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Bacteriorhodopsin in Liposomes: Quantitative Evaluation of ΔpH Changes Induced by Variations of Light Intensity and Conductivity Parameters

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Summary. A description of ion movement and energy transduction in terms of a kinetic variant of nonequilibrium thermodynamics was subjected to experimental tests in bacteriorhodopsin liposomes. The effects of variation of light intensity, proton permeability, proton-potassium ion exchange activity, and potassium ion permeability on the steady-state pH gradient were in quantitative agreement with the predictions of the theoretical description. It is suggested that the theoretical description will be useful in other, more complex systems to gain detailed information about their energy transduction and ion permeation properties.

Abbreviations

S13; 5-chloro-3-*tert*-butyl-2′-chloro-4′-nitrosalicylanilide 1799; (hexofluoro-acetonyl) acetone

Symbols

	Meaning	*Manipulated by*
A_v	effective energy input of the photon	constant
ΔpH	pH gradient (out-in)	several methods
L_e	nonproton electric permeation coefficient	valinomycin
L^l_H	electric proton leakage coefficient	protonophore
L_n	electroneutral proton permeation coefficient	nigericin
L_v	activity of bacteriorhodopsin	light intensity
n	number of protons pumped per photocycle	constant
α	inward pumping fraction	preparation method

Bacteriorhodopsin has played an important role in demonstrating the feasibility of one of the fundamental concepts of the chemiosmotic hypothesis (Mitchell, 1961), i.e. the proton as coupling agent in bioenergy transduction (Slater, 1977). Furthermore, it may well become the first proton pump the mechanism of which is elucidated at the molecular level (Honig, Ebrey, Callender, Dinur & Ottolenghi, 1979). Its potential as an alternative for mineral oil is still under discussion (Singh & Caplan, 1980). Because bacteriorhodopsin has been well characterized and because it can be easily purified to homogeneity (Stoeckenius, Lozier & Bogomolni, 1979), it may in its reconstituted form serve as a model system to study different aspects of biological energy transduction. In principle any enzyme that catalyzes active transport in which an ion gradient or a membrane potential is involved may fruitfully be studied after its reconstitution into a closed lipid bilayer already containing bacteriorhodopsin (co-reconstitution, *see* e.g. Hellingwerf, 1979). There are few other possibilities to study the pure enzyme with regard to the quantitative dependence of the rate of transport on different energization parameters.

A special type of kinetic theory was developed (Van Dam & Westerhoff, 1977; Westerhoff & Van Dam, 1979; *see also* Hill, 1979) to evaluate the coupling between different transport systems and metabolic reactions, and to facilitate the assertion of the influence of transport systems on the larger metabolic conglomerates they are part of. In fact, this theory is a mixture of linear irreversible thermodynamics (Onsager, 1931; Katchalsky & Curran, 1967; Rottenberg, 1979) and kinetics: on the one hand it speaks in terms of free energy differences as the driving forces for reactions, whereas on the other hand, and contrary to the earlier application of irreversible thermodynamics to bioenergetics, characteristics of the underlying mechanisms are retained in the formulae that describe the total system. The theory has been elaborated and in part tested for mitochondrial oxidative phosphorylation (Van Dam & Westerhoff,

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1977; Van Dam, Westerhoff, Krab, Van der Meer & Arents, 1980). Although the outcome of the tests was positive, they could not be rigid enough due to the complexity of the mitochondrial system. The most rigid tests can only be carried out in well-defined and pure systems. Then one can be sure that only one system is considered at a time. Reconstituted bacteriorhodopsin liposomes (Hellingwerf, 1979) form such a system.

The theoretical description has, therefore, been elaborated for the system of reconstituted bacteriorhodopsin liposomes (Westerhoff, Scholte & Hellingwerf, 1979). Semi-quantitative tests (Hellingwerf, Arents, Scholte & Westerhoff, 1979) have not only led to semi-quantitative confirmation of the validity of the theoretical description, but also to some conclusions about the functioning of bacteriorhodopsin itself. For instance, this theoretical description quantitatively predicts how the steady-state pH gradient in bacteriorhodopsin liposomes depends on the intensity of illumination, the proton conductivity of the liposomal membrane, and the K^+/H^+-exchange activity of the membrane. In this article these predictions will be shown to be confirmed by the appropriate experimental tests.

Elements of this work have been published as part of a Ph.D. thesis (Hellingwerf, 1979).

Materials and Methods

Bacteriorhodopsin liposomes were prepared as decribed (Hellingwerf, Scholte & Van Dam, 1978*a*) by sonication for 60 times 15 sec (each followed by 45 sec silence, room temperature). Unless stated otherwise, the transmembrane pH difference was determined from [^{14}C] aminomethane uptake using the flow dialysis procedure (Colowick & Womack, 1969) with the interpretation method described earlier (Hellingwerf et al., 1979). Valinomycin was added at the concentrations indicated in order to quickly attain the steady states. The vessel also contained an Ingold 10 403 3095 pH electrode. The internal volume of the bacteriorhodopsin liposomes was determined from their retention of K^+ upon passage through a Sephadex G50 coarse column equilibrated with iso-osmotic choline chloride. Unless otherwise indicated the flow medium consisted of 250 mM K_2SO_4, 1 mM (K)EDTA, pH 6.0. The lamp was replaced by a 150 W, 20 V Xenon lamp (Osram), equipped with two heat filters and a flexible light guide. Illumination intensity was about 0.17 kW/m^2 onto the cylindrical vessel 0.75 cm in diameter. Where indicated, light intensity was varied with neutral density filters (Oriel Corporation, Stanford, Conn.). The total ethanol concentration was kept below 2.0% (vol/vol). At this concentration ethanol had no effect on the decay rate of the pH gradient after stopping illumination, nor on the initial rate of proton uptake after onset of illumination.

Proton uptake into the bacteriorhodopsin vesicles was assayed either with the pH meter described by Hellingwerf et al. (1979), or in a 1 ml vessel, equipped with a magnetic stirrer. Illumination was then carried out with the lamp described above. The number of protons corresponding to a measured pH shift (Radiometer phm 64 research pH meter, Ingold 10 403 3059 pH electrode) was determined by adding known amounts of oxalic acid, before ionophores had been added. 1-α-Phosphatidyl choline (type V-e from egg yolk, 100 g/liter in chloroform/methanol, 9/1) was purchased from Sigma Chemical Company. Soybean phospholipids were isolated as described by Kagawa and Racker (1971). [^{14}C]Aminomethane (250 μCi/ml, 2.23 mM) and [^{3}H]H_2O (5 mCi/ml) were purchased from Amersham Radiochemical Centre, England. 5-Chloro-3-*tert*-butyl-2'-chloro-4'-nitrosalicylanilide (S13) was a gift from Dr. P. Hamm, Monsanto Company, St. Louis, Mo. Nigericin and valinomycin were gifts from Dr. W.C. Pettinga, Eli Lilly and Company, Indianapolis, Ind. α,α'-bis(hexafluoro-acetonyl)acetone (1799) was a gift from Dr. P. Heytler, Dupont de Nemours and Co., Wilmington, Del. Chlorpromazine was purchased from Serva, Heidelberg. All other reagents were of analytical grade. Twice-distilled water was used. All experiments were carried out at 300 K.

Results

The quantitative description of bacteriorhodopsin reconstituted in liposomes (Westerhoff et al., 1979) yielded a theoretical relation between the steady-state pH gradient, the effective thermodynamic force exerted by absorbed photons (called A_v), the light intensity, and the different permeability parameters of the liposomal membrane. An additional parameter L_v, present in this relation, indicates the activity of bacteriorhodopsin (in terms of cycle turnovers/min/unit of effective photon energy). Hellingwerf et al. (1979) established that L_v is not only proportional to bacteriorhodopsin concentration, but also to light intensity. A_v, equaling that portion of the energy of the absorbed photon that is made available for the proton pump, was independent of light intensity, though its exact value is still unknown (Hellingwerf, 1979; Hellingwerf et al., 1979). To test this relation and thereby the quantitative description, we will consider variation of light intensity, proton permeability, and proton-potassium exchange activity. We rewrite Equation 23 in Westerhoff et al. (1979) as the dependence of the inverse of the pH gradient on all other parameters:

$$\frac{A_v}{\Delta \mathrm{pH}} = \frac{(n^2 L_v + L_{\mathrm{H}}^l)\left(1+\frac{L_n}{L_e}\right)+L_n}{-n(1-2\alpha)L_v}. \qquad (1)$$

The equation predicts a linear relation between the inverse of the pH gradient ($1/\Delta$pH) and the inverse of the light intensity (which is proportional to L_v; Hellingwerf et al., 1979), the slope of which is affected by the proton permeability of the membrane (L_{H}^l), the electroneutral proton exchange or symport activity (L_n) and the other anion and cation permeabilities (L_e). There is also an effect exerted by the photon (A_v), the number of protons pumped per photochemical cycle (n), and the orientation of bacteriorhodopsin (α = the fraction of bacteriorhodopsin

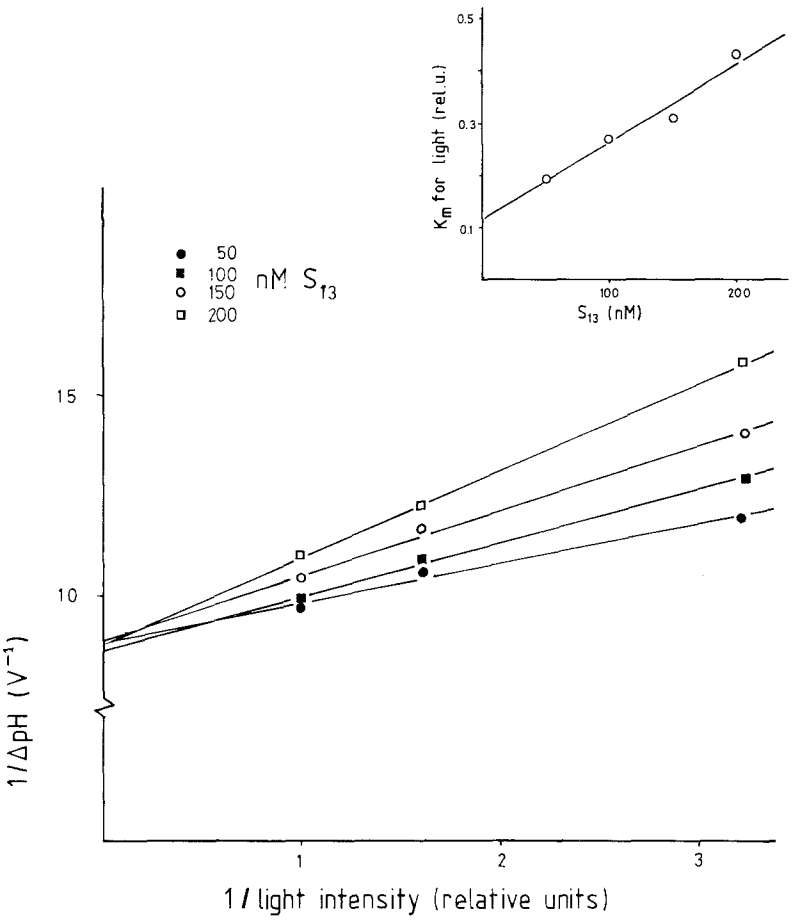

Fig. 1. Effect of protonophore on the relation between pH gradient and light intensity. 0.70 ml of bacteriorhodopsin liposomes (25 g/liter egg phosphatidyl choline, 2.5 g/liter bacteriorhodopsin, 250 mM K_2SO_4, 1 mM (K) EDTA, pH 6.0) were incubated with valinomycin (0.20 mg/g lipid). S13 concentrations were: (●) 0.050 μM, (■) 0.10 μM, (○) 0.15 μM, and (□) 0.20 μM. The insert shows the $K_m \equiv$ the inverse of the intercept of the (1/light intensity) axis, as a function of S13 concentration. Ten minutes were allowed for every condition to reach its steady state. The pH gradient is expressed in volts, using that at 300 °K one pH unit corresponds to 59.5 mV

with *in vivo* orientation). A_v is constant (Hellingwerf et al., 1979), α is constant for the preparation (about 0.8; Hellingwerf, Tegelaers, Westerhoff, Arents & Van Dam, 1978*b*) and n is assumed to be constant (a constant pump stoichiometry is one of the assumptions in the theoretical model (Westerhoff et al., 1979)). For simplicity's sake we may therefore equate them to A_v, 1, and 1, respectively. (Note, however, that the value of n is still under discussion and may, in fact, equal 2. *See* e.g. Ort and Parson, 1979 and Keszthelyi and Ormos, 1980). Eq. (2) is the result of these simplifications:

$$\frac{A_v}{\Delta \mathrm{pH}} = \left(L_{\mathrm{H}}^{l}\left(1+\frac{L_n}{L_e}\right)+L_n\right)\frac{1}{L_v}+\left(1+\frac{L_n}{L_e}\right). \qquad (2)$$

This equation predicts that the inverse of the pH gradient varies linearly with the inverse of the light intensity. We determined the pH gradient from the steady-state uptake of aminomethane, using flow dialysis as the assay method. A doubly reciprocal plot of the pH gradient versus the light intensity does indeed yield a straight line (correlation coefficient exceeding 0.990; Westerhoff et al.,1981).

In view of the evaluation of the efficiency of energy conversion in bacteriorhodopsin liposomes, it is relevant to study the effect of increased proton permeability, the permeability unique in its straightforward dissipation of proton-motive energy (Hellingwerf et al., 1979). Eq. (2) states that an increase in proton permeability L_{H}^{l} will increase the slope of the straight line in the doubly reciprocal plot without affecting the (1/ΔpH) axis intercept. We varied L_{H}^{l} by titrating with the protonophore S13 (Williamson & Metcalf, 1967). The interpolation necessary for an accurate interpretation of the flow dialysis results (*cf.* Hellingwerf et al., 1979) and the duration of the experiments, limit the number of experimental points per experiment. We therefore omitted the points without protonophore, so that waiting for the highest steady-state ΔpH became unnecessary. The results (Fig. 1) show that the slope of (1/ΔpH) versus (1/light intensity) indeed increases with increasing protonophore concentration, whereas the (1/ΔpH) axis intercept is not affected, as was predicted.

The linear doubly reciprocal relation between pH gradient and light intensity reflects a light-saturation effect. In an earlier paper (Hellingwerf et al., 1979)

we interpreted light saturation at these low-light intensities on the initial rate of proton uptake and the steady-state proton uptake as evidence for a back-pressure effect of the protonmotive force on the bacteriorhodopsin pump rate. In the same article an equation was derived that evaluates the half-saturating light intensity:

$$\text{“}K_m \text{ for light”} = L_H^l + \frac{L_n L_e}{L_n + L_e}. \quad (3)$$

An increase in proton permeability of the liposomal membrane (L_H^l) is predicted to increase the K_m for light intensity: the back pressure decreases with increasing proton leakage. Qualitatively this effect is already observed in Fig. 1, but the insert shows that the “K_m” values measured are indeed linearly related to the amount of protonophore added.

Here the assumption was used that proton permeability increases linearly with added protonophore concentration. To investigate this, bacteriorhodopsin liposomes were incubated in a vessel containing a pH electrode. Then they were illuminated until the steady state of proton uptake (‘static head’) had been reached. After the light was switched off, the proton efflux was followed and, especially at that point where a certain number of protons (n) had not yet leaked back out of the liposomes, the efflux rate was determined. This experiment was then repeated at different protonophore concentrations, the efflux rate being measured at the same value of n. Although the pH gradient is generally not proportional to the proton uptake (Arents et al., *in preparation*) it certainly is a function of it, so that identical values of n mean identical values of the pH gradient. It follows that the determined efflux rates are direct, proportional measures of proton permeabilities (L_H^l) and should, if the above assumption is correct, also increase linearly with the protonophore concentration. Fig. 2 confirms this, at least for the concentration range of the protonophore in which it was used in the experiments described here. Consequently in Fig. 1 the S_{13} concentration can be considered as linearly related to the proton permeability. That the proton efflux rate is linear, but not proportional to the S_{13} concentration, reflects the endogeneous permeability for protons of the liposomal membranes.

To examine in more detail whether the theory would be in accordance with experimental results at varying protonophore concentrations we did the experiment suggested by Eq. (2), if it was written in the form of Eq. (4):

$$\frac{A_v}{\Delta \text{pH}} = \left(\frac{1}{L_v} + \frac{L_n}{L_v L_e}\right) L_H^l + \left(\frac{L_n}{L_v} + \frac{L_n}{L_e} + 1\right). \quad (4)$$

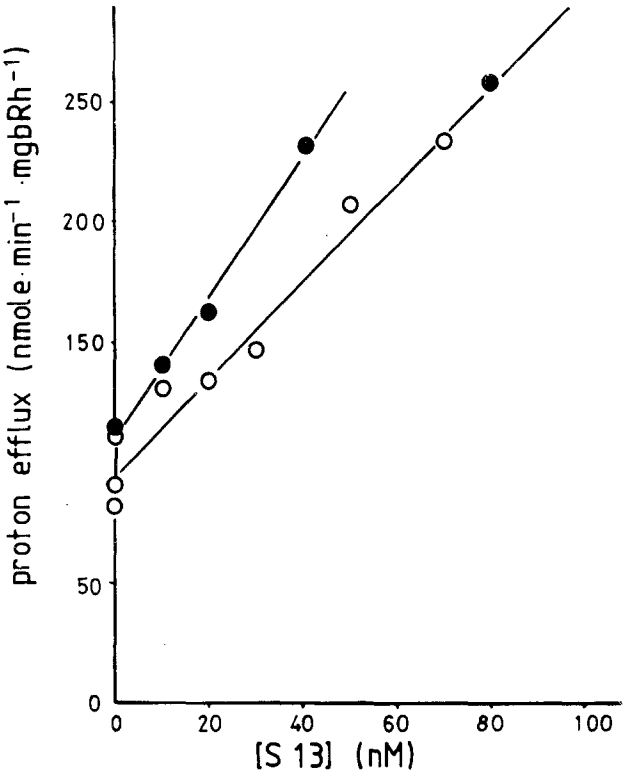

Fig. 2. The proton permeability coefficient of the liposomal membrane depends linearly on the concentration of added S13. Bacteriorhodopsin liposomes (30 g/liter egg phosphatidyl choline, 3 g/liter bacteriorhodopsin, 250 mM K_2SO_4, 1 mM (K) EDTA, pH 6.0) were prepared. 0.15 ml was mixed with 0.85 ml of the same medium. 0.2 mg valinomycin/g phospholipid was added. By interrupted illumination the passive proton efflux in the dark at a certain residual proton uptake was measured at increasing S13 concentrations (closed dots). The points indicated by the open dots were obtained the next day after overnight incubation at 4 °C and with a slightly different dilution of the liposomes, i.e. 0.10 ml + 0.90 ml. The lines are linear least-squares fits with correlation coefficients 1.00 and 0.98, respectively (the dot at the highest proton efflux rate was not included in the calculation)

We measured the pH gradient by two independent methods which gave analogous, positive results. Fig. 3 shows the experiment in which we measured the steady-state pH gradient by measuring steady-state proton uptake into bacteriorhodopsin liposomes containing a buffer mixture with pH-independent buffer capacity (Hellingwerf, 1979). The inverse of the pH gradient varies linearly with proton permeability, both slope and intercept being affected by the light intensity. The parallel flow dialysis experiment gave similar results that scattered less at high ΔpH values.

Having examined the effects of variation of the rate of pumping of bacteriorhodopsin (by light intensity) and of the proton back leakage (by protonophore), we evaluated how an increased permeability for “neutral” protons would affect the pH

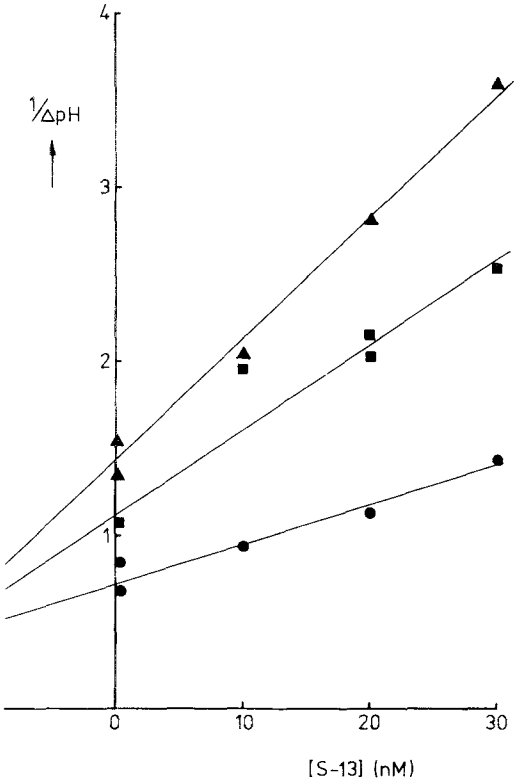

Fig. 3. Relation between pH gradient and protonophore concentration at different light intensities. Bacteriorhodopsin liposomes containing a buffer mixture with pH-independent buffer capacity (80 g/liter soybean phospholipid, 8 g/liter bacteriorhodopsin, 30 mM (NaK) tartrate, 12.5 mM (K) citrate, 50 mM (K) phosphate, 50 mM (K) oxalate, 37.5 mM (Na) β-glycerol phosphate, 62.5 mM (Na) pyrophosphate, 1 mM (K) EDTA, 165 mM K_2SO_4, 165 mM Na_2SO_4) were diluted sixfold with a medium containing 0.22 M K_2SO_4 plus 0.33 M Na_2SO_4, pH 6.8. For determinations of the intraliposomal buffer capacity the extravesicular medium was replaced by the latter medium, using the Sephadex-column centrifugation method (Penefski, 1977). Then the internal buffer capacity was determined from the difference in final and initial external pH change after addition of a known amount of acid (*cf.* Mitchell & Moyle, 1967) and was found to be in close agreement with that calculated from the buffer capacity of the buffer mixture and the internal volume determined by potassium retention on a Sephadex column. In all incubations 3 μM valinomycin was present. Intravesicular pH changes were calculated from proton uptake and the buffer capacity essentially as described by Mitchell and Moyle (1967). Light intensities were 100% (●), 62% (■), and 31% (▲). The pH gradient is expressed in units (1 unit is equivalent to 59 mV at room temperature)

gradient. To investigate this the K^+/H^+-exchanger nigericin (Ovchinnikov, Ivanov & Shkrob, 1974) was used. It is expected that the electroneutral proton permeability coefficient ($L_n = L_{KOH} + L_{HCl}$) increases linearly with the number of active nigericin molecules. We checked this by measuring how, at a given magnitude of the pH gradient, the rate of decay of the pH gradient is stimulated by a certain concentration of added nigericin. We use proton uptake as a unique function of the pH gradient (*cf.* the

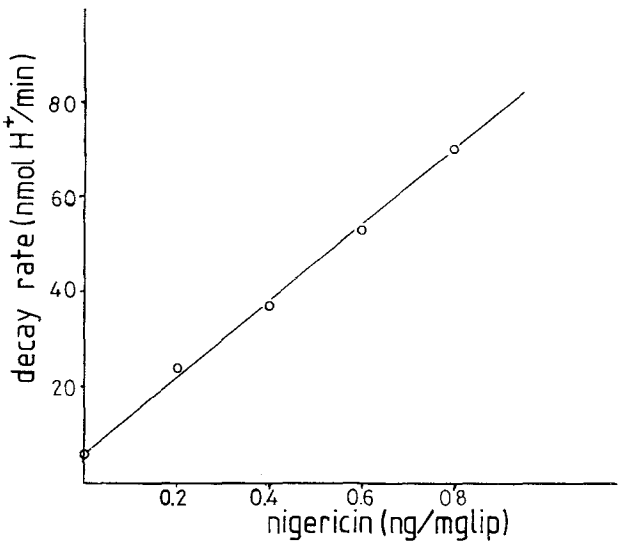

Fig. 4. L_n, the electroneutral proton permeability coefficient is linear with added nigericin concentration. 0.7 ml of a suspension of bacteriorhodopsin liposomes (25 g/liter egg phosphatidyl choline, 2.5 g/liter bacteriorhodopsin in 250 mM K_2SO_4, 1.0 mM (K) EDTA, pH 6.0) was placed in the flow dialysis vessel. In light on, light off, nigericin addition, light on, etc., cycles the proton efflux in the dark period at a residual proton uptake of 0.058 mmol/g bRh (maximum extent was 0.17 mmol H^+/g bRh) was measured as a function of the concentration of added nigericin. The ethanol added was checked to have no effect of itself

experiments of Fig. 2). Fig. 4 gives the results of these experiments: up to (added) nigericin concentrations of 0.8 ng/mg the decay rate is linearly related to the added nigericin concentration. Thus nigericin can be used to vary L_n in a linear way. It also appears that the endogenous (i.e. in the absence of added ionophores) proton permeability, which is the sum of electric (H^+ or OH^-, denoted by $L^l_{H\text{-endogenous}}$) and electrically silent (HCl or KOH, denoted by $L_{n\text{-endogenous}}$) proton permeability is low in comparison to the variation induced by the titration with nigericin. Consequently the point where $L_n = 0$ (i.e. added nigericin equivalent to $-L_{n\text{-endogenous}}$) must lie close to (and on the left of) the decay rate axis. The following reformulation of Eq. (2) stresses the predictions that are relevant for the nigericin titrations:

$$\frac{A_v}{\Delta \mathrm{pH}} = \left(\frac{1}{L_v} + \frac{L^l_H}{L_e L_v} + \frac{1}{L_e}\right) L_n + \left(1 + \frac{L^l_H}{L_v}\right). \tag{5}$$

The inverse of the pH gradient should depend linearly on the concentration of added nigericin. Fig. 5 (open dots) shows that this is confirmed by experimental results.

After this analysis of the effect of light intensity and two types of ionophores separately, experiments were set up to see whether our theory could be used to analyze the combined (inter-?)action of iono-

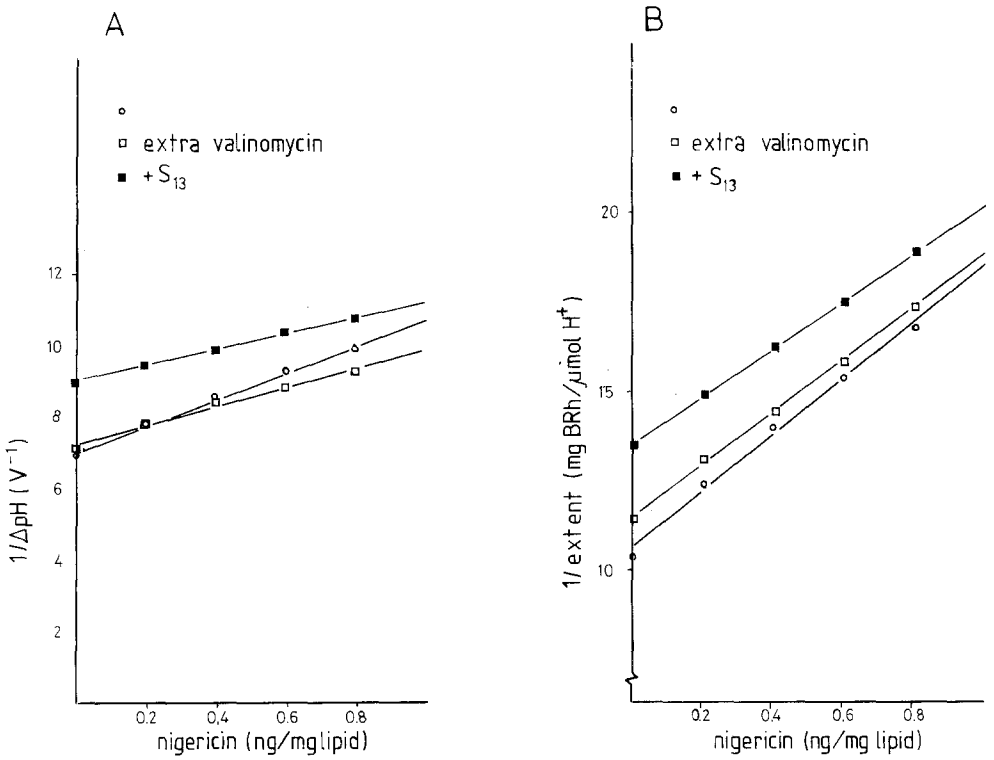

Fig. 5. Effect of nigericin on the steady-state pH gradient. 0.7 ml of the suspension of bacteriorhodopsin liposomes that was also used in the experiment of Fig. 4, was preincubated for 15 min with valinomycin (0.20 mg/g lipid (○), or in the case of (□) "extra valinomycin", 1.0 mg/g lipid) or in the presence of both 0.20 mg/g lipid valinomycin and 0.20 μM S13 (■). At $t=0$ min 20 μl of the [^{14}C]aminomethane stock and 7 μl of the [^{3}H]H_2O stock were added. At $t=15$ min illumination was started. Nigericin additions followed at $t=40$, 50, 60 and 70 min. At $t=80$ min illumination was stopped and the decay of the pH gradient was followed until $t=100$ min. The internal volume of the liposomes was determined at 0.62 μl/mg lipid. *A*. Shows the result in terms of ΔpH calculated from the uptake of aminomethane, the straight lines are linear least-squares best fits with correlation coefficients 0.998, 0.989 (extra valinomycin), and 0.999 (S13) respectively. *B*. Shows the results read from the simultaneous pH recordings

phores. Eq. (5) suggests some possible experiments: the slope of the line in the plot $(1/\Delta\text{pH})$ versus nigericin concentration should be decreased by valinomycin. Furthermore the point at $L_n=0$, which (*see above*) almost equals the $(1/\Delta\text{pH})$ intercept, should be unaffected by this K^+ ionophore (Ovchinnikov et al., 1974). Fig. 5*a* confirms this; the very slight decrease in ΔpH at zero nigericin (*see also* Hellingwerf et al., 1978*b*) being explicable by the expected partial inactivation of bacteriorhodopsin by the added valinomycin (Rott & Avi-Dor, 1977). In Fig. 5*b* the proton uptake monitored in the same experiment is plotted in a fashion similar to Fig. 5*a*. Qualitatively the same results are obtained, although a minor drift in the pH electrode caused an error in the proton uptake determination, as can be concluded from comparison of the points at zero nigericin with the corresponding points in Fig. 5*a*. Generally speaking the steady-state extent of proton uptake can be used as a quantitative, but not as a linear measure of the steady-state pH gradient. Only if the buffer capacities are pH-independent (Arents et al., *in preparation*), the pH gradient will be a linear function of the proton uptake.

An analogous experiment tracing the combined action of nigericin and the protonophore S13 is also possible. Eq. (5) predicts that S13 will increase both the slope and the $(1/\Delta\text{pH})$ intercept of the plot of $(1/\Delta\text{pH})$ versus nigericin concentration. Here an anomaly is found: although the $(1/\Delta\text{pH})$ intercept increases, thereby indicating protonophore activity, the slope decreases (*cf.* Fig. 5).

Thus in this case the theoretical description does not appear to describe the combined effect of the ionophores used in this experiment. At this point two possible interpretations of this apparent shortcoming of the theory were considered: (1.) The theory does not correctly describe the combined effect of variation of the different permeability parameters L_n and L_H^i. (2.) The theory is correct, but the ionophores used have other effects than the assumed linear variation of the corresponding permeability parameters.

The first possibility might, for instance, be due to

the breakdown of the postulated proportional relations between flows and forces (free energy gradients; Westerhoff et al., 1979) in the ΔpH range studied. The second possibility might be a result of direct interaction between the ionophores (Anraku, 1979). The experimental approach is obvious: see whether variation of the pH gradient in the same ΔpH range, but induced by varying a different coefficient, would give results in agreement with, or in contrast to, Eq. (5). Inspection of the equation shows that a decrease in intensity of the illumination (L_v) is predicted to have almost the same effect as an increase in the proton conductance of the membrane (L^l_H).

Fig. 5 shows that to tackle this problem one may just as well measure the extent of proton uptake, which is a unique though not linear, function of the pH gradient: also in Fig. 5*b* S13 decreases rather than increases the slope. Therefore, we measured the effect of nigericin addition on this parameter at full and at reduced light intensity. Table 1 shows that, while S13 again had the anomalous effect, the reduction of light intensity did indeed increase both the (1/extent) intercept as well as the slope of the line the latter being monitored by the difference in inverse extent that is induced by nigericin. Thus the first of the two above-mentioned interpretations could be ruled out, and the second interpretation had to be examined.

First it was checked that the anomaly was not due to an S13-specific effect. The lipid-soluble protonophore 1799 (*cf.* Table 1) and the water-soluble 2,4-dinitrophenol gave similar results. Then the conditions of the experiments were critically re-examined in view of possible ionophore interactions. In all experiments we used a small amount of valinomycin in order to reduce the time needed to attain the steady-state ΔpH. This is necessary to reduce experimental variation due to pH drift of the pH electrode or the interpolation that is necessary in the flow dialysis experiments (Hellingwerf et al., 1979). The observed anomaly could be explained, however, if an interaction between valinomycin and S13 existed that would increase the activity of the former (*cf.* O'Brien, Nieva-Gomez & Gennis, 1978; Yamaguchi & Anraku, 1978; Anraku, 1979). On top of the predicted effect on the line in Fig. 5 there would be the effect of apparently adding extra valinomycin. The intercept would still increase, but the effect on the slope would become unpredictable.

In order to prove this point, we omitted valinomycin in an experiment analogous to the experiment of Table 1, except for the fact that soybean phospholipids replaced the egg phosphatidylcholine. With the former lipids steady states are reached more

Table 1. Effect of light intensity and two types of protonophore on the nigericin-induced decrease of proton uptake[a]

Condition	Extent of proton uptake (nmol H^+/mg bacteriorhodopsin)		Δ(1/extent) (g bRH/mmol H^+)
	−nigericin	+nigericin	
Control	119	96	1.8
Reduced illumination	74	56	4.4.
S13	80	75	0.85
1799	90	85	0.60

[a] 0.2 ml of a suspension of bacteriorhodopsin liposomes (25 g/liter egg phosphatidyl choline, 2.5 g/liter bacteriorhodopsin, 250 mM K_2SO_4, 1 mM (K) EDTA, pH 6.0) was diluted with 0.8 ml of the same medium and illuminated in the presence of 1.0 µg valinomycin and, if indicated, 4.0 ng nigericin, 0.20 nmol S13, or 1.0 nmol 1799. Where indicated light intensity was reduced to 24% by use of a neutral density filter.

Table 2. Anomalous effect of S13 on the nigericin-induced ΔpH decrease correlates with the presence of valinomycin[a]

Valinomycin	S13	Extent of proton uptake (nmol H^+/mg bacteriorhodopsin)		Δ(1/extent) (g bRH/mmol H^+)
		−nigericin	+nigericin	
+	−	98, 100	82	2.0, 2.2
+	+	70	63, 65	1.7, 1.3
−	−	70, 73	43	9.0, 9.6
−	+	60	32, 30	15, 16

[a] 0.10 ml of a suspension of bacteriorhodopsin liposomes (20 g/liter soybean phospholipids, 2 g/liter bacteriorhodopsin in 150 mM KCl) was diluted in 0.9 ml 150 mM KCl (final pH 5.3). Where indicated 0.40 µg valinomycin, 1.0 ng nigericin, or 1.0 nmol S13 was added.

quickly. Table 2 proves that, in the absence of valinomycin, S13 has the straightforward effect of reenforcing the nigericin effect on 1/extent. With egg phosphatidylcholine these results could be confirmed, be it that the experiments showed more scatter (reflecting the effects of electrode drift being more relevant in these slower experiments). The suggested (Anraku, 1979) mechanism for interaction between valinomycin and a negatively charged protonophore is that the anionic form of the protonophore shields the charge of the K^+-loaded valinomycin and thus facilitates its permeation through the bilayer. A last method to check our explanation was to use a positively charged protonophore. Table 3 shows that such an uncoupler (chlorpromazine) does indeed increase the effect of nigericin on 1/ΔpH, even in the presence of valinomycin. Because in this Table the pH gradient itself, rather than the extent of proton

Table 3. The positively charged protonophore chlorpromazine does increase the nigericin dependence of $(1/\Delta pH)$[a]

Chlorpromazine (mM)	ΔpH (mV)		Δ(1/ΔpH) (V^{-1})
	−nigericin	+nigericin	
0	133	99	2.6
0.10	130	88	3.7
0.30	122	74	5.3

[a] The upper compartment of the flow dialysis vessel contained 0.7 ml of a suspension of bacteriorhodopsin liposomes (25 g/liter egg phosphatidyl choline, 2.5 g/liter bacteriorhodopsin, 250 mM K_2SO_4, 1 mM (K) EDTA, pH 5.9), 3.5 μg valinomycin, 20 μl [^{14}C]aminomethane, 7 μl [^{3}H]H_2O, and the indicated concentrations of chlorpromazine. Total amount of ethanol at most 12 μl. After 15-min preincubation and 30-min illumination, 14 ng nigericin was added. After another 35 min the light was turned off. The internal volume of the vesicles was taken as 0.62 ml/g lipid (*cf.* Fig. 5).

uptake, is shown, the solution of the anomaly cannot be an artifact due to a nonlinear relation between extent of proton uptake and pH gradient.

Discussion

The experiments presented in this article show that our kinetic irreversible thermodynamic description of bacteriorhodopsin liposomes quantitatively predicts the variations in pH gradient caused by variations in light intensity, in proton permeability of the membrane, and in H^+/K^+ exchange activity. This conclusion is important, because it was obtained in the well-defined system of bacteriorhodopsin reconstituted with purified phospholipids. Consequently, analogous quantitative descriptions may now be applied to other, more complicated, systems in which their correctness cannot be checked directly because too many processes occur at the same time (e.g. mitochondria; Westerhoff & Van Dam, 1979). In addition this conclusion supports the conclusions about bacteriorhodopsin that were tentatively drawn in an earlier, more qualitative, study (Hellingwerf et al., 1979).

To some (Erecinska & Wilson, 1979) the validity of this mechanistic irreversible thermodynamic description may come as a surprise, because the validity of the underlying proportional relations between flows and the free energy gradients that drive them has only been proven for processes that are close to equilibrium ($\Delta G \ll RT$). In the experiments shown here the pH gradients were in the order of five times RT. The explanation of this paradox is manifold. Firstly, it has not been proven for (biochemical) processes that outside the RT region the relations between flows and forces are never proportional. Secondly, Rottenberg (1973), Heinz (1978) and Van der Meer, Westerhoff and Van Dam (1980) have shown that under relevant conditions enzyme-catalyzed reactions may exhibit perhaps unexpectedly large regions where flows are proportional to, or at least linear with, their driving forces. In any case such linear relations have been established experimentally in mitochondria (e.g. Padan & Rottenberg, 1973; Nicholls, 1974; Azzone, Pozzan, Massari & Bragadin, 1978; Van Dam et al., 1980), submitochondrial particles (though the linear range may be limited; Sorgato & Ferguson, 1979) and even in (bacteriorhodopsin) liposomes (Kell & Morris, 1980; Arents et al., *in preparation*).

The relations between the flows and Gibbs free energy differences that were used (Westerhoff et al., 1979) to derive the equations tested in this article, were proportional. The question arises whether the equations tested in this article would have looked different if they had been derived from linear, nonproportional equations. Then the results presented here would support the earlier, proportional, rather than the linear, nonproportional, relations. As the derivation by Van der Meer et al. (1980) suggests that for leakage reactions the flow-force relations are still proportional, it can be shown (Westerhoff et al., *in preparation*) that the form of the equations remains exactly the same, the only difference being a shift in value of A_v and a multiplication of both L_v and A_v by some factor. L_v remains proportional to light intensity.

The second aspect of this article is that it illustrates the advantage of the present type of irreversible thermodynamics over the earlier one (for a review *see* Rottenberg, 1979) to describe biological energy transduction. Whereas the earlier type finds phenomenological relations between the flows and the forces in a system (and may therefore be called phenomenological irreversible thermodynamics), the present type finds mechanistic relations, i.e. relations in which the activities of the underlying mechanisms are still explicitly present in the form of characteristic parameters (Westerhoff & Van Dam, 1979). Evidently, the earlier type does not predict the effect of variations in light intensity, proton permeability of the membrane, or H^+/K^+ exchange on the steady-state protonmotive force. The present, mechanistic, type of theory does. Moreover, its predictions have now been verified experimentally.

After this discussion of the more technical aspects it seems appropriate to turn to the biological

relevance of the developed description and its tests. This relevance is at least twofold:

1. It allows one to quantitatively predict the effect of membrane-permeation processes on energy parameters such as the protonmotive force. These processes potentially include more than only the nonphysiological ones assayed in this paper (i.e. proton permeation via ionophores). The quantitative description is expected to be equally useful with physiologically relevant transport, such as that of phosphate and adenine nucleotides across the inner mitochondrial membrane (Van Dam & Westerhoff, 1977), glucose transport across epithelial tissue, and possibly bacterial transport (Westerhoff & Van Dam, 1979). Moreover, an extension to metabolic systems seems possible (Van Dam et al., 1978).

2. It allows one to establish whether two transport catalysts have a direct interaction that is relevant in their translocating activity. In this paper the interaction of valinomycin with anionic protonophores (Anraku, 1979) was taken as an example.

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