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Mosaic nonequilibrium thermodynamics describes biological energy transduction

(bacteriorhodopsin/ion movement/linearity/protonmotive force/liposomes)

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ABSTRACT A procedure, called "mosaic nonequilibrium thermodynamics," for describing ion movement and energy transduction in biological membranes is tested in a model system: bacteriorhodopsin liposomes. The important steps in the theoretical derivations are summarized; one of the experimental tests of the postulated fundamental flow–force relationships is shown. Furthermore, how the quantitative method, even if used only qualitatively, facilitates analysis and understanding of experimental results (in this case, the effect of medium composition on the development of pH gradient and membrane potential in the bacteriorhodopsin liposomes) is shown. The main advantage of this method lies in its quantitative description of the effect of variation of system parameters on the performance of, in this case, the reconstituted proton pump bacteriorhodopsin. As an example, the method is shown to explain quantitatively the dependence of the steady-state pH gradient on the light intensity. Even in more refined analyses of experiments, the quantitative theoretical description is in full accordance with the experimental results; this is illustrated by considering the effect of valinomycin on the dependence of the initial rate of proton uptake into bacteriorhodopsin liposomes on light intensity. It is concluded that mosaic nonequilibrium thermodynamics describes ion movement and energy transduction in the model system of bacteriorhodopsin liposomes and, therefore, may be applied to any other biological system performing such processes.

Problems such as the detailed elaboration of the chemosmotic mechanism (1) in terms of the localization of the relevant proton gradient (2–5) or in terms of the actual H^+/O and H^+/P ratios under conditions of steady-state oxidative phosphorylation (6–9) can hardly be tackled with qualitative methods. In the analysis of isolated enzyme-catalyzed reactions, enzyme kinetics has been a very useful theoretical framework. In systems that consist of numerous enzymes operating in a metabolic steady state, a theoretical tool of similar strength would be welcome. However, the mathematics of a full description of the enzyme kinetics of such a system gets too complex to be analytically solvable, and computer simulation studies, though sometimes useful, most often have the disadvantage that real understanding of the system is lost. Some simplification with regard to the full enzyme kinetic description is necessary. Wilson *et al.* (10, 11) have tried to extend enzyme kinetics to mitochondrial oxidative phosphorylation (see also ref. 12). However, their method stops where the present questions begin; it does not explicitly consider proton movement as an intermediary process in oxidative phosphorylation.

A quantitative description that does take proton movement in mitochondrial energy transduction into account has been developed from linear nonequilibrium thermodynamics by Caplan and coworkers (13–15). Although this description can account for some of the experimentally observed relations between chemical reaction rates and Gibbs free energy gradients, including the electrochemical potential difference for protons across the mitochondrial inner membrane (16, 17), it does not offer the possibility to gain insight into the biological mechanisms that underly the processes. Only approximate information about the H^+/O and H^+/P stoichiometries and the relevance of localized proton gradients can be distilled from experiments by use of this method (16, 17).

Work by Onsager (18), Spiegler (19), Kedem and Katchalsky (20), and Katchalsky and coworkers (20, 21) suggested that the gap between this type of linear irreversible thermodynamic description and the actual mechanisms by which the processes occur can be bridged. For the elemental processes that together formed the system, the seemingly uninformative phenomenological proportionality coefficient can be expressed in terms of the rate constants of the chemical reactions and frictional coefficients for transmembrane flux. Even the kinetic constants of enzyme-catalyzed reactions can be made explicit in the proportionality constants (22–24). The step from this incorporation of kinetic parameters into elemental processes to the evaluation of their bearing on the description of a complex system of reactions (possibly catalyzed by separate enzymes) was set by Kedem and Katchalsky (ref. 25; see also refs. 26–28). The principle is to define the system in terms of its constituent, mutually independent, "elemental" reactions. Then for each of the latter, the relationship between rate (flow) and free energy gradient (force) is written down. Finally, it is realized that the flows through parallel reactions and the forces across serial reactions can be summed (if certain steady-state conditions are met). Thus, the numerous equations describing all of the elemental processes can be reduced to fewer relationships between the experimentally determinable flows and forces. The difference with the relationships obtained by Caplan *et al.* is that the proportionality constants between flows and forces now contain the rate constants and frictional coefficients of the elemental processes of which the system consists.

Several groups have elaborated upon the method for oxidative phosphorylation (9, 29–34) and light-driven proton transport in bacteriorhodopsin liposomes (35, 36). Thus, equations have been derived that contain parameters characteristic for all of the elemental processes, such as the H^+/O and H^+/P stoichiometries of the proton pumps involved in oxidative phosphorylation, the proton permeability of the inner mitochondrial membrane, and the extent to which the linear flow–force relationships of the process are displaced from the near-equilibrium proportional relationships (9). Thus, these equations re-

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Abbreviation: PtdCho, phosphatidylcholine.

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flect the mosaic nature of the complete system, and we suggest naming the approach accordingly: "mosaic nonequilibrium thermodynamics."

It is important to check whether the mosaic nonequilibrium thermodynamic theory does indeed describe ion transport and biological energy transduction in a quantitatively correct manner, but it would be inappropriate to test the method in the system to be analyzed by it later because a circular argument might be the result. Therefore, the best defined model system for proton-mediated bioenergy transduction was selected to serve as a test system for the theory. In summary, (*i*) the fundamental flow–force relationships postulated in the derivation of the theory should be tested; (*ii*) the theory should help us in interpreting experimental results in a semiquantitative sense; (*iii*) the theory should predict what effect variation of a free energy gradient has on the fluxes, and (*iv*) the theory should predict quantitatively the effect of variation of the activity of one of the elemental processes on the relevant measurable parameters.

This paper reports that the theory fulfills all these criteria in the system of reconstituted bacteriorhodopsin (37) liposomes. Accordingly, the mosaic nonequilibrium thermodynamic method as developed by others (29, 31, 33, 34) and by us (30, 32, 35) is among the few theoretical methods that are founded on solid experimental evidence. Only part of this evidence is presented here; the rest has been submitted as parallel papers (38, 39).

MATERIALS AND METHODS

Egg phosphatidylcholine (PtdCho) was obtained from Sigma, octadecylamine (stearylamine) was obtained from Merck. Valinomycin was obtained from Boehringer. Twice-distilled water was used, except in the experiment reported in Fig. 5, in which water filtered through a Milli-Q purification system (Millipore) was used. Flow dialysis (36) was modified: 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 onto the vessel (cylindrical, 0.75 cm in diameter) was about 45 W/m^2 (white light). Proton uptake was measured either in pH meter 1 [100% light intensity of $\approx$0.65 kW/m^2 white light as described (36)] or in pH meter 2 [100% light intensity of $\approx$0.17 kW/m^2 (39)]. If indicated, light intensity was reduced through the use of neutral density filters (Oriel, Stamford, CT). For liposomes prepared in buffer mixture K12 (a buffer mixture with pH-independent buffer capacity for pH values between 6 and 8), the pH gradient was calculated from proton uptake, the inner volume of the liposomes, and the measured buffer capacity of the buffer mixture (39). If indicated, the extravesicular medium was replaced by a different medium (e.g., the isotonic medium K12) by centrifugation through a Sephadex G-50 (coarse) column [packed in 5-ml disposable syringes; preswollen in and washed with the required medium (40)]. Buffer mixture K12 consists of 0.10 M citrate/0.060 M tartrate/0.10 M phosphate/0.125 M pyrophosphate/0.075 M β-glycerol phosphate/0.10 M oxalate/0.050 M malate/0.112 M glucose/0.64 M K^+/0.72 M Na^+. Medium K12 consists of 0.32 M K_2SO_4/0.36 M Na_2SO_4. All other materials and methods were as described (36).

RESULTS

The relationships derived by the mosaic thermodynamics method depend on the underlying elemental reactions. The most important element of the chemosmotic coupling mechanism (1) used in our derivations is the notion that the total process of energy transduction and ion movement in bacteriorhodopsin liposomes is the resultant of a number of ion movements that are independent of each other except for their mutual influence through membrane potential and ion gradients. We consider first the light-induced proton pump bacteriorhodopsin, next the proton back-leakage, and finally the leakage of ions other than protons (Fig. 1). In a more detailed description, additional permeation processes, partial orientation of the bacteriorhodopsin, and a H^+/photon stoichiometry not equal to 1 are taken into account, but to simplify this presentation, these additional factors are not considered here (cf. ref. 35). Fig. 1 also shows the fundamental relations that are postulated to exist between the Gibbs free energy gradients and the proton and ion fluxes. The proton leakage current is postulated to be proportional to the protonmotive force (electrochemical potential difference for protons across the liposomal membrane) (21). For the passive or facilitated ion leakage (J_e), a similar relation is postulated. The rate at which bacteriorhodopsin pumps protons (J_H^ν) is postulated to be decreased by the protonmotive force in a linear way. The fact that bacteriorhodopsin does pump protons is caused by the force exerted by the absorbed photons (A_ν).

Writing the light-driven proton flow catalyzed by bacteriorhodopsin as linearly dependent on a photon force and on the protonmotive force resembles similar postulates for ion pumps driven by chemical reactions (27, 41). However, this postulate is not trivial. Keizer (42, 43) and Hill (23) have discussed the thermodynamic treatment of light and concluded that only black-body radiation or light with well-defined frequencies may be treated by conventional irreversible thermodynamics. Although there is no theoretical justification for treating a photon beam within the scope of Onsager's (18) irreversible thermodynamics, we still propose to use analogous equations. They turn out to fit the experimental results, which may either be considered to be coincidential or to inspire a search for theoretical justification.

In contrast to our treatment, Rottenberg (44) embodies light intensity in the photon force. Hill's (23) and our (35) choice to take the photon force as independent of light intensity is supported by experimental data (36): an increase in light intensity causes an increase in the number of active bacteriorhodopsin molecules (L_ν; Fig. 1) rather than an increase in the force exerted by each absorbed photon.

Another assumption implicit in Fig. 1 is that no additional processes interfere with the variables in which we are interested. Heat and water flow, which may indeed occur, can be left out of consideration in view of their negligible coupling to the processes we treat (32).

The other relevant considerations in the derivation of testable equations from these fundamental equations are that the actual proton flow is the sum of the two proton flows depicted in Fig. 1 and that account is taken of the steady-state condition that reigns in any particular experiment (20).

As an example, we show the results of an experiment that measures the development of the membrane potential and the

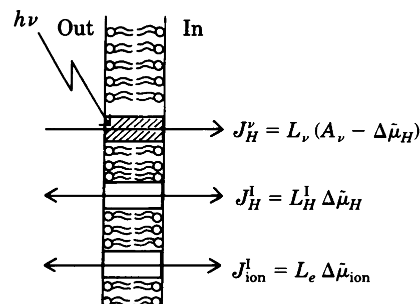

FIG. 1. The ion fluxes across the membrane of bacteriorhodopsin liposomes and their description in terms of flux–force relationships.

pH gradient in bacteriorhodopsin liposomes in different media. Generally, the electrical capacity of this type of biological membrane is low compared to the buffer capacities (35). Therefore, total ion movement can be considered to be almost electroneutral after the translocation of only a few charges. The following relationship between the membrane potential and the developing pH gradient can then be derived:

$$(F\Delta\psi)^{*e} = \frac{L_\nu + L_H^1}{L_\nu + L_H^1 + L_e}\left((\Delta\tilde{\mu}_H)^{*e*h} - Z(\Delta \mathrm{pH})^{*e}\right). \quad [1]$$

[$Z = -RT \ln(10)$ and F are unit conversion factors.] In this equation the asterisks mark the steady-state condition meant, *e for the steady-state condition of net electroneutral flow, *e*h for the steady-state condition of net electroneutral flow and zero net proton flow (the steady state reached after prolonged illumination). Note that the parameters that represent the activity of the elemental processes—i.e., L_ν for the activity of bacteriorhodopsin, L_H^1 for the capacity of the proton leakage pathway, and L_e for the capacity of the leakage pathways of the other ions (Fig. 1)—are still present in an equation that relates measurable parameters.

Eq. 1 states that, once the steady-state condition of net electroneutral flow has been reached, the membrane potential will decrease upon increase of the pH gradient. In fact, the membrane potential will be a fraction of the difference between the final protonmotive force and the pH gradient at any time. The magnitude of this fraction varies with the medium in which the bacteriorhodopsin liposomes are suspended (i.e., with the nonproton electric permeability). In conditions in which a somewhat permeant ion such as chloride is present (Fig. 2*A*), this fraction will only be small. If, on the contrary, the ions present are relatively impermeant (Fig. 2*B*), the membrane potential will amount to a significant fraction of this difference. Consequently, in the latter case soon after illumination has been started, the protonmotive force will approach its final steady-state value. There is yet another method to influence the development in time of pH gradient and membrane potential: increase the buffer capacity within the bacteriorhodopsin liposomes. Then the development of the pH gradient will be slower and, therefore, the decrease in membrane potential with time will occur only slowly (compare Fig. 2*C* with Fig. 2*A*). We conclude that the theory also can be used in semiquantitative analysis of experiments and may lead to the identification of the ion permeation processes responsible for the observed behavior of energetic parameters.

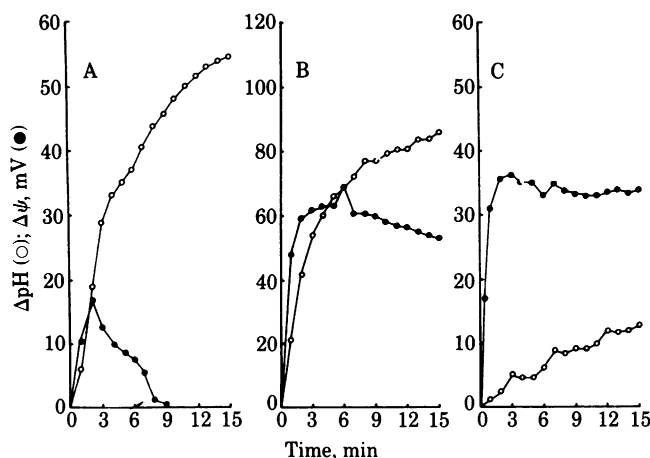

FIG. 2. The development in time of membrane potential and pH gradient in bacteriorhodopsin liposomes in different suspension media. (*A*) Reconstitution conditions: egg PtdCho, 115 g/liter; stearylamine, 2.3 g/liter, bacteriorhodopsin, 2.3 g/liter; sonication, 675 s; medium, 150 mM KCl (pH 6.2). Of the resulting suspension, 0.75 ml plus 3.5 μCi (1 Ci = 3.7×10^{10} becquerels) of aminomethane or 5 μCi of thiocyanate was used in the flow dialysis. (*B*) Reconstitution conditions: egg PtdCho, 40 g/liter; bacteriorhodopsin, 4 g/liter; sonication, 900 s; medium, 250 mM potassium phosphate, pH 7.0/1 mM $MgSO_4$. The external medium was replaced by 219 mM K_2SO_4, pH 6.5/1 mM $MgSO_4$. Of this suspension, 0.97 ml with 7.5 μCi of aminomethane or thiocyanate was used in the flow dialysis. (*C*) Reconstitution conditions: egg PtdCho, 120 g/liter; stearylamine, 2.4 g/liter; bacteriorhodopsin, 6 g/liter; sonication, 450 s; medium, 75 mM potassium citrate (pH 6.0). Of the liposomal suspension, 0.75 ml and 3.5 μCi of aminomethane or 5 μCi of thiocyanate were subjected to flow dialysis.

To justify the use of the theory in quantitative analysis of experimental results, its elemental, postulated relationships should be checked experimentally as far as this is possible. We first showed that proton leakage in bacteriorhodopsin liposomes is linear to the pH gradient (39). Then the rate of light-induced proton uptake was monitored as a function of time and plotted as a function of the pH gradient calculated from proton uptake and the intraliposomal (pH independent) buffer capacity. Fig. 3 shows that the rate of proton uptake into bacteriorhodopsin liposomes depends linearly on the pH gradient. In connection with the linear dependence of proton leakage on the pH gradient, this means that also the pumped proton flux (J_H^ν; the inward flow, corrected for the back leakage) depends linearly on the pH gradient. Because in these experiments the influence of the membrane potential was eliminated (unpublished data) by the addition of excess valinomycin (a K^+ conductor), this supports the postulated (Fig. 1) linear relation between the rate at which bacteriorhodopsin pumps protons and the protonmotive force.

Apart from the influence of the pH gradient and other energy gradients on the rate of the processes, the mosaic thermodynamic theory also accounts for the effects of changes in the activities of the various elemental systems on ion movement and the steady pH gradient and membrane potential. Eq. 2 shows the predicted dependence of the steady-state pH gradient on

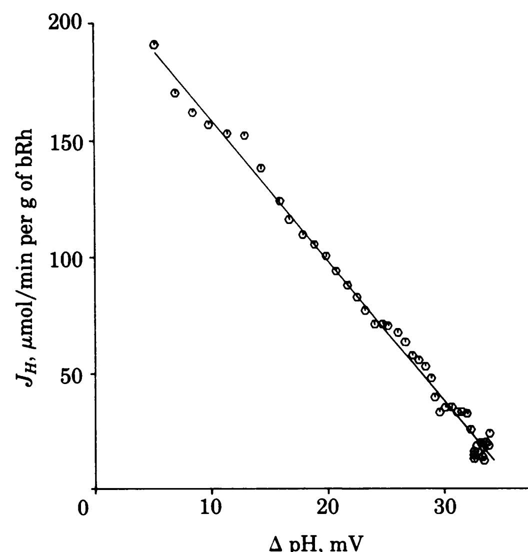

FIG. 3. The relationship between illumination-induced proton influx and pH gradient is linear. Reconstitution conditions: egg PtdCho, 30 g/liter; stearylamine, 0.6 g/liter; bacteriorhodopsin (bRh), 3 g/liter; sonication, 900 s; medium, buffer mixture K12 (pH 6.8). The external medium was replaced by medium K12. Bacteriorhodopsin liposomes (light adapted; 1.1 mg of lipid) were preincubated (pH 6.1) in pH meter 2 in the presence of 7 μg of valinomycin.

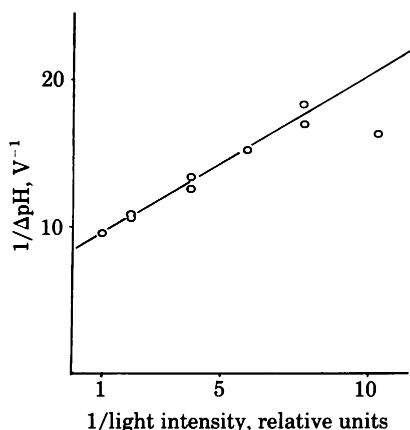

FIG. 4. The reciprocal of the steady-state pH gradient depends linearly on the reciprocal of the light intensity. Reconstitution conditions: soybean phospholipids, 50 g/liter; bacteriorhodopsin, 5 g/liter; medium, 150 mM KCl/1.0 mM EDTA (K salt), pH 6.0; sonication, 675 s at 0°C. Of this preparation, 0.7 ml was preincubated in the flow dialysis vessel with 7 μg of valinomycin, 25 μl of stock [^{14}C]aminomethane, and 7 μl of stock [^{3}H]H_2O. Then illumination with three light intensities was carried out. At $t = 70$ min, 50 μM S13 was added and illumination was stopped. Three different runs with partially identical light intensities were carried out one after the other. By leaving out the far right point, the correlation coefficient (linear least squares line) was 0.991.

the different parameters that characterize the activities of the elemental systems:

$$(\Delta pH)^{*e*h} = \frac{Z\,L_\nu A_\nu}{L_\nu + L_H^1}. \qquad [2]$$

We chose the easiest way to vary the activity (L_ν) of the light-driven bacteriorhodopsin proton pump—variation of the light intensity (36). In accordance with the prediction of the theory, the reciprocal of the pH gradient varies linearly with the reciprocal of the light intensity (Fig. 4).

We also checked whether variations of the different parameters influenced the initial rate of proton uptake by bacteriorhodopsin liposomes (after the onset of illumination) in a way predicted by the theory. The results were again in agreement with the theory. An example of such an experiment is shown by Fig. 5. This experiment checks Eq. 3,

$$\frac{A_\nu}{-J_{H,i}} = \frac{1 + (L_e + L_H^1)/L_\nu}{L_e}, \qquad [3]$$

which gives a relationship between the initial rate of proton uptake into bacteriorhosopsin liposomes ($-J_{H,i}$) and light intensity (L_ν). Again a hyperbolic relationship is expected between the two parameters (see also ref. 36), giving rise to the definition of a half-saturating light intensity (the "K_m for light"). Detailed analysis of Eq. 3 shows that the light intensity at which half-saturation occurs should increase with the nonproton electric permeability of the membrane (L_e). Fig. 5B shows how the K_m for light derived from Fig. 5A depends on the permeability of the membrane for ions other than protons. To vary this latter permeability, the experiment was carried out at different concentrations of valinomycin.

The dependence of the K_m for light on the electric permeability of the liposomal membrane reflects the phenomenon (Fig. 1) that the rate at which bacteriorhodopsin pumps protons is decreased by the protonmotive force in the form of the membrane potential. This and analogous experiments (36) have led us to inspect the kinetics of the photochemical cycle of bacteriorhodopsin. Indeed, it could be shown (45) that the rise and decay kinetics of "O_{660}" (37) absorbance vary with the magnitude of the protonmotive force.

DISCUSSION

In a large number of experiments, only some of which have been presented in this report, we have shown that our thermodynamic description of energy transduction and ion permeation in bacteriorhodopsin liposomes based on the chemosmotic cou-

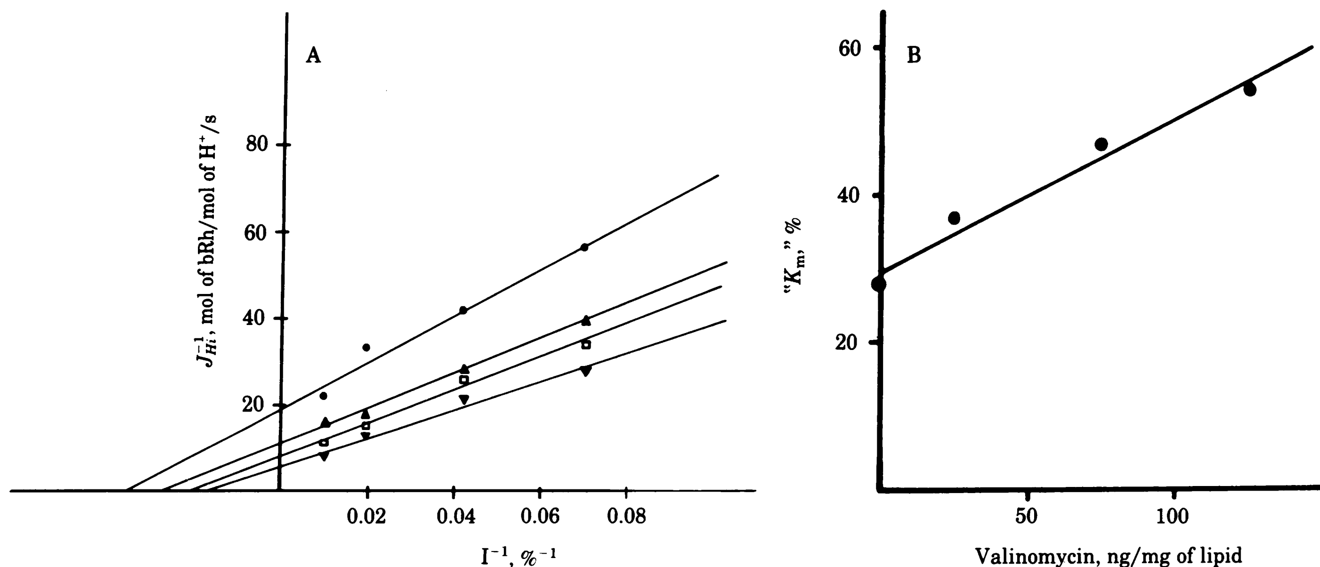

FIG. 5. (A) The combined effect of variation in light intensity and variation in potassium permeability on the initial rate of proton uptake. Reconstitution conditions: egg PtdCho, 25 g/liter; bacteriorhodopsin (bRh), 2.5 g/liter; medium, 250 mM K_2SO_4/1.0 mM EDTA (K salt), pH 7.0; sonication, 900 s. Of this preparation, 0.2 ml was incubated with 3.7 ml of the medium in pH meter 1 and preilluminated 10 times for 15 s (each illumination period was followed by 15 s of darkness) at 100% light intensity. After 1 hr of darkness, 10 s of illumination followed by dark periods of at least 10 min were carried out at different light intensities. After the series of four light intensities had been measured, valinomycin was added to the indicated concentrations and the preillumination procedure was repeated, now for 50 s, followed by 50 s of darkness and a half hour of dark decay. Valinomycin concentrations were 0 (●), 25 (▲), 75 (□), and 125 (▼) μg/g of lipid. (B) "K_m" from A vs. valinomycin concentration.

pling mechanism (35) is in quantitative accordance with experimental observation. The experiments were aimed to test the theory at different points with the following results.

(*i*) The theory accounts quantitatively for the effects of variation of light intensity (Fig. 3), proton permeability, H^+/K^+ exchange, and K^+ permeability on the steady-state pH gradient (also ref. 38).

(*ii*) The theory accounts quantitatively for the effects on the initial rate of proton uptake (Fig. 5, unpublished data).

(*iii*) At least some of the postulated linear relationships between flows and forces (free energy gradient) are actually linear (Fig. 3; ref. 39).

(*iv*) Complex experimental situations such as the combined variation of light intensity and a permeability parameter, are quantitatively described by the theory (Fig. 5; ref. 38; unpublished data).

(*v*) The theory has predictive value. Its predicted (35) product-inhibition-like effect of the protonmotive force on the rate of pumping by bacteriorhodopsin was afterwards established experimentally (Fig. 4; refs. 36 and 45).

(*vi*) The theory has analytic value. It can for instance be used to establish direct interaction between two ionophores (38).

(*vii*) The theory also can be used to semiquantitatively interpret and thereby "understand" experimental observations, such as the antagonistic effect of valinomycin and nigericin on the steady-state pH gradient and the development in time of membrane potential and pH gradient (ref. 36; Fig. 2). In addition it has heuristic value; with bacteriorhodopsin liposomes, mitochondria (9), and intact liver cells (46), it guided us to choose the experiments that were critical to the questions asked.

No theoretical justification can as yet be given to treat a nonmonochromatic photon beam (with the exception of blackbody radiation) in terms of an Onsager (18) type of irreversible thermodynamics (23, 42, 43). One wonders whether the fit between our description and experimental findings is a coincidence, or a sign of still unexplored theoretical principles. Experimental investigations of the question of whether other light-driven systems are also describable by mosaic nonequilibrium thermodynamics and theoretical examinations (cf. refs. 23, 42, 43) of possible links between thermodynamics and photoprocesses may shed light on this problem.

We conclude that the mosaic nonequilibrium thermodynamic method (9, 29–36) is potentially of great use in the detailed analysis of complex problems in bioenergetics.

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