin. Using the concentration value of 1 g bacteriorhodopsin/20 g lipids, an intraliposomal volume of 0.7 ml/g phospholipids [18], a medium of 150 mM KCl in water, and zero equilibrium values for $\Delta\mu_K$ and $\Delta\mu_{Cl}$ it can be calculated that zero proton uptake steady-state values of $\Delta\mu_K$ and $\Delta\mu_{Cl}$ cannot differ by more than 1.5 mV from zero. A second consideration might be that, apart from water, K^+ and Cl^- are the main determinants of the osmolarity of both the inner and the outer compartment, so that a change in value of $(\Delta\mu_K + \Delta\mu_{Cl})$ by 1% would probably be followed by a change of the internal volume by 5% due to osmotic swelling or shrinking of the liposomes so that the final $(\Delta\mu_K + \Delta\mu_{Cl})$ would still equal zero. A third consideration is that electrocompensatory flows of K^+ and Cl^- will have opposite effects on $(\Delta\mu_K + \Delta\mu_{Cl})$. Clearly for not too small values of $(\Delta\mu_H)^{**}$

$$|(\Delta\mu_K + \Delta\mu_{Cl})^{**}| < |(\Delta\mu_{Cl})^{**}| << |(\Delta\mu_H)^{**}| \tag{22}$$

so that Eqn. 21 can be approximated by:

$$(\Delta\mu_H)^{**} = \frac{-n(1-2\alpha)L_\nu A_\nu}{(n^2L_\nu + L_H^1)\left(1 + \frac{L_n}{L_e}\right) + L_n} \tag{23}$$

Also $(\Delta\psi)^{**}$ can be evaluated: From Eqns. 13, 22 and 23 we derive

$$F(\Delta\psi)^{**} = \frac{-n(1-2\alpha)L_\nu A_\nu}{(n^2L_\nu + L_H^1)\left(1+\frac{L_e}{L_n}\right) + L_e} \tag{24}$$

And to be complete, from Eqns. 23 and 24:

$$(\Delta\tilde{\mu}_H)^{**} = \frac{-n(1-2\alpha)L_\nu A_\nu}{(n^2L_\nu + L_H^1) + \frac{L_nL_e}{L_n+L_e}} \tag{25}$$

The changes brought about by valinomycin and nigericin are instructive. If for simplicity it is assumed that:

$$L_{HCl} << L_{KOH} \tag{26}$$

$$L_{Cl}^1 << L_K^1 \tag{27}$$

then Eqns. 23—25 reduce to:

$$(\Delta\mu_H)^{**} = \frac{-n(1-2\alpha)L_\nu A_\nu}{(n^2L_\nu + L_H^1)\left(1+\frac{L_{KOH}}{L_K^1}\right) + L_{KOH}} \tag{28}$$

$$(F\Delta\psi)^{**} = \frac{-n(1-2\alpha)L_\nu A_\nu}{(n^2L_\nu + L_H^1)\left(1+\frac{L_K^1}{L_{KOH}}\right) + L_K^1} \tag{29}$$

$$(\Delta\tilde{\mu}_H)^{**} = \frac{-n(1-2\alpha)L_\nu A_\nu}{n^2L_\nu + L_H^1 + \frac{L_{KOH}L_K^1}{L_{KOH}+L_K^1}} \tag{30}$$

Now if the proton conductivity of the H^+/K^+ exchange and K^+ leak in series can be neglected relative to the proton conductivity of membrane and bacteriorhodopsin, i.e. if

$$\frac{L_{KOH}L_K^1}{L_{KOH}+L_K^1} << n^2L_\nu + L_H^1 \tag{31}$$

then the above equations can all be equally well approximated by

$$(\Delta\mu_H)^{**} \approx \frac{\Delta\tilde{\mu}_H^{max}}{1+\frac{L_{KOH}}{L_K^1}} \tag{32}$$

$$(F\Delta\psi)^{**} \approx \frac{\Delta\tilde{\mu}_H^{max}}{1+\frac{L_K^1}{L_{KOH}}} \tag{33}$$

$$(\Delta\tilde{\mu}_H)^{**} \approx \Delta\tilde{\mu}_H^{max}\left(\equiv\frac{-n(1-2\alpha)L_\nu A_\nu}{n^2L_\nu + L_H^1}\right) \tag{34}$$

Thus at low valinomycin or nigericin concentrations, the effect of these ionophores on $(\Delta\tilde{\mu}_H)^{**}$ can be neglected, as their effect on $(\Delta\mu_H)^{**}$ is balanced by their effect on $(F\Delta\psi)^{**}$. At higher concentrations the antagonistic effect of the

two ionophores should be accompanied by an effect of lowering $(\Delta\tilde{\mu}_H)^{**}$.

2.4. Time-dependent changes in membrane potential and pH difference across the membrane. Membrane potential and pH difference are caused by the proton pump activity of the bacteriorhodopsin and only secondarily influenced by the passive processes also described in Fig. 2 and by possible feedback of $\Delta\tilde{\mu}_H$ on the pump. It follows that for some time after illumination is started, membrane potential and pH difference can be calculated from the number of protons accumulated inside the liposomes. For this calculation the electrical and buffer capacities of membrane and internal space, respectively, must be used. In steady-state measurements of ΔpH in our standard preparation (Arents, J.C. et al., unpublished results) it was observed that a ΔpH of 1.0 unit is caused by the uptake of about 100 μmol of H^+/g bacteriorhodopsin. This enables us to calculate an average buffer capacity β of about 2.5 mol H^+ · mol BRh^{-1} · pH $unit^{-1}$, which is equivalent to about 6 F/g phospholipid. Remembering the electrical capacity of the system calculated earlier (3 F · g^{-1} phospholipid) and using a value for a truly initial (i.e. $\Delta\tilde{\mu}_H = 0$) J_H the first part of Fig. 4 can be drawn. This Fig. 4 is to represent the present model's prediction of the time dependence of ΔpH and $\Delta\psi$ induced by illumination. Of course this prediction is only valid for a period much shorter than a second as already after 3 s compensatory ion movement abolishes about 98% of the increase in $F\Delta\psi$ (see section 2.1.). Luckily after 3 s of illumination the electroneutral flow condition approximately holds, so that Eqn. 13 becomes valid. Neglecting $\Delta\mu_K^*$ and $\Delta\mu_{Cl}^*$ and using Eqn. 25 this equation can be put into the following form:

$$(F\Delta\psi^*) = \frac{n^2 L_v + L_H^1}{L_{eg}} [(\Delta\mu_H)^{**} - (\Delta\mu_H)^*] + \frac{L_n L_e}{(L_n + L_e) L_{eg}} (\Delta\tilde{\mu}_H)^{**} \qquad (35)$$

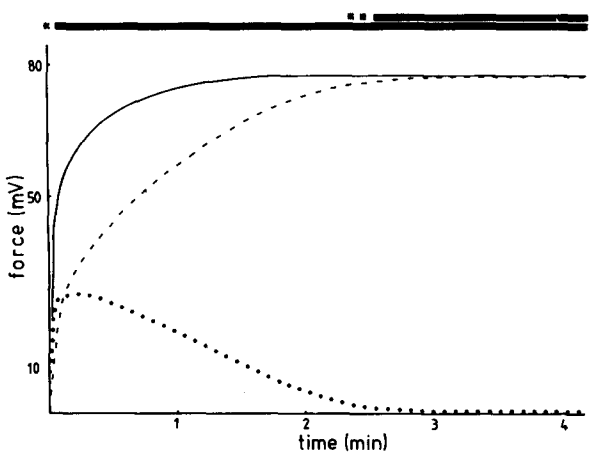

Fig. 4. Predicted time dependence of $\Delta\tilde{\mu}_H$ (———), $\Delta\mu_H$ (- - - - -), and $F\Delta\psi$ (· · · · · ·) in standard bacteriorhodopsin liposomes. For the initial phase, pH and electrical capacities of 6 and 3 F/g phospholipid, respectively, were assumed, together with a truly initial proton uptake of 850 μmol H^+/g bacteriorhodopsin per min. The black bars in the upper part of the figure indicate the assumed steady-state conditions; *, the steady state of electroneutral flow, and **, the steady state of zero net proton uptake. Further details are given in the text.

By adding $(\Delta\mu_H)^*$ one finds:

$$(\Delta\tilde{\mu}_H)^* = (\Delta\tilde{\mu}_H)^{**} - \frac{L_e}{L_{eg}}[(\Delta\tilde{\mu}_H)^{**} - (\Delta\mu_H)^*] + \frac{L_n L_e}{(L_n + L_e)L_{eg}}(\Delta\tilde{\mu}_H)^{**} \quad (36)$$

The implications of these equations become clear, if the (usual) case is considered, in which:

$$\frac{L_n L_e}{(L_n + L_e)} << n^2 L_\nu + L_H^1 \quad (37)$$

(cf. Eqn. 31). For not too low values of $[(\Delta\tilde{\mu}_H)^{**} - (\Delta\tilde{\mu}_H)^*]$ the last term in Eqn. 35 can then be neglected. It can be concluded that during the electroneutral steady state a $(\Delta\psi)^*$ exists which decreases with increasing $(\Delta\mu_H)^*$ for as long as protons are taken up. The size of this initial membrane potential relative to the final proton-motive force depends on the permeability of the membrane for K^+ and Cl^- relative to the permeability for protons: only at low permeability of K^+ and Cl^- a large initial $\Delta\psi$ is expected. In view of the predicted decrease in $(F\Delta\psi)^*$ paralleling the increase in $(\Delta\mu_H)^*$ the question may arise whether $(\Delta\tilde{\mu}_H)^*$ has already reached its maximal value at the beginning of the electroneutral steady state. Eqn. 36 without its last term shows that this is not the case, unless the permeability of the membrane for K^+ and Cl^- is much smaller than that for protons.

Assuming that no gross changes in buffer capacity of the intraliposomal space occur, we can now construct the part of Fig. 4 to the right of the 1—3 s gap: since protons are still being taken up, though at a slower rate, $(\Delta\mu_H)^*$ keeps increasing after $t = 3$ s. Its rate of increase, however, slowly decreases due to back leakage of protons and feedback pressure of $(\Delta\tilde{\mu}_H)^*$ on the pump, which results in a lowered J_H. As the leakage of ions other than protons has reached a high level due to the high membrane potential and because the proton influx dwindles, $\Delta\psi$ slowly diminishes. During the whole period after illumination is started the proton-motive force itself continuously increases, with as asymptote $(\Delta\tilde{\mu}_H)^{**}$ given by Eqn. 25.

2.5. Feedback on photochemical cycle velocity. As mentioned in section 1 the model presented assumes a feedback effect of $\Delta\tilde{\mu}_H$ on the rate at which bacteriorhodopsin pumps protons across the membrane. As this rate is supposed to be coupled to the flow through the bacteriorhodopsin photochemical cycle by a fixed stoichiometric number n, an interesting experiment would be to show the inhibitory effect of $\Delta\tilde{\mu}_H$ on J_ν. However, it is technically difficult to measure J_ν, $\Delta\mu_H$ and $\Delta\psi$ at the same time, so that a different approach should be chosen.

To find out which experiments may be conclusive and which will not, it is useful to apply the calculation method to a slightly altered model. This model, presented in Fig. 3b will be called the ideal current source model (ics model), whilst the model described in Figs. 2 and 3a and section 1 will be called the voltage source model (vs model). Derivation of the analogues of Eqns. 8 to 37 can be accomplished following the recipe presented in section 2.1—2.4. In the following these analogues will be used without derivation.

The first distinction between the ics and the vs models is:

$$\text{vs: } J_\nu = L_\nu(A_\nu + n(1 - 2\alpha)\,\Delta\tilde{\mu}_H) \tag{8}$$

$$\text{ics: } J_\nu = L_\nu A_\nu \tag{8}'$$

but these variables are difficult to measure. It may be more convenient to measure under steady-state conditions. One possibility is the measure J_ν as a function of protonophore, or valinomycin plus nigericin concentration during the $J_H = 0$ steady state. Inserting Eqn. 25 for $\Delta\tilde{\mu}_H$ into Eqn. 8 we derive: (using for simplicity $\alpha = 1$)

$$J_\nu^{**} = L_\nu A_\nu \left[\frac{1}{1 + \frac{n^2 L_\nu}{\gamma_H}}\right] \tag{38}$$

with:

$$\gamma_H = L_H^1 + \frac{L_n L_e}{L_n + L_e} \tag{39}$$

For the ics model:

$$J_\nu^{**} = L_\nu A_\nu \tag{38}'$$

Also Eqns. 18 and 23 for the initial rate of proton uptake and the steady-state transmembrane pH difference, respectively, take a different form in the model without proton back pressure:

$$J_{Hi}^{*} = \frac{n(1 - 2\alpha)\,L_\nu A_\nu}{1 + \frac{L_H^1}{L_e}} \tag{18}'$$

$$(\Delta\tilde{\mu}_H)^{**} = \frac{-n(1 - 2\alpha)\,L_\nu A_\nu}{L_H^1\left(1 + \frac{L_n}{L_e}\right) + L_n} \tag{23}'$$

The absence of L_ν (the proton pump's activity parameter) in the denominators of these equations is characteristic.

Further derivation of equations relating the fluxes of Cl^- and K^+ to the thermodynamic forces in the system are not given here, since no experimental tests on these equations have been carried out. With the use of the appropriate ion-selective electrodes also those equations can, in principle, be subjected to critical tests.

In the following article [18] results will be presented of the critical tests to which the above-derived equations were subjected. It will be shown that the description of bacteriorhodopsin vesicles, given here, can survive these tests. A clear thermodynamic picture of bacteriorhodopsin will evolve from these experiments: bacteriorhodopsin acting as a voltage source, switched on by illumination.

Acknowledgements

The authors wish to thank Karel van Dam for inspiration, encouragement and profitable criticism on the manuscript and Joseph Tager for his help in preparing the manuscript.

References

1 Katchalsky, A. and Curran, P.F. (1974) Non-equilibrium Thermodynamics in Biophysics, 2nd edn., Harvard University Press, Cambridge
2 Mitchell, P. (1961) Nature 191, 144—148
3 Popper, K.R. (1969) Conjectures and Refutations, Routledge and Kegan Paul, London
4 Kuhn, Th.S. (1970) The Structure of Scientific Revolutions, 2nd edn., The University of Chicago Press, Chicago
5 Boyer, P.D., Chance, B., Ernster, L., Mitchell, P., Racker, E. and Slater, E.C. (1977) Annu. Rev. Biochem. 46, 955—1026
6 Mitchell, P. (1976) Biochem. Soc. Trans. 4, 399—430
7 Azzone, G.F., Pozzan, T. and Massari, S. (1978) Biochim. Biophys. Acta 501, 307—316
8 Van Dam, K., Wiechmann, A.H.C.A., Hellingwerf, K.J., Arents, J.C. and Westerhoff, H.V. (1978) in Proc. of the 11th FEBS Meeting Copenhagen (Nicholls, P., Møller, J.V., Jørgensen, P.L. and Moody, A.J., eds.), Vol. 45, pp. 121—132, Pergamon Press, Oxford
9 Gould, J.M. and Cramer, W.A. (1977) J. Biol. Chem. 252, 5875—5882
10 De Groot, S.R. (1952) Thermodynamics of Irreversible Processes, North-Holland, Amsterdam
11 De Groot, S.R. and Mazur, P. (1962) Non-equilibrium Thermodynamics, North-Holland, Amsterdam
12 Mitchell, P. (1966) Chemiosmotic Coupling in Oxidative and Photosynthetic Phosphorylation, Glynn Research, Bodmin
13 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
14 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
15 Slater, E.C., Rosing, J. and Mol. A. (1973) Biochim. Biophys. Acta 292, 534—553
16 Racker, E. (1973) Biochem. Biophys. Res. Commun. 55, 224—230
17 Kayushin, L.P. and Skulachev, V.P. (1974) FEBS Lett. 39, 39—42
18 Hellingwerf, K.J., Arents, J.C., Scholte, B.J. and Westerhoff, H.V. (1979) Biochim. Biophys. Acta 547, 561—582
19 Mueller, P., Rudin, D.O., Ti Tien, 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
20 Bangham, A.D., De Gier, J. and Greville, G.D. (1967) Chem. Phys. Lipids 1, 225—246
21 Bakker, E.P., Rottenberg, H. and Caplan, S.R. (1976) Biochim. Biophys. Acta 440, 557—572
22 Michel, H. and Oesterhelt, D. (1976) FEBS Lett. 65, 175—178
23 Lakshminarayanaiah, N. (1969) Transport Phenomena in Membranes, Academic Press, New York
24 Davies, M. (1965) Biophys. J. 5, 651—654
25 Scibona, G., Scuppa, B., Fabiani, C. and Ciavola, C. (1978) Biochim. Biophys. Acta 506, 30—41
26 Onsager, L. (1931) Phys. Res. 37, 405—426
27 Nicholls, D.G. (1977) Eur. J. Biochem. 77, 349—356
28 Dunlop, P.J. and Gosting, L.J. (1959) J. Phys. Chem. 63, 86—93
29 Rottenberg, H. (1973) Biophys. J. 13, 503—511
30 Van der Meer, R., Akerboom, T.P.M., Groen, A.K. and Tager, J.M. (1978) Eur. J. Biochem. 84, 421—428
31 Nicholls, D.G. (1974) Eur. J. Biochem. 50, 305—315
32 Sone, N., Yoshida, M., Hirata, H. and Kagawa, Y. (1977) J. Biol. Chem. 252, 2956—2960
33 Bakker-Grünwald, T. and Van Dam, K. (1973) Biochim. Biophys. Acta 292, 808—814
34 Racker, E. and Kandrach, A. (1971) J. Biol. Chem. 246, 7069—7071
35 Fettiplace, R., Andrews, D.M. and Haydon, D.A. (1971) J. Membrane Biol. 5, 277—296
36 Montal, M. and Mueller, P. (1972) Proc. Natl. Acad. Sci. U.S. 69, 3561—3566
37 Montal, M. (1976) Annu. Rev. Biophys. Bioeng. 5, 119—175
38 Coster, H.G.L. (1965) Biophys. J. 5, 669—686
39 Papahadjopoulos, D. and Watkins, J.C. (1967) Biochim. Biophys. Acta 135, 639—652