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VOLTAGE-DEPENDENT PROTON FLUXES IN LIPOSOMES

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Liposomes containing buffered KCl were prepared from bacterial lipids, were diluted into K^+-free media and were treated with valinomycin to induce the formation of a diffusion potential ($\Delta\psi$). Upon formation of such a potential, substantial proton influx was observed, as assayed by the quenching of 9-aminoacridine fluorescence. Complete reversal of fluorescence quenching occurred when the potential was collapsed by addition of KCl or when methylamine was added. Studies of proton influx as a function of the theoretical magnitude of the $\Delta\psi$ indicated that the phenomenon occurred only above a $\Delta\psi$ of about -60 mV. Establishment of a Na^+ diffusion potential also resulted in proton influx. Treatment of K^+-loaded liposomes with *N,N'*-dicyclohexylcarbodiimide did not reduce the $\Delta\psi$-dependent proton influx. Moreover, proton influx could be demonstrated upon imposition of a diffusion potential in liposomes prepared from a synthetic lipid. The proton fluxes associated with generation of a diffusion potential in liposomes may complicate studies of reconstituted systems in which proton translocation should occur, and may affect the magnitude of the electrochemical proton gradient that is operant under some conditions.

Introduction

Many important bioenergetic processes are catalyzed by membrane-associated proteins and involve proton translocation across the membrane. Some of these proteins, e.g., transport proteins, have no activity in isolation from the membrane, and others, e.g., the proton-translocating F_1F_0 ATPase, retain an assayable activity but cannot carry out their energy-dependent functions without a membrane. The requirement for a closed membrane system for energy-transducing processes is one of the predictions of the chemiosmotic hypothesis [1,2], and results from the nature of the direct form of energy utilized. According to Mitchell's hypothesis [1,2], this form of energy is an electrochemical gradient of protons whose maintenance would require the presence of a closed, relatively proton-impermeable membrane. Thus, for experimental study of the purified catalysts of bioenergetic work, the development of reconstituted systems has been invaluable (as reviewed, for example, in Refs. 3–5). The purified proteins are reconstituted in proteoliposomes which can be energized either by the inclusion of a proton pump, such as bacteriorhodopsin [6] or respiratory chain complexes [7], or by imposition of a ΔpH or a valinomycin-induced K^+ diffusion potential (e.g., Refs. 8 and 9).

Since proton movements are intrinsic parts of the processes under investigation, the proton per-

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Abbreviations: $\Delta\psi$, transmembrane electrical potential; ΔpH, transmembrane pH gradient; DCCD, *N,N'*-dicyclohexylcarbodiimide; diS-C_3-(5), 3,3-dipropylthiodicarbocyanine; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Taps, 3-{[2-hydroxy-1,2-bis(hydroxymethyl)ethyl]amino}-1-propanesulfonic acid.

meability of the liposomes under unenergized and energized conditions is extremely important. Several studies have addressed the question of passive proton permeability of liposomes [10–15], producing a broad range of values depending upon the experimental approach. Using small imposed pH gradients with at least some liposomal preparations, high intrinsic proton permeabilities have been found [14]. It has also been noted that an exchange between alkali cations and protons can be induced by suspending, for example, K^+-loaded liposomes in choline buffer [16]. However, attention has not been specifically focussed upon the appearance of pathways for proton conductance upon energization of liposomes either in the absence or presence of reconstituted proteins. As part of the initiation of an effort to reconstitute proton-translocating catalysts isolated from the membranes of alkalophilic bacteria, we undertook an examination of the proton movements that might occur upon establishment of a $\Delta\psi$, positive out, by addition of valinomycin to K^+-loaded liposomes in a K^+-free medium. The characteristics and implications of the substantial proton influx that is observed under these conditions are the subject of this report.

Materials and Methods

Preparation of lipids from Bacillus alcalophilus and commercial lipids. Bacillus alcalophilus (ATCC 27647) was grown in L-malate-containing medium at pH 10.5 as previously described [17]. Cells were harvested in the late logarithmic phase of growth and lipids were extracted with chloroform/methanol [18] and washed with acetone/ether as described by Newman and Wilson [19]. The final lipid preparation was suspended in 2 mM 2-mercaptoethanol at 50 mg/ml and stored in 2 ml aliquots under N_2 gas at $-80°C$. While a characterization of the extracted lipids has not been completed, other similar alkalophiles have been found to possess a ratio of polar to non-polar lipids of 6:4, with the latter lipids composed mainly of diacylglycerols [20]. L-α-Phosphatidylcholine, α-oleyl-β-palmitoyl, a synthetic phosphatidylcholine, was purchased from Sigma Chemical Co. The solvent was evaporated and the dried lipids were suspended in 2 mM 2-mercaptoethanol and stored as described above for the *B. alcalophilus* lipids.

Preparation of liposomes. Liposomes were prepared by the bath-sonication of lipids as described by Newman and Wilson [19]. Aliquots (0.2 ml) of lipids, prepared as described above, were placed in a pyrex test tube (15 × 125 mm) to which small volumes of concentrated solutions were added to achieve the compositions indicated for specific experiments. The level of the bath was adjusted to give maximal agitation of the solution and the lipids were sonicated under N_2 gas for 15 min. Liposomes were prepared daily in this way and were then kept at room temperature during the course of the experiment.

Fluorescence assays. The quenching of the fluorescence of 9-aminoacridine was used to monitor changes in the pH across the liposomal membrane [21]. Liposomes (10 μl) were added to 2 ml of buffer containing 5 μM 9-aminoacridine at room temperature. After a baseline was obtained, valinomycin was added to the final concentration indicated for individual experiments. Subsequently, changes in fluorescence were followed with a Perkin-Elmer spectrofluorimeter using an excitation wavelength of 420 nm and an emission wavelength of 520 nm. The percentage of fluorescence quenching was calculated with the fluorescence in the absence of liposomes at 100 and the dark current value at zero.

For quantitative correlation of changes in 9-aminoacridine fluorescence with changes in the ΔpH, liposomes prepared in 140 mM buffered KCl were diluted 200-fold into buffered choline chloride. Nigericin was added to 2 μM; under these conditions, nigericin has been shown to completely exchange intraliposomal $^{86}Rb^+$ for H^+ [22]. Assuming the exchange of K^+ for H^+ in the calibration experiment was 1:1, a 200-fold gradient of H^+, acid in, was generated at the point of maximal fluorescence quenching (Fig. 1B). At that point, various known increments of NaCl were added causing a progressive dissipation of the ΔpH. As shown in Fig. 1A, a linear relationship was found between the observed fluorescence quenching and the calculated ΔpH remaining after each increment of NaCl.

The quenching of the fluorescence of the cyanine dye DiS-C_3-(5) was used to monitor qualitatively

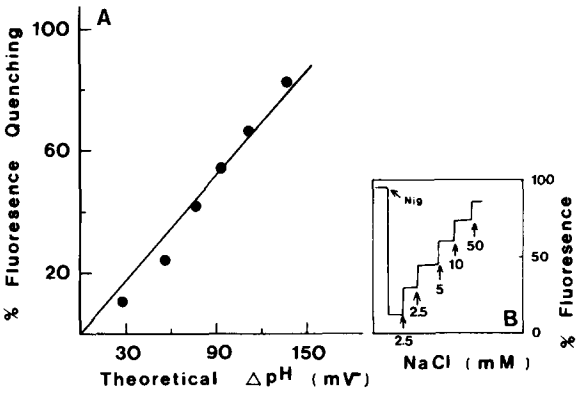

Fig. 1. The relationship between the magnitude of quenching of 9-aminoacridine fluorescence and the change in the ΔpH. Liposomes containing 140 mM KCl, 10 mM Taps, pH 9.0, were prepared from *B. alcalophilus* lipids as described under Materials and Methods. An aliquot (10 μl) was diluted 200-fold into 140 mM cholineCl, 10 mM Taps, pH 9.0, containing 5 μM 9-aminoacridine. After a baseline was established, nigericin was added to a final concentration of 2 μM and the quenching of fluorescence was recorded (fig. 1B). That quenching was progressively reversed by the addition of the increments of NaCl indicated in (B). The percentage fluorescence quenching was then plotted as a function of the ΔpH (converted to mV equivalents). The calibration experiment could not be conducted in an Li^+-containing medium because nigericin catalyzed exchange of the Li^+ for intraliposomal H^+.

the presence of a $\Delta\psi$, positive out [21,23]. The protocols were as described above except that a final dye concentration of 1 μM was employed, and fluorescence was assayed using an excitation wavelength of 620 nm and an emission wavelength of 670 nm.

Materials. 9-Aminoacridine, valinomycin and synthetic lipid were obtained from Sigma Chemical Co. $^{86}Rb^+$ was purchased from New England Nuclear Company. Nigericin was a gift from Dr. R.L. Hamill of the Eli Lilly Company. DiS-C_3-(5) was a generous gift from Dr. A. Waggoner. All other chemicals were obtained commercially at the highest possible purity. The bath sonicator, 80 W, 80 kHz generator model G80-80-1 and tank model t80-80-I-RS, was purchased from Laboratory Supplies Co., Inc. (Hicksville, N.Y.).

Results

Liposomes that were loaded with 140 mM KCl, buffered to pH 9.0, during preparation were diluted into either 140 mM KCl, 140 mM choline chloride, or 140 mM LiCl, also buffered to pH 9.0 with 10 mM Taps. The quenching of the fluorescence of 9-aminoacridine was monitored before and after the addition of 1 μM valinomycin. A slight fluorescence quenching was observed immediately upon addition of the liposomes, after which the fluorescence remained constant (Fig. 2). When valinomycin was added, the fluorescence was still unchanged in liposome suspensions that had been diluted in KCl, whereas, upon addition of valinomycin to liposomes diluted in K^+-free solutions, there was a rapid quenching of fluorescence that was greater in choline chloride than in LiCl. However, using many different liposome preparations, a more stable baseline was routinely observed upon dilution into LiCl; therefore, this condition was used in most of the subsequent experiments. Addition of either 10 mM KCl, to

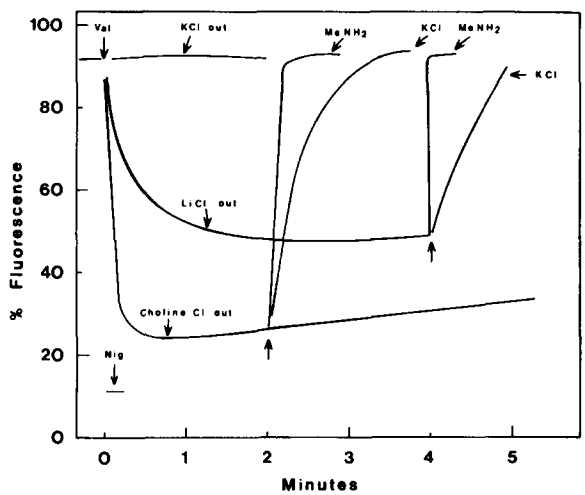

Fig. 2. Quenching of 9-aminoacridine fluorescence upon establishment of a diffusion potential across the liposomal membrane. Liposomes containing 140 mM KCl, 10 mM Taps, pH 9.0, were prepared from *B. alcalophilus* lipids as described under Materials and Methods. An aliquot of liposomes (10 μl) was diluted 200-fold into similarly buffered solutions containing 5 μM 9-aminoacridine and 140 mM of either KCl, LiCl or choline chloride. Valinomycin was added, where indicated, to a final concentration of 1 μM and the changes in fluorescence were monitored. The additions, methylamine or KCl, that were made at the points shown by upward arrows were made to a final concentration of 10 mM. A short horizontal line on the left side of the figure indicates the percentage fluorescence after the addition of nigericin (2 μM) to a liposome sample that was diluted in buffered choline chloride.

collapse the potential, or 10 mM methylamine, a weak permeant base, restored the fluorescence to its original level. On the other hand, addition of 10 mM LiCl to the liposomes suspended in LiCl had no effect on the fluorescence quenching. The maximal apparent proton influx was calculated to correspond to a ΔpH, acid in, of approx. 1.5 pH units. There was some variability in the rate of fluorescence quenching between different liposome preparations. Indeed, in an occasional leaky preparation from which K^+ efflux occurred without the addition of valinomycin (as assessed by $^{86}Rb^+$ efflux), fluorescence quenching also occurred, without added valinomycin, upon dilution into K^+-free medium. However, the magnitude and pattern of the 9-aminoacridine quenching was completely reproducible and was unaffected by pH in a range from pH 7.0 to 10.0, and by the substitution of sulfate for chloride as the anion. Treatment of the liposomes with 250 μM DCCD for 3.5 h at room temperature before dilution and valinomycin addition also had no effect on the quenching of 9-aminoacridine fluorescence. Although data will not be shown, when fluorescence of the $\Delta\psi$ probe DiS-C_3-(5) was monitored instead of 9-aminoacridine, using the same experimental conditions, a quenching of fluorescence was observed, indicating that a $\Delta\psi$, positive out, had been produced; the quenching occurred rapidly and did not reverse over a period of minutes. In a control experiment, liposomes were prepared with $^{86}Rb^+$ inside (6.6 mM ^{86}RbCl, 60 mCi/mmol) in addition to 140 mM K^+. The extent of valinomycin-dependent $^{86}Rb^+$ efflux was examined upon dilution into K^+-free solutions, as described by García et al. [22]; the results obtained were qualitatively and quantitatively similar to those that had been observed in that prior study which had utilized liposomes prepared with lipids from *Escherichia coli*. That is, only a small percentage of the intraliposomal $^{86}Rb^+$ is released as a $\Delta\psi$, positive out, is generated; upon addition of nigericin, rapid efflux of all the cation is observed.

When the quenching of 9-aminoacridine fluorescence was examined as a function of the theoretical magnitude of the $\Delta\psi$, a sigmoidal relationship was found (Fig. 3). Liposomes containing 140 mM KCl buffered with 10 mM Taps, pH 9.0, were diluted into solutions containing various ratios of KCl and LiCl (whose sum was 140 mM), also buffered with 10 mM Taps, pH 9.0. The $\Delta\psi$ expected upon addition of valinomycin was calculated from the Nernst equation. At theoretical $\Delta\psi$ values of -60 mV or less, there was little or no quenching of 9-aminoacridine fluorescence. At higher $\Delta\psi$ values, the amount of quenching increased dramatically with increases in the $\Delta\psi$ until a $\Delta\psi$ of about -120 mV. The shape of the curve was identical when choline chloride, instead of LiCl, was used in the dilution buffer.

A series of experiments was then conducted to explore whether valinomycin could mediate sufficient Na^+-flux, when Na^+ rather than K^+ was present inside the liposome, to cause proton influx. Indeed, addition of valinomycin to liposomes containing 140 mM NaCl (buffered to pH 9.0), and diluted into similarly buffered LiCl (140 mM) resulted in a quenching of the fluorescence of 9-aminoacridine (Fig. 4); within a given experiment, the rate of proton influx depended upon the valinomycin concentration. As shown in Fig. 4, the proton influx was reversed by the addition of extraliposomal KCl. If a high concentration of

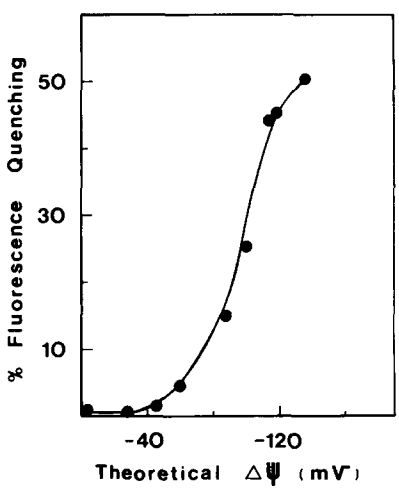

Fig. 3. Quenching of 9-aminoacridine fluorescence as a function of the magnitude of the imposed diffusion potential. Experimental details were the same as described in the legend to Fig. 2 except that the liposomes were diluted 200-fold into buffered solutions containing various proportions of LiCl and KCl (final concentration, 140 mM). The $\Delta\psi$ expected under each condition was calculated from the Nernst equation. The results were identical when the dilutions were conducted in combinations of choline chloride and KCl or when sulfate salts were used instead of chloride salts.

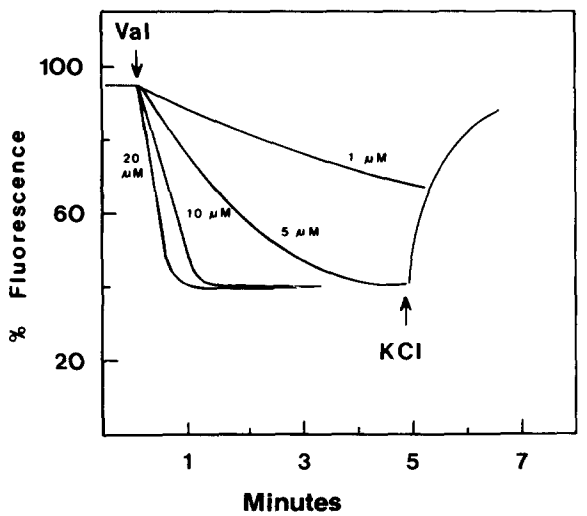

Fig. 4. Quenching of 9-aminoacridine fluorescence in response to a Na^+ diffusion potential. Liposomes containing 140 mM NaCl, 10 mM Taps, pH 9.0, were prepared from *B. alcalophilus* lipids as described under Materials and Methods. Aliquots (10 μl) were diluted 200-fold into 140 mM LiCl, 10 mM Taps, pH 9.0, containing 5 μM 9-aminoacridine. Valinomycin was added at the indicated concentrations. Where shown, by the upward arrow, 25 mM KCl was added.

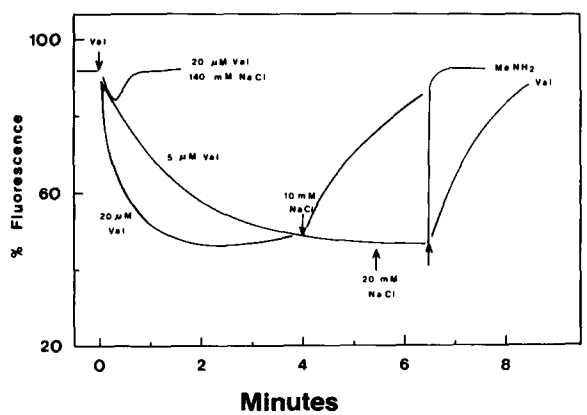

Fig. 5. The effect of sodium on the quenching of 9-aminoacridine fluorescence in response to a K^+ diffusion potential. Liposomes containing buffered KCl were prepared as in the legend to Fig. 2, and were diluted into 140 mM NaCl, 10 mM Taps, pH 9.0 (top trace), or 140 mM LiCl, 10 mM Taps, pH 9.0. Valinomycin was added either to 5 or to 20 μM, and fluorescence was monitored. Subsequent additions, as indicated, were: 10 mM NaCl added to liposomes which had been treated with 20 μM valinomycin; 20 mM NaCl added to liposomes that had been treated with 5 μM valinomycin followed after about 1 min by the addition of either 15 μM valinomycin or 10 mM methylamine.

valinomycin (20 μM) was used to initiate K^+ efflux and proton influx with K^+-containing liposomes, the proton influx was sensitive to extraliposomal Na^+. As shown in Fig. 5, when K^+-containing liposomes were diluted into 140 mM NaCl prior to the addition of 20 μM valinomycin, only a small, rapidly reversed quenching of 9-aminoacridine was observed. Similarly, after dilution into LiCl (140 mM) and addition of 20 μM valinomycin the observed proton influx could be reversed by adding 10 mM NaCl. However, if the same experiment was conducted with 5 μM valinomycin, even 20 mM NaCl failed to reverse the quenching unless more valinomycin was added (Fig. 5). The proton influx was completely sensitive to methylamine. Addition of choline chloride had no effect, and when the dilution buffer contained choline chloride instead of LiCl, added LiCl had no effect (data not shown).

Finally, the quenching of 9-aminoacridine fluo-

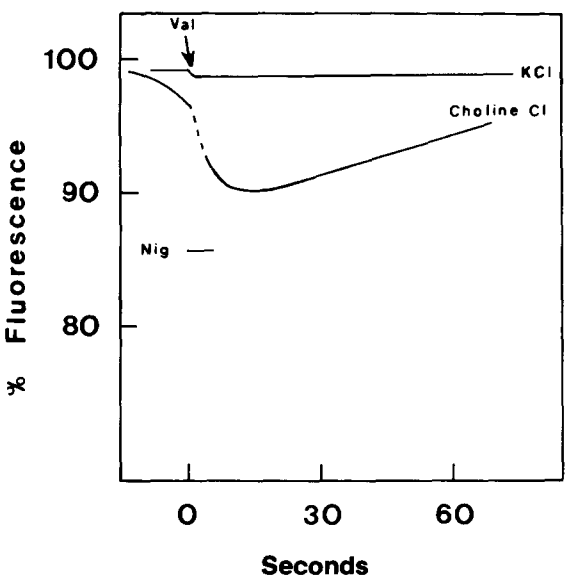

Fig. 6. Quenching of 9-aminoacridine fluorescence in response to a K^+ diffusion potential in liposomes made from a synthetic phosphatidyl-choline. Liposomes containing 140 mM KCl, 10 mM Hepes, pH 7.0, were prepared from a synthetic phosphatidylcholine as described under Materials and Methods. Aliquots (10 μl) were diluted 200-fold into either 140 mM KCl or 140 mM choline chloride, 10 mM Hepes, pH 7.0. Valinomycin was added to 20 μM and the fluorescence was monitored. The horizontal line indicates the percentage fluorescence of liposomes diluted in buffered choline chloride and treated with 2 μM nigericin.

rescence was examined upon establishment of a valinomycin-mediated K^+ diffusion $\Delta\psi$ in liposomes prepared from commercially obtained synthetic lipid. The liposomes prepared from such lipid, and loaded with 140 mM KCl, exhibited a smaller total change in fluorescence observed upon treatment with nigericin than had those prepared from *B. alcalophilus* lipids (Fig. 6). The liposomes prepared entirely with the synthetic lipid were also somewhat leaky; a slow rate of fluorescence quenching was observed upon dilution of these liposomes into choline chloride (buffered to pH 7.0 with Hepes), even before the addition of valinomycin. After valinomycin was added, there was a very rapid quenching of fluorescence which substantially reversed during a minute of incubation.

Discussion

The results of this study show that upon establishment of a valinomycin-mediated K^+-diffusion potential across a liposomal membrane, there is an appreciable influx of protons, as assayed by the quenching of 9-aminoacridine fluorescence. Indeed, if the low concentrations of Tris used in the buffers exerted the kind of weak base effect described by Sone et al. [24], the proton influx in the current study may be an underestimate. The lipid preparations might contain small amounts of contaminating proteins which could conduct the protons. This is probably not a major factor in the observed effects, however, since extensive DCCD treatment of the liposomes had no effect at all on the proton influx, and the phenomenon was qualitatively reproducible in apparently smaller liposomes prepared from a synthetic lipid. The actual path of proton movements has not been established; indeed we cannot rule out the possibility that valinomycin participates in these movements. Less proton conductance has been observed in controls of some reconstituted systems (e.g., Ref. 25) in which both lower valinomycin concentrations and theoretical $\Delta\psi$ values were used. If valinomycin at the concentrations used in the current study were acting as a protonophore, it would be important, since many studies in both liposomes, vesicles, and cells (e.g., Refs. 14, 19 and 26) use such concentrations. However, several considerations weigh against this possibility. Occasional leaky liposomes, prepared from bacterial lipids, and the relatively leaky liposomes that were prepared from synthetic lipids exhibited proton influx upon dilution into K^+-free media even in the absence of valinomycin. These results are similar to those reported earlier, for other lipid preparations, by Scarpa and De Gier [16]. Also, Deamer and Nichols [14] found that valinomycin, at 1 μM, did not abolish a pH gradient that was imposed across liposomal membranes. Moreover, in the current experiments the observed proton influx bore a specific relationship to the magnitude of the voltage. The proton influx occurred only above a threshold $\Delta\psi$ and could be mediated by a Na^+ diffusion potential in the presence of sufficient Na^+ and valinomycin. Notably, diffusion potential-dependent proton influx has similarly been shown in liposomes made from *E. coli* lipids [27], has been suggested in connection with transport phenomena in isolated membrane vesicles [28], and has recently been observed using starved whole cells of *B. alcalophilus* [29] and *Bacillus firmus* [30].

In the current study, a $\Delta\psi$, positive out, was qualitatively demonstrated, both by the limited $^{86}Rb^+$ efflux and by a fluorescence assay, in spite of the proton influx. It was, of course, inappropriate to standardize the fluorescence using theoretical $\Delta\psi$ values, as is usually done to quantify this method, and lipophilic cations were technically unsuitable in the filtration procedures used for the liposomes. However, using the uptake of lipophilic cations the $\Delta\psi$ generated artificially across starved whole cells has been found to correlate within 10% with the theoretical value [30] even though it co-existed with a ΔpH, acid in. Therefore, the proton influx that occurs in response to an imposed $\Delta\psi$ of sufficient magnitude does not appreciably reduce that $\Delta\psi$, at least at steady-state. Perhaps the protons that move inward transiently lower the $\Delta\psi$, facilitating the outward movement of more K^+. Indeed, the possibility should be considered that a respiration-generated $\Delta\psi$ of some threshold magnitude may similarly open a proton conductance pathway which may, in turn, be compensated by enhanced proton extrusion via respiration. Such a $\Delta\psi$-dependent 'suck-back' of protons has been described by Setty et al. [31]. The proton 'leak' pathways assessed in unenergized or

charge-compensated systems may not reflect those that occur when a $\Delta\psi$ exists across the membrane.

There are important bioenergetic consequences of proton influx upon establishment of a diffusion potential. First, an apparently gated conductance of protons could easily be confused with proton conductance associated with porter function or ATP synthesis. The Na^+-dependent proton fluxes, for example, interfere with attempts to assay a Na^+/H^+ antiporter by following proton movements in proteoliposomes. This underscores the importance of determining whether experimentally observed proton conductance is completely sensitive to a process-specific inhibitor. Second, the presence of a $\Delta pH_{acid\ in}$ may have to be taken into account as part of the electrochemical proton gradient that is operant when diffusion potentials are established. The kinetics of $\Delta\psi$ generation versus the proton influx versus the rate of a particular bioenergetic process will be critical, and cannot be assessed by the methods used here. If the $\Delta\psi$-dependent proton influx actually occurs more slowly than a particular bioenergetic process in reconstituted liposomes, then the relevant electrochemical proton gradient would be the diffusion potential only. However, if proton influx occurs more rapidly than or concomitant with the bioenergetic process, then the true driving force is less than the $\Delta\psi$. Moreover, a substantial chemical gradient of protons or changes in pH_{in} per se could affect the activity of some membrane-associated proteins. It should be possible to assess many of these effects by conducting experiments below the threshold $\Delta\psi$ at which proton influx occurs.

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While this paper was in press, a report by Krishnamoorthy and Hinckle [32] appeared, describing in detail the non-linear dependence of H^+/OH^- fluxes on the $\Delta\psi$, generated in mitochondria or liposomes.

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