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Nigericin-mediated H^+, K^+ and Na^+ transports across vesicular membrane: *T*-jump studies

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The decay of ΔpH across vesicular membranes by nigericin-mediated H^+ and metal ion (M^+) transports has b studied at 25 °C after creating ΔpH by temperature jump (*T*-jump). In these experiments K^+ or Na^+ were cho as M^+ for the compensating flux. Theoretical expressions derived to analyse these data suggest a method for estimating the intrinsic rate constants for the translocation of nig-H (k_1) and for the translocation of nig-M (k_2) across membrane, from the pH dependence of the ΔpH decay. The following could be inferred from the analysis of data. (a) At pH ~ 7.5 and 250 mM ion concentrations, nigericin-mediated H^+ and M^+ transport rates are lower in a medium of K^+ than in a medium of Na^+, although ionophore selectivity of nigericin towards K^+ is 25–45-times higher than that towards Na^+. However, at lower [M^+] (~ 50 mM) the transport rates are higher in a medium of K^+ than in a medium of Na^+. Such behaviours can be understood with the help of parameters determined in this work. (b) The intrinsic rate constants k_1 and k_2 associated with the translocations of nig-H and nig-K or nig-Na across membrane are similar in magnitude. (c) At pH ~ 7.5 translocation of nig-H is the dominant rate-limiting step in a medium containing K^+. In contrast with this, at this pH, translocation of nig-M is the dominant rate-limiting step when metal ion is Na^+. (d) $k_1 \approx k_2 \sim 6 \cdot 10^3\ s^{-1}$ could be estimated at 25 °C in vesicles prepared from soyabean phospholipid, and lipid mixtures of 80% phosphatidylcholine (PC) + 20% phosphatidylethanolamine and 92% PC + 8% phosphatidic acid. (e) The apparent dissociation constants of nig-M in vesicles were estimated to be $\sim 1.5 \cdot 10^{-3}$ M for K^+ and $6.4 \cdot 10^{-2}$ M for Na^+ (at 50 mM ion concentrations) using $\sim 10^{-8.45}$ M for the apparent dissociation constant of nig-H.

Introduction

The study of H^+ transport across biomembranes is useful in the study of energy coupling [1]. Carrier-mediated transport is an important mechanism of H^+ transport [2]. Nigericin is an electro-neutral carrier of H^+. It cotransports metal ions M^+ in the opposite direction to maintain electrical neutrality across membranes [3]. The four basic steps of nigericin mediated H^+ transport across membranes shown in Fig. 1a are: (i) exchange of H^+ with nigericin bound M^+ at the inner aqueous medium-membrane interface and fast equilibration with the nigericins in the inner layer, (ii) translocation of nig-H across membrane with intrinisic rate constant k_1, (iii) exchange of nigericin bound H^+ with M^+ at the outer aqueous medium-membrane interface and fast equilibration with the nigericins in the outer layer and (iv) translocation of nig-M back across membrane with intrinsic rate constant k_2. Instead of at the interfaces, the M^+/H^+ exchanges could occur in the aqueous medium itself with subsequent transfer of nig-H or nig-M between the aqueous medium and the membrane (Fig. 1b) similar to the mechanism proposed for O-Methyl red-mediated H^+ transport [4]. The motivations behind the present study are as follows: (1) It is important to identify the rate-limiting steps in the H^+ transport mechanism and to determine the rate constants associated with them. This has not been possible for nigericin-mediated H^+ transport so far, since all the relevant dissociation constants, transport data with metal ions K^+ and Na^+ and the mathematical equations needed for the analysis of data are not available in the literature. The limited H^+ flux data with K^+ ions reported [5], is not adequate. (2) The equations given by Grzesiek and Dencher [6] for the analysis of ΔpH decay by H^+ transport in liposomes

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do not take note of the details of the transport mechanism. We wanted to derive the equations after including such details in the case of nigericin-mediated H^+ transport and demonstrate the utility of such derivations. (3) From the simple mechanism of Fig. 1 and from our equations, we had inferred that at high pH or high concentrations of M^+, nigericin-mediated Na^+ transport rate in liposomes can be higher than that for K^+ even though ionophore selectivity of nigericin towards Na^+ is ~25–45-times smaller than that towards K^+ [2,3]. This possibility has not been recognised so far to the best of our knowledge. We wanted to see whether we can have such situations in the pH region 6–8 of biological interest.

Recently the fast H^+ transport across membrane in liposomes has been studied by creating a pH gradient, ΔpH, across membrane in temperature-jump (T-jump) experiments [5,7] or in stopped-flow studies [6,8–10] and monitoring the decay of ΔpH with the help of fluorescence from entrapped pyranine. T-jump relaxation [11] has also been used in experiments where changes in the optical absorption of the H^+ carrier, O-Methyl red, itself was monitored [4]. Since the observations in these two techniques are made in the absence of electric fields across the membrane, they are particularly useful in the study of electroneutral transport [5]. The equations given in the Appendix can be used for the analysis of stopped-flow relaxation data also.

In order to understand the various factors which could affect the H^+ transport in biological membranes it is more appropriate to use model membrane systems of biological origin. Vesicles prepared from soyabean phospholipids (SBPL) have been used by several workers [1,5–7,12–15] and more information about this system is always desirable. The reconstitution of vesicles from SBPL is relatively easy. Furthermore, pyranine or buffers trapped inside these vesicles do not leak out. Therefore, substantial work on model membranes is likely to be carried out using SBPL. These reasons prompted us to obtain most of the data on SBPL vesicles. In addition, relevant data on vesicles prepared from mixtures of phosphatidylcholine (PC), phosphatidylethanolamine (PE) and phosphatidic acid (PA) have also been obtained in this work. Vesicles prepared from PC alone were not used in our studies, since the amplitude data suggested the leaking of buffers through the membrane in these preparations.

Materials and Methods

L-α-Phosphatidylcholine (~99%) from frozen egg yolk, asolectin, L-α-phosphatidic acid sodium salt from egg yolk lecithin, dimyristoyl-L-α-phosphatidylethanolamine, and dimyristoyl-L-α-phosphatidylcholine (DMPC) were obtained from Sigma Chemical Co. Total phosphate concentration in asolectin vesicle solutions were determined using the method described by

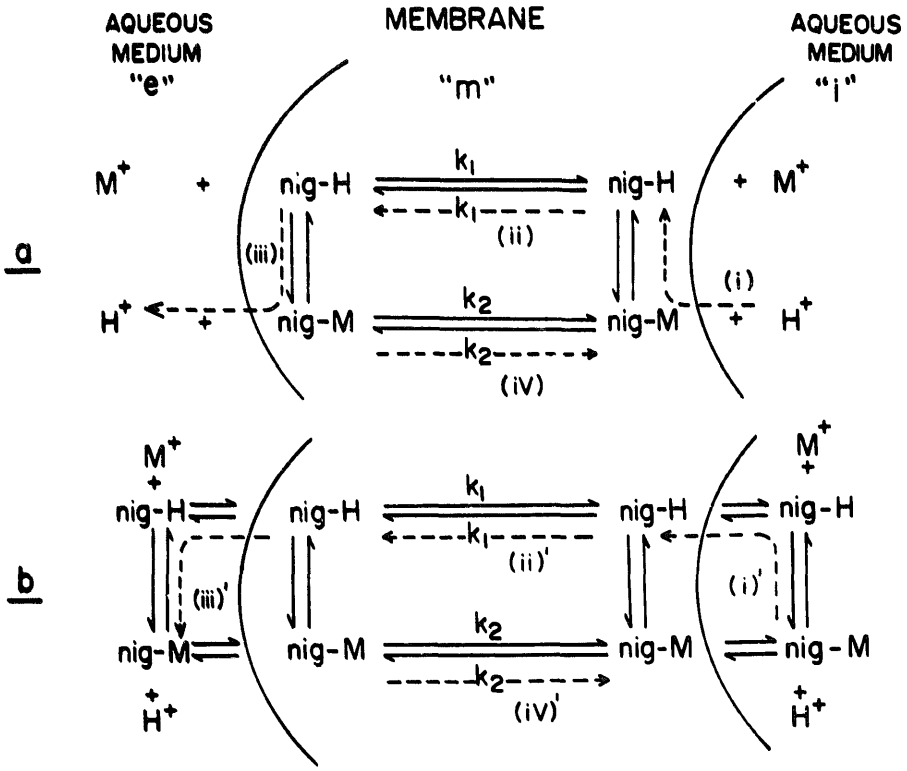

Fig. 1. Suggested mechanisms of nigericin-mediated H^+ transport across membrane in vesicle solutions containing metal ions M^+. The broken lines indicate net flux in the T-jump experiments.

Ames [16]. DMPC was used as the standard in such estimates. For asolectin concentration of 1 mg/ml, total phosphate concentration was estimated to be ~ 1 mM. In this paper, 1 mg/ml lipid solutions are taken to have a lipid concentration of 1 mM. The SBPL vesicles were prepared from purified, acetone washed asolectin [15], by sonicating in nitrogen atmosphere in a buffer solution chosen to be trapped inside vesicles, using a bath type sonicator. When pyranine had to be trapped inside vesicles the buffer solutions had pyranine. Removal of pyranine from outside vesicles, and change of the buffer medium outside vesicles to another buffer medium, were carried out by passage through a Sephadex G-50 column eluted with the second buffer solution [5]. The optical densities of vesicle solutions at 700 nm (due to light scattering) were monitored to ensure the near constancy of vesicle size distribution (approx. 300 Å) in different preparations [5]. Stock solutions of 1 mM nigericin in ethanol were used in the preparation of vesicle solutions with specified amount of nigericin. *N*-(Acetamido)-2-aminoethanesulphonic acid (Aces), tris(hydroxymethyl) aminoethane (Tris) (from Sigma) and potassium dihydrogenphosphate (phosphate) (Ranbaxy Lab.) at specified concentrations were used to buffer the pH. The pH of the buffer solutions were adjusted by adding dilute HCl/KOH/NaOH. The pyranine concentration in the buffer solutions was 2 mM when it was inside and 7 μM when it was outside vesicles in experiments. Similar procedures were used in the preparation of vesicle solutions from lipid mixtures.

A Cary-17D spectrophotometer and a Shimadzu RF-540 spectrofluorimeter were used to measure optical densities at 700 nm and steady state fluorescence, respectively. The *T*-jump instrument is described elsewhere [17]. A particular set of data was obtained using the same vesicle preparation to avoid scatter of data due to small differences between two preparations.

Results

Buffer capacity studies

Buffer capacity data obtained by adding known amounts of HCl/KOH/NaOH to SBPL vesicle solutions and monitoring the small changes in pH with the help of a combination pH electrode and a recorder show contributions from two types of ionisable groups (pK = 6.9 and 7.8) in both the outer and inner layers of vesicles (Fig. 2). The groups in the inner layer and the entrapped buffer contributed to the buffer capacity only after the addition of nigericin. The solid lines in Fig. 2 were calculated using the parameters given in Table I and Eqn. A-6. Nigericin (~ 0.5 μM in typical experiments) makes negligible contributions to buffer capacity data. The asymmetry in the buffer capacities of the outer and inner layer [13] is associated with the asymmetry in the concentrations of buffer groups of pK 7.8. The endogeneous contribution to internal buffer capacity b_i (equivalent to ~ 50 mM phosphate at pH 6.9, estimated using Table I and $V_e/V_i = 2.25 \pm 0.2$ for [lip] = 1 M) agrees well with those determined from kinetic experiments (discussed below) using (i) Fig. 3e and Eqn. A-14, (ii) Fig. 4a and Eqn. A-18 or Eqn. 1, and (iii) Fig. 4b and Eqn. A-18. (V_e/V_i = (volume external to vesicles)/(volume internal to vesicles), were estimated using the fluorescence from pyranine solutions entrapped inside the vesicles).

Buffer capacities were also determined for typical lipid mixtures of PC + PA, PC + PE and SBPL + PE. 8% PA in the lipid mixture gave a contribution of ~ 80

TABLE I

The pK and $\partial pK/\partial T$ of buffer groups determined in this work [a] or taken from the literature [b] at 0.1 ionic strength and 25 °C relevant in SBPL vesicles in the pH range 6–8

Weak acid	Subscript [c] 'j'	pK_j	$\partial pK_j/\partial T$	Concentration in 1 mg/ml of lipid [A_j] in mM
Tris in '1' [b]	1	8.16	−0.031	
Phosphate in '1' [b]	1	6.86	−0.0028	
Phosphate in '6'	6	6.9	−0.0028	
Aces in '1' [b]	1	6.77	−0.02	
Aces in '6'	6	6.9	−0.02	
Pyranine	0	7.25	−0.00624	
Endogeneous buffer	2	6.9	0.0	0.012
Endogeneous buffer	3	7.8	−0.007	0.034
Endogeneous buffer	4	7.8	−0.007	0.020
Endogeneous buffer	5	6.9	0.0	0.012

[a] The errors in pK_j and $\partial pK_j/\partial T$ are ±0.05 and ±0.0002. Simultaneous equations obtained by substituting δS_f observed at pH = 6.9 for different concentrations of vesicles and buffers in Eqn. A-13, were used in the determination of $\partial pK_j/\partial T$.

[b] Taken from Ref. 28.

[c] '1' and '6' are outside and inside vesicles, '2' and '3' are in the outer layer, '4' and '5' are in the inner layer of membrane.

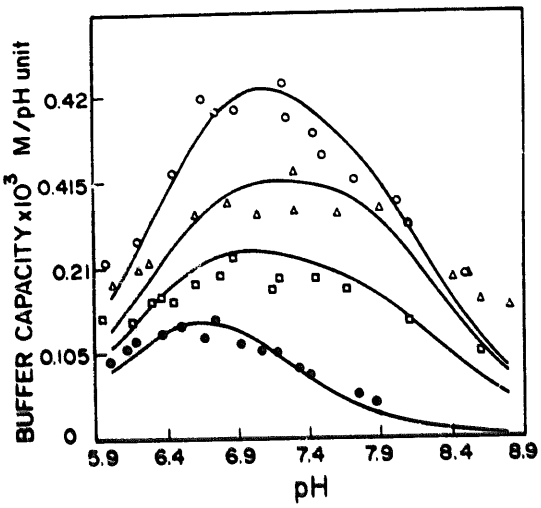

Fig. 2. Buffer capacities of aqueous solutions containing: (a) 0.25 mM Aces (●); (b) 6.4 mM SBPL (with 2 mM or 80 mM phosphate buffer inside vesicles) and 0.25 mM Aces (□); (c) 6.4 mM SBPL (with 2 mM phosphate buffer inside vesicles), 0.25 mM Aces and 0.5 μM nigericin (△); (d) 6.4 mM SBPL (with 80 mM phosphate buffer inside vesicles), 0.25 mM Aces and 0.5 μM nigericin (○); in the pH range 6–8.8 at 25 °C. The solutions had 100 mM KCl. The solid lines are calculated using Table I and Eqn. A-6.

mM from a group of pK 8.0 to the internal buffer capacity b_i. Similarly 20% PE gave a contribution of ~ 60 mM from a group of pK ~ 6.9 to b_i, apart from a larger contribution from a group of much higher pK (> 8.5). It may be noted that Papahadjopoulos [18] had also observed a group with pK in the range 6.5–7.5 in some of the preparations from PE. In view of the wide variations in the pK of groups, depending on the environment [18,19], we have not attempted to identify the buffer groups in SBPL vesicles even though the constituents of asolectin are known [20].

T-jump studies

The informations obtained in the T-jump relaxation studies are: (I) identification of fluorescence changes associated with the ΔpH decay due to H^+ transport across membrane; (II) clarification of whether the ΔpH decay rate is increased by (a) increasing the equilibration rate associated with the transfer of nig-M and nig-H between aqueous medium and membrane and by (b) increasing the rate of H^+/M^+ exchange between nigericin and aqueous medium; (these data can also be used to check the endogeneous contributions to the internal buffer capacities); (III) pH dependence of the ΔpH decay rate; (IV) confirmation that no H^+ or buffer leaks were created in the vesicular membrane due to T-jump or pH changes during the experiments. (If such leaks were created, the interpretation of data will be erroneous.) Theoretical equations for the analysis of T-jump data are discussed in the Appendix.

The quartz windows of the T-jump cell enable us to excite the pyranine in vesicle solutions at 466 nm using a light source and a Bausch and Lomb high intensity monochromator. The fluorescence above 500 nm is mainly due to unprotonated form of pyranine [21] and was detected in a perpendicular direction using a filter. A photomultiplier and fast amplifiers were used to convert the fluorescence into electrical signals. A home made digital data acquisition instrument was used for recording the time dependence of fluorescence, and for reducing noise by signal averaging and numerical filtering. At least four relaxation traces were obtained on the same sample to ensure the reproducibility of data.

(I) The vesicle solutions in the T-jump cell were subjected to temperature jump of 1.5 C° by fast Joule heating (with heating time constant ~ 5 μs) by discharging a previously charged condenser. Since the pK of a buffer is temperature dependent, a T-jump will

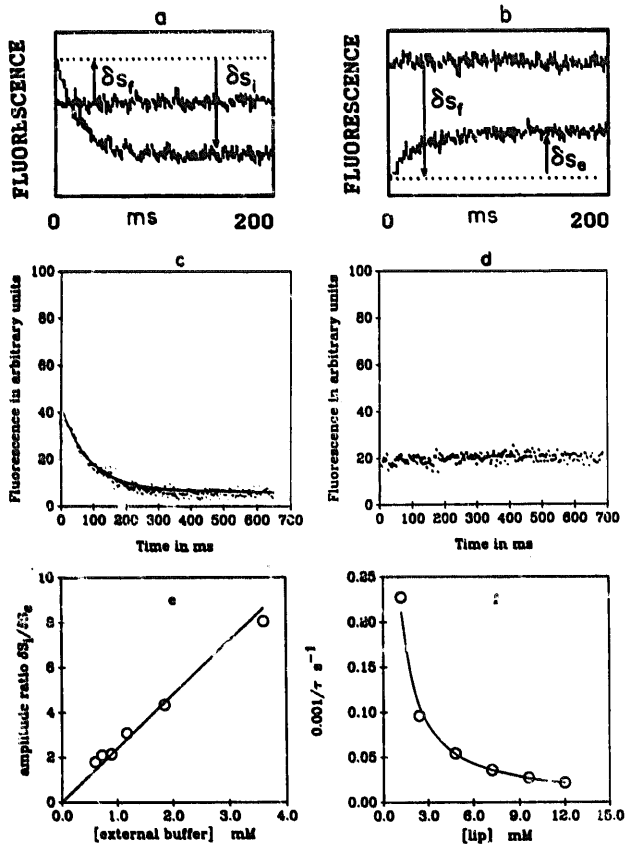

Fig. 3. T-jump relaxation traces observed in SBPL vesicle solutions containing 100 mM KCl at pH 6.9 and with: (a) 2 mM pyranine inside; (b) 7 μM pyranine outside; horizontal traces correspond to fluorescence levels before T-jump ([lip] = 12.5 mM, $[Nig]_0$ = 1.8 μM, 2 mM phosphate buffer inside vesicles, 0.55 mM Aces buffer outside at 25 °C); (c) with 2 mM pyranine inside and the matching single exponential superposed ([lip] = 3.2 mM, $[Nig]_0$ = 0.5 μM, 2 mM phosphate inside and 50 mM Aces outside at 20 °C). (d) Difference between the experimental relaxation and superposed single-exponential in (c). (e) Variation of slow amplitude ratio $\delta S_i/\delta S_e$ with external buffer concentration (=[endogenic buffer on the outer layer]+[Aces buffer outside]) in experiments similar to (a) and (b). (f) Lipid concentration dependence of relaxation rate, τ^{-1}, observed with $[Nig]_0$ = 1.7 μM and with buffer concentrations as given for (c).

produce an associated pH jump also in a solution of buffer species (Eqns. A-8 and A-9). The time constant associated with the establishment of pH jump is determined by the equilibration time of the fast proton exchange equilibria (Eqns. A-1 and A-2) or heating time, whichever is longer. In our experiments the latter is the case. The fast pH jumps and change in the dissociation constant of pyranine with temperature cause changes in the concentration of unprotonated form of pyranine and hence fast changes in fluorescence. The horizontal traces in Figs. 3a and 3b correspond to fluorescence before T-jump. The fast changes in fluorescence, δS_f in these figures, are the algebraic sum of changes due to the above two processes and are accounted for using Eqn. A-13 and $\partial \mathrm{p}K_j/\partial T$ given in Table I. When $\partial \mathrm{p}K/\partial T$ associated with buffer species inside and outside vesicles are different, the pH jump in the aqueous medium inside vesicles will be different from the pH jump outside (Eqn. A-9). In the specific situation when phosphate buffer is inside and Aces is outside, pH inside will be higher than outside by ΔpH (~ 0.025 pH units) as a consequence of this difference in pH jumps. Equilibrium (corresponding to $\Delta\mathrm{pH} = 0$ in our vesicles) will be reached by H^+ transport across vesicular membrane, decreasing the pH inside (which can be observed by a decrease in fluorescence when pyranine is inside as in Fig. 3a) with an associated increase in pH outside (which can be observed by an increase in fluorescence if pyranine is outside as in Fig. 3b). The directions of slower changes in fluorescence (in the millisecond time scale) with amplitudes δS_i and δS_e in Figs. 3a and 3b, and the similarity in the magnitudes of time constants τ associated with them, are consistent with their assignment to ΔpH decay due to H^+ transport across membrane. The possibility that the slower changes in fluorescence are associated with the binding of pyranine to lipid or to lipid-bound nigericin can be excluded, since in that case as far as the direction of fluorescence change is concerned, it would not have mattered whether pyranine was inside or outside vesicles, contrary to the observations of Figs. 3a and 3b. On increasing the buffer concentration outside, pH outside will be strongly buffered and ΔpH decay will cause changes in pH mainly inside vesicles. A typical trace obtained under such conditions and the matching trace from a calibrated exponential generator are shown in Fig. 3c. A dual-channel oscilloscope was used for displaying these two traces. Fig. 3d shows that the difference between the two traces is a constant with respect to time, and confirms the single-exponential nature of the experimental trace. The τ associated with the ΔpH decay, was determined from the time constant of the matching exponential. Further confirmation of the assignment of slow fluorescence changes to H^+ transport was obtained by noting that the amplitude ratio $\delta S_i/\delta S_e$ increased linearly with the buffer

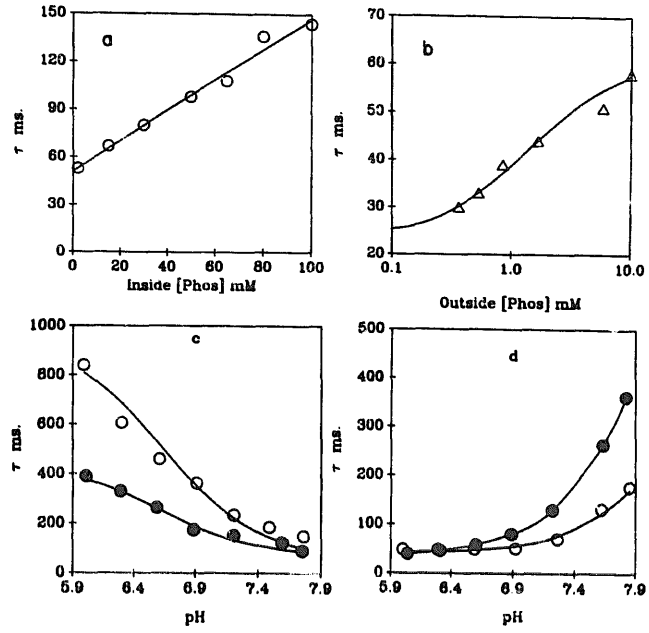

Fig. 4. Increase of τ with increase in phosphate buffer concentration (a) inside (with [lip] = 3.4 mM, $[\mathrm{Nig}]_0 = 0.5$ μM, and Aces outside 30 mM), and (b) outside (with [lip] = 13 mM, $[\mathrm{Nig}]_0 = 3.3$ μM and Aces inside 80 mM) at pH 6.9 and 25°C in SBPL vesicle solutions containing 100 mM KCl. The slope and intercept of the solid line of (a) are used in Eqn. A-18 to get an endogeneous contribution to b_i equivalent to 53 mM phosphate. $k_{+\mathrm{H}} = 4.25 \cdot 10^6$ s^{-1} obtained by fitting the data to Eqn. A-18 has been used in calculating the solid line of (b). Dependence of τ in SBPL vesicle solutions containing: (c) 50 mM NaCl (○), and 250 mM M^+ NaCl (●); (d) 50 mM KCl (○), and 250 mM KCl (●) ([lip] = 5.5 mM, $[\mathrm{Nig}]_0 = 0.8$ μM, 2 mM phosphate inside and 25 mM Aces outside at 25°C). The solid lines were calculated using the tables and Eqn. 1.

capacity outside vesicles, b_e (see Fig. 3e and Eqn. A-14).

(IIa) Since nigericin is preferentially solvated in the lipid we can expect [nig] $\ll 1$ μM in the aqueous medium in the experiments corresponding to Fig. 3f. Vesicle concentration [ves] ~ 2 μM can be estimated from the lipid concentrations [lip]. Thus [ves] $\gg$ [nig] in our experiments. The transfer of nig-H and nig-M between aqueous medium and vesicular membrane (Fig. 1b) can be treated as bimolecular reactions between ion bound nigericins and vesicles. The equilibration rate for such a process cannot decrease on increasing [ves]. Fig. 3f shows that τ^{-1} decreases with increase in [ves]. Therefore, we conclude that the above process does not limit the rate of ΔpH decay.

(IIb) The rate of exchange of nigericin bound H^+ and M^+ with those in aqueous medium can be increased by increasing the concentrations of buffers or [M^+]. (See discussions on the role of buffers in proton transfer [22,23].) Figs. 4a and 4b show that τ increases (or ΔpH decay rate decreases) with increase in buffer concentrations inside and outside vesicles. Even after the expected increases in τ with increases in buffer concentrations (Eqn. A-18) are factored out, the H^+

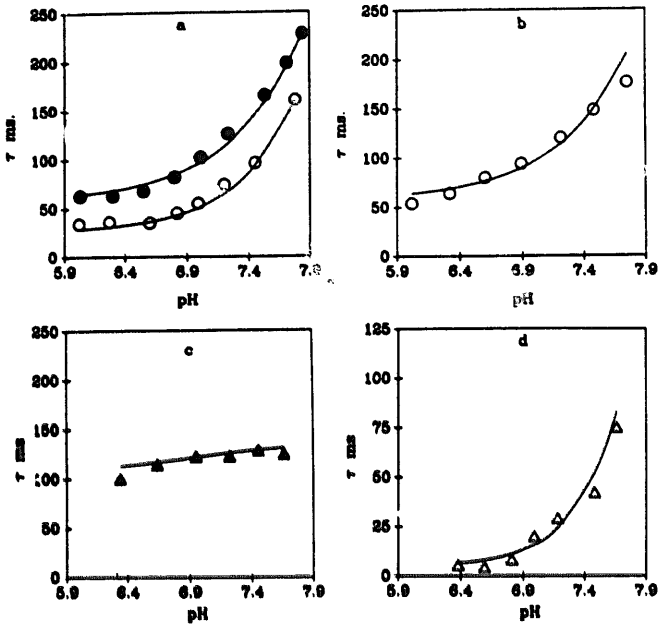

Fig. 5. pH dependence of τ in: (a) SBPL vesicles with 50 mM Aces outside and 2 mM phosphate (○) or 50 mM phosphate inside (●); (b) SBPL vesicles with 50 mM phosphate outside and 50 mM Aces inside (○); (c) 80% PC+20% PE vesicles with 50 mM Aces outside and 50 mM phosphate inside; (d) 92% PC+8% PA vesicles with 5 mM Aces outside and 2 mM phosphate inside. The solutions had 100 mM KCl. ([lip] = 3.4 mM and $[Nig]_0 = 0.5$ μM for (a) and (b); [lip] = 5.6 mM, 3.1 mM and $[Nig]_0 = 0.9$ μM, 0.7 μM for (c) and (d), respectively). Solid lines calculated with the help parameters given in the tables and Eqn. 1.

transport rate does not show an increase with increase in buffer concentrations. The solid lines in Figs. 4a and 4b were calculated using Eqn. A-18 and parameters given in Table I, without invoking any increase in k_{+H} with buffer concentrations. (It was not necessary to invoke the electrogenic H^+ flux, suggested for large pH jumps (~ 2 pH units) by Bramhall [24] in explaining our results.) Fig. 4d shows that increasing $[K^+]$ also increases τ. Therefore, we conclude that increasing the rate of H^+/K^+ exchange between nigericin and aqueous medium, does not enhance τ^{-1} or the rate of ΔpH decay.

The observed decrease of τ (Fig. 4c) with increase in $[Na^+]$ might create the impression that ion exchange rate may limit the ΔpH decay rate when the metal ion is Na^+. However, this possibility is ruled out, since τ increased and k_{+H} did not require to be increased with buffer concentrations even when the metal ion was Na^+.

(III) Figs. 5a–5d show typical data on pH dependence of τ in vesicle solutions containing 100 mM KCl. The data obtained with SBPL vesicles (Figs. 5a and 5b) show that when pH was decreased from 7.7, τ decreased initially, but tended to have a constant value in the pH region 6–6.8. With vesicles prepared from a mixture of 80% PC and 20% PE, τ (Fig. 5c) was nearly constant in the pH region of study (6.5–7.7). The behaviour observed with vesicles prepared from a mixture of 92% PC and 8% PA (Fig. 5d), was similar to that with SBPL vesicles but with a smaller τ at pH ~ 6.5. (Data below pH 6.5 were not reliable in this case, since the amplitude data indicated the possibility of leakage of buffers at low pH.)

Figs. 4c and 4d show that the pH dependence of τ observed in a medium containing Na^+ is opposite to that seen when the metal ion in the medium is K^+. The data show that τ in a medium of Na^+ is lower than the τ in a medium of K^+ at pH ~ 8. In contrast with this, at pH^+ ~ 6, τ in a medium of Na^+ is higher than the τ in a medium of K^+. Only the latter observation can be intuitively correlated with the ionophore selectivity [2,3,25]. Since in a medium of Na^+, τ decreases with $[Na^+]$ (Fig. 4c) and in a medium of K^+, τ increases with $[K^+]$ (Fig. 4d), the above mentioned contra-intuitive situation could be encountered even at pH lower than ~ 8 by a suitable choice of $[M^+]$.

(IV) Since τ depends on the constitution and concentration of buffers inside vesicles (Eqns. A-18 or A-30), the success of the T-jump technique mainly rests on the validity of the assumption that these do not change during the experiments due to leaks in the vesicular membrane. Leaks can be detected by the discrepancy between observed and calculated amplitudes, since the magnitudes of pH jumps (Eqn. A-9) and hence δS_f and δS_i (Eqns. A-13 and A-14) also

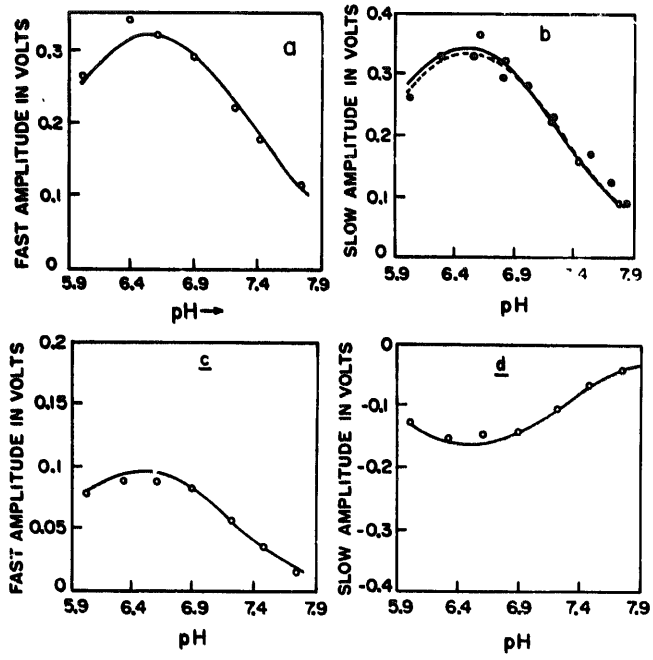

Fig. 6. pH dependence of amplitudes observed on giving 1.5 C° T-jump to solutions containing 100 mM KCl: (a) fast amplitudes with 7 μM pyranine and 20 mM Aces in the absence of vesicles; (b) slow amplitudes in experiments corresponding to Fig. 5a; (c) fast amplitudes in experiments corresponding to Fig. 5b; (d) slow amplitudes in experiments corresponding to Fig. 5b. The solid lines are calculated with the help of data given in tables and $e_r = 0.04$ using Eqns. A-13 and A-14.

depend on the composition and concentrations of buffers inside and on H^+ leaks. Fig. 6a shows the pH dependence of fast fluorescence changes (converted into voltage in our instrument) observed from pyranine solutions in Aces buffer solutions. The solid line superposed on experimental points had been calculated using Eqn. A-13 and confirms the validity of parameters used in the formulae for predicting the pH dependences of amplitudes. Figs. 6b–6d show the pH dependent amplitude data corresponding to the experimental conditions of Figs. 5a and 5b. The agreement between the experimental amplitudes and those calculated using Eqns. A-13 and A-14 (solid lines in Figs. 6b–6d) and the reproducibility of τ data on repeating T-jump on the same sample show that there were no leaks in our experiments.

Discussion

The results given above have enabled the assignment of the observed relaxation (with time constant τ in the millisecond region) to decay of ΔpH across membrane by nigericin-mediated H^+ transport. Furthermore, Figs. 3f, 4a and 4b have shown that neither the transfer of nig-H and nig-M from aqueous medium to lipid nor H^+/M^+ exchanges between the aqueous medium and nigericin limit the rate of ΔpH decay.

In typical experiments we had [lip] ~ 3.3 mM and nigericin concentration in vesicle solutions $[Nig]_0 \sim 0.5$ μM, which corresponds to local concentration [nig] ~ 0.3 mM in membrane. Thus, [nig] was small compared to the total concentration of buffer species contributing to internal buffer capacity b_i ($\geqq$ equivalent of 50 mM phosphate). Therefore, the variation of τ^{-1} from the changes in the contribution of $[Nig]_0$ to b_i on varying $[Nig]_0$ is negligible. The experimentally observed τ^{-1} increases with an increase in $[Nig]_0$, [5], or a decrease in [lip] (Fig. 3f). Since increase of $[Nig]_0$ and decrease of [lip] both result in an increase of the carrier concentration in membrane, [nig], a model in which τ^{-1} depends on [nig] is appropriate.

If there had been leaks in the vesicular membrane, experimental τ^{-1} had to be analysed after including a correction factor. The amplitudes (Fig. 6) and reproducibility of τ data eliminate this possibility of leaks. The pH-dependent fluidity in membranes, presumably controlled by ionisable groups on the membrane, had been inferred in EPR experiments on lipid mixtures [26]. Since the buffer capacity data do not show any variations in the pK of endogeneous groups on changing M^+ from Na^+ to K^+ we do not expect the pH depedence of the fluidity factor to change as much as seen in the τ data of Fig. 4c and 4d by the mere change of M^+. Thus the dominant changes in τ are not due to the changes in fluidity factor.

Information from the pH dependence of τ

The above conclusions have been used in writing Eqns. A-19–A-30 in the Appendix. For the data in Figs. 4c, 4d and 5, buffer concentrations outside vesicles were high and $b_i V_i / b_e V_e \ll 1$ in Eqns. A-18 and A-26. With this approximation Eqn. A-30 can be written as,

$$\tau^{-1} = \{0.95 \cdot [Nig]_0/[lip]\} \cdot \{(\ln 10) \cdot [H^+]/b_i\} f(K_c,[H^+]) \quad (1)$$

$$f(K_c,[H^+]) = [k_1 \cdot k_2/(k_1 \cdot [H^+] + k_2 \cdot K_c)][K_c/(K_c + [H^+])] \quad (2)$$

Comparing Eqn. A-18 with Eqn. 1 we note that k_{+H} is replaced by a complex expression when the details of the transport mechanism are included in the derivation of an expression for τ^{-1}. We note that from a good set of data on the pH dependence of τ, we can get estimates of both k_1 and k_2 with the help of Eqn. 1, if $f(K_c,[H^+])$ is pH dependent.

In Eqn. 1 the contribution to $b_i/[H^+]$ from a buffer group (of $pK = pK_j$) increases with increase in pH, with a tendency to reach a constant value (proportional to $1/K_j$) only for $pH > pK_j + 1$ (see Eqn. A-8). The endogeneous contribution to b_i, estimated from data similar to Fig. 4a and the amplitude data (Fig. 6) show that the buffer groups listed in Table I are relevant in the expression for $b_i/[H^+]$. The b_i data did not change on changing the metal ion in the medium from K^+ to Na^+. Therefore, if $b_i/[H^+]$ alone were to determine the pH dependence of τ, it should have inreased with pH in the entire pH range of our study. Figs. 4d, 5a, 5b and 5d do show an increase in τ with pH, but prominently only for pH > 7. The differences in the patterns of pH dependences seen in Figs. 5c and 5d on changing the lipid composition also show that the endogeneous contribution to b_i is an important factor contributing to the pH dependence of τ. However, $b_i/[H^+]$ alone cannot explain (i) the unusual decrease in τ with increase in pH when Na^+ was the metal ion in solutions (Fig. 4c) and (ii) the insignificant change in τ with pH in the range 6–6.8 when M^+ was K^+ (Figs. 4d and 5). Therefore, we conclude that $f(K_c,[H^+])$ is significantly pH dependent.

The τ calculated using Eqns. 1 and 2 with $k_1 = k_2$ (solid lines in Figs. 4c, 4d, 5a–5d) are in good agreement with the data throughout the pH range of our study. The estimates of K_c and k_1 ($= k_2$) given in Table II used in these calculations are reasonable as discussed below.

Eqn. A-23 requires that K_c should increase with increase in $[M^+]$. Table II shows such a trend. The estimated K_c are not exactly proportional to $[M^+]$ probably since activity coefficients do change with ion concentrations. Using Table II and the pK associated

TABLE II

Parameters [a,b] used in the calculation of the solid lines of Figs. 4c, 4d and 5a–5d using Eqn. 1, which account for the observed pH dependence of τ

Apart from the endogenic contributions given below, b_i includes contributions from buffers trapped inside.

System	Contribution to b_i (mM)	pK	pK_c	$k_1(=k_2)$ (10^3 s^{-1})
92% PC+8% PA with 0.1 M K^+	80	8.0	6.85	8.5
80% PC+20% PE with 0.1 M K^+	60	6.9	6.85	6.6
SBPL with 0.1 M K^+	30	6.9		
	45	7.8	6.78	7.9
SBPL with 0.05 M K^+	[c]	[c]	6.90	6.6
SBPL with 0.25 M K^+	[c]	[c]	6.65	4.8
SBPL with 0.05 M Na^+	[c]	[c]	8.20	5.6
SBPL with 0.25 M Na^+	[c]	[c]	7.80	4.8

[a] The errors in the estimates of k_1 and pK_c, are $\sim \pm 1.2 \cdot 10^3$ s^{-1} and $\sim \pm 0.05$, respectively.

[b] pK is associated with the buffer group contributing to b_i and having a concentration given in the second column. pK_c is $-\log_{10}(K_c)$.

[c] Taken to be the same as that observed with 0.1 M K^+ since the experiments showed variations within the limits of errors.

with nigericin (= 8.45) [2], the apparent metal ion dissociation constants of nig-M can be estimated with the help of Eqn. A-23. ($K_M \sim 6.4 \cdot 10^{-2}$ M and $1.5 \cdot 10^{-3}$ M for Na^+ and K^+, respectively, at 50 mM ion concentrations). The order of magnitude of the ratio of these K_M is close to that expected from the data reported in the literature: the binding of K^+ to nigericin is nearly 50-times stronger than that of Na^+ [25]. The above explanation also requires the rate constants associated with translocations of nig-H, nig-K and nig-Na across membrane to have similar magnitudes ($k_1 = k_2 \sim 6 \cdot 10^3$ s^{-1}). This is reasonable since nig-H and nig-M are electrically neutral and can have only small differences in their energies of solvation in the lipid medium and in their sizes. It may be noted that the rate constant associated with the translocation of protonated FCCP (which is also electrically neutral) in planar bilayers also has a similar value [22]. However, we cannot compare our results with those reported for monensin mediated Na^+ transport [27], since these authors have not analysed their data by a mechanism similar to Fig. 1 including H^+/Na^+ exchange.

Rate-limiting species

Since the pK of nigericin is ~ 8.45, concentration of nigericin species to which an ion is not bound, [nig$^-$], is negligible compared to [nig-H] + [nig-M] in the pH range of our study. Thus using Eqns. A-21 and A-23 we get [nig-M]/[nig-H] $\approx K_c/[H^+]$. At sufficiently low pH, [nig-H] $\approx$ [nig]$_0$ and so [nig-M] $\approx$ [nig]$_0 \cdot K_c/[H^+]$. Similarly, at high pH, [nig-M] $\approx$ [nig]$_0$ and [nig-H] $\approx$ [nig]$_0 \cdot [H^+]/K_c$. Therefore, using Eqns. 1 and 2 we find that at low pH when $K_c \ll [H^+]$, τ^{-1} is proportional to [nig-M]/$\{k_1/k_2 + $[nig-M]/[nig]$_0\}$ and at high pH when $K_c \gg [H^+]$ it is proportional to [nig-H]/$\{k_2/k_1 + $[nig-H]/[nig]$_0\}$. For nigericin we have $k_1 \approx k_2$. Therefore, we can say that when $K_c <$ $[H^+]$ translocation of nig-M and when $K_c > [H^+]$ translocation of nig-H across membrane dominantly limit the rate of ΔpH decay. (If $k_1 \ll k_2$ and $k_1 \gg k_2$, respectively, in the above two situations, the above conclusions would require much extreme pH conditions than that determined merely by the magnitude of K_c.) However, translocations of nig-H and nig-M, respectively, in the above two situations are also important in determining the rates of ΔpH decay in the pH range 6–8 of our study.

Using the estimates of K_c (Table II) we note that at pH $\sim$ 7.5 the dominant rate-limiting species is nig-H in a medium containing K^+ and nig-Na in a medium containing Na^+. At this pH, at sufficiently low metal ion concentrations (say $\sim$ 50 mM), [nig-H] in a medium of K^+ will be greater than [nig-Na] in a medium of Na^+ and hence the net H^+ transport rate and the associated M^+ transport rate (which cause the ΔpH decay) will be higher in a medium of K^+. This pattern follows the ionophore selectivity data [2,3,25]. However, on increasing the ion concentration say to 250 mM, [nig-H] will decrease in a medium of K^+ and [nig-Na] will increase in a medium of Na^+ to such an extent that the transport rates in a medium of K^+ will be lower than that in a medium of Na^+. Such a behaviour seen in Figs. 4c and 4d could not have been guessed easily from ionophore selectivity data.

Furthermore, at very low pH, the estimates of K_M given above yield [nig-K] > [nig-Na] when $[K^+] = [Na^+]$. Therefore, when $K_c < [H^+]$ for both K^+ and Na^+ the rate of nigericin-mediated ΔpH decay and the associated H^+ and M^+ ion transport rates will be higher with K^+ ions rather than with Na^+ ions (since nig-M is the dominant rate-limiting species at such pH). This is consistent with the conventional understanding [2,3]. In contrast with this, since the binding of Na^+ to nigericin is an order of magnitude weaker than K^+, it is easier to displace the nigericin bound Na^+ by H^+. Therefore, when $[K^+] = [Na^+]$, the concentration [nig-H] will be higher with Na^+ ions rather than with K^+ ions. Thus, when $K_c > [H^+]$ for both the metal ions the rate of nigericin-mediated ΔpH decay and the associated M^+ transport across membrane will be higher (by as much as 20–50-times depending on the ion concentration) with Na^+ rather than with K^+ (since nig-H will be the dominant rate-limiting species at such pH), even though ionophore selectivity of nigericin towards K^+ is 25–45-times higher than that towards Na^+ [2,3,25]. This fact, which has the support of our experimental data

(Figs. 4c and 4d) and theoretical expressions, does not appear to have been widely recognised. In liposomes, the data of Table II can be useful in chosing K^+ or Na^+ to obtain maximum nigericin-mediated ion transport rates at given pH and ion concentration.

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Appendix

Equilibria

The proton dissociation equilibria relevant in the discussion of vesicle solutions are,

$$B_jH^+ \rightleftharpoons B_j + H^+ \quad \text{(A-1)}$$

$$H_2O \rightleftharpoons H^+ + OH^- \quad \text{(A-2)}$$

with 'j' referring to buffer species (e.g. $j = 0$ refers to pyranine). The apparent proton dissociation constants K_j can be used to obtain the concentrations of the protonated form of buffer species $[B_jH^+]$ in terms of the total buffer species concentration $[A_j]$:

$$[B_jH^+] = [A_j]\cdot[H^+]/(K_j + [H^+]) \quad \text{(A-3)}$$

$$[A_j] = [B_jH^+] + [B_j] \quad \text{(A-4)}$$

Buffer capacity and pH change due to proton transport

If $\delta[H^+]_e^t$ is the expected increase in $[H^+]_e$ outside vesicles (region 'e') due to net H^+ transport from inside vesicles (region 'i') in the absence of fast protonation equilibria, the pH change δpH_e in 'e' after the equilibration of the fast protonation equilibria can be expressed in terms of the buffer capacity of 'e', b_e.

$$\delta pH_e = -\delta[H^+]_e^t / b_e \quad \text{(A-5)}$$

$$b_e = (\ln 10)\left\{\sum_j \frac{[A_j]\cdot K_j[H^+]_e}{(K_j + [H^+]_e)^2} + [H^+]_e + [OH^-]_e\right\} \quad \text{(A-6)}$$

where the summation extends over the buffer species which are in fast H^+ exchange equilibrium with the aqueous medium in 'e'. Using the conservation of protons, we can write,

$$\delta pH_i \cdot b_i \cdot V_i = -\delta pH_e \cdot b_e \cdot V_e \quad \text{(A-7)}$$

b_i is the buffer capacity and δpH_i is the pH change in 'i'. V_i and V_e are the aqueous solvent volumes in regions 'i' and 'e'.

pH-jump associated with T-jump in a mixture of buffer species

When a mixture of buffers in fast proton exchange equilibrium with the aqueous medium is subjected to a T-jump δT, the proton dissociation constants of the buffer species change by δpK_j [28], requiring a change in the equilibrium concentration of H^+.

$$\delta pK_j = (\partial pK_j/\partial T)\cdot\delta T \quad \text{(A-8)}$$

Equilibrium concentration of H^+ will be reached by the change in concentrations $\delta[C_m]$ of the various species participating in the fast protonation equilibria. For small changes $\delta[C_m]$ when second-order terms such as $\delta[C_m]\cdot\delta[C_n]$ could be neglected, the expression for δpH_x, the change in pH of the region 'x', can be derived using proton conservation relations.

$$\delta pH_x = (\ln 10)\left\{\sum_j \frac{[A_j]\cdot K_j\cdot[H^+]_x}{(K_j + [H^+]_x)^2}\cdot\delta pK_j + [OH^-]_x\cdot\delta pK_w\right\} b_x^{-1} \quad \text{(A-9)}$$

$$K_w = [H^+]_x\cdot[OH^-]_x \quad \text{(A-10)}$$

In Eqn. A-9, b_x is the buffer capacity of the region 'x' (see Eqn. A-6) and the summation extends over the buffer species which are in fast H^+ exchange equilibrium with the aqueous medium in 'x'. At the concentrations used in our experiments the second term of Eqn. A-9 can be neglected.

Amplitudes of fluorescence changes in T-jump experiments

If S is the total signal intensity due to fluorescence from pyranine in the unprotonated and protonated forms (B_0, and B_0H^+) and if δS is the change in signal intensity (or amplitude) due to changes in their concentrations $\delta[B_0]$ and $\delta[B_0H^+]$, such that $\delta S \ll S$,

$$\delta S/S = (e_1\cdot\delta[B_0] + e_2\cdot\delta[B_0H^+])/(e_1\cdot[B_0] + e_2\cdot[B_0H^+]) \quad \text{(A-11)}$$

when the amount of light absorbed by the solution is small compared to the incident light [11]. In eq Eqn. A-11 e_1 and e_2 are constants of proportionality reflecting the fluorescence efficiency of the two species. At the wave lengths of observation we have $e_1 \gg e_2$.

$$e_r = e_2/e_1; \quad S_t = S(1 - e_r) \quad \text{(A-12)}$$

(a) Fast amplitudes δS_f. Following a T-jump, the relative concentrations of protonated and unprotonated forms of pyranine in the region 'x' change due to (i) change in the proton dissociation constant of pyra-

nine, δpK_0 and (ii) pH jump, δpH_x (see Eqn. A-9) in 'x'. The fast change in the pyranine fluorescence (fast amplitude), δS_f has contributions from both the processes. Using Eqns. A-9–A-12 we can write,

$$\delta S_f = (\ln 10)\cdot S_t\cdot(\delta pH_x - \delta pK_0)\cdot\frac{[H^+]}{(K_0+[H^+])}\cdot\frac{K_0}{(K_0+e_r\cdot[H^+])} \quad \text{(A-13)}$$

The correction e_r due to non negligible fluorescence from the protonated form of pyranine becomes important in low pH situations.

(b) Slow amplitudes. At equilibrium pH inside and outside are equal in our vesicles. (This had been inferred from the experiments which showed negligible change in the pH dependence of pyranine fluorescence on entrapping it inside vesicles.) If the pH jump in 'e' is not equal to that in 'i', a pH gradient ΔpH will be established across the vesicular membrane. ΔpH will decay and equilibrium between 'e' and 'i' will be established by H^+ transport across membrane. Using the conservation relation Eqn. A-7 and the expression Eqn. A-9 for fast pH jumps, the amplitude δS_i of the slower fluorescence change due to ΔpH decay observed with pyranine in 'i' can be expressed as,

$$\delta S_i = \frac{(\ln 10)\cdot S_t\cdot(\delta pH_i - \delta pH_e)}{(1+b_i\cdot V_i/b_e\cdot V_e)}\cdot\frac{[H^+]}{(K_0+[H^+]_i)}\cdot\frac{K_0}{(K_0+e_r\cdot[H^+]_i)} \quad \text{(A-14)}$$

The expression for the slow amplitude δS_e observed with pyranine in 'e' is similar to Eqn. A-14 but with subscripts 'i' and 'e' interchanged.

Validity of Eqn. A-14. The expression Eqn. A-14 is valid if ΔpH decay is controlled by the $[H^+]$ gradient alone. In nigericin-mediated H^+ transport, exchange of metal ion M^+ by H^+ ensures that there is no build up of electric potential across membrane which could have opposed ΔpH decay. Since $[M^+] \gg [H^+]$ and the deviations from equilibrium concentrations are small in our experiments, analysis similar to Donnan membrane equilibrium can be used to show that a quasi-equilibrium associated with cation transports will be reached when $[H^+]$ outside and inside are nearly equal. Therefore, Eqn. A-14 can be used to analyse our data.

Relaxation times

In homogeneous reactions in solutions, when the deviation from equilibrium is small, the time dependence of approach to equilibrium can be expressed as a sum of exponentials [11]. Procedures for obtaining expressions for relaxation times in these cases are well known [29,30]. We can treat the physical process of H^+ transport across membrane as a chemical reaction in which the proton outside is converted into a proton inside vesicles or vice-versa, with subsequent reactions involving buffer species. The rate equation for small deviations from equilibrium concentrations is,

$$-d\{\Delta Y_i\}/dt = k_{+H}\cdot\Delta[H^+]_i - k_{-H}\cdot\Delta[H^+]_e \quad \text{(A-15)}$$

$$Y_i = \sum[B_jH^+]_i + [H^+]_i - [OH^-]_i \quad \text{(A-16)}$$

when the H^+ transport is controlled by the $[H^+]$ gradient and a fast M^+ transport prevents the build up of electric potential across membrane. In Eqn. A-16 the summation is over the buffer species 'j' involved in fast proton exchange equilibria in 'i' (fast compared to H^+ transport rates). k_{+H} and k_{-H} are the rate constants associated with H^+ transport from 'i' to 'e' and from 'e' to 'i'. With $\delta K_w = 0$ during the process of H^+ transport Eqn. A-15 can be rewritten using Eqns. A-5–A-7.

$$-d(\Delta Y_i)/dt = \Delta Y_i/\tau \quad \text{(A-17)}$$

$$\tau^{-1} = (\ln 10)\{1 + b_iV_i/(b_eV_e)\}k_{+H}\cdot[H^+]_i/b_i \quad \text{(A-18)}$$

Since the chemical species in Y_i are in fast H^+ exchange equilibria with the aqueous medium 'i', the concentrations of each of these species in 'i' (including pyranine in 'i') approach equilibrium values with the time constant τ. Contributions to τ from OH^- transport can be included by adding a term with $k_{+OH}\cdot[OH^-]_i$ to Eqn. A-18. Such an expression is similar to that given by Grzesiek and Dencher [6]. However, the factor involving V_i/V_e in Eqn. A-18 had not been considered by these authors.

Nigericin-mediated proton transport. Eqn. A-18 does not include information about the mode of H^+ and M^+ transports. For nigericin-mediated transport, Fig. 1 can be used to determine the relaxation behaviour. In Results we have shown that H^+/M^+ exchange between nigericin and aqueous medium or exchange of nigericin between the aqueous and lipid phases are not the rate-limiting steps of ΔpH decay. Thus, for the rate-limiting step,

$$-d\Delta\{Y_i + [\text{nig-H}]_i\}/dt = k_1\cdot\Delta[\text{nig-H}]_i - k_1\cdot\Delta[\text{nig-H}]_e \quad \text{(A-19)}$$

$$-d\Delta\{[\text{nig-H}]_i + [\text{nig-M}]_i\}/dt = k_1\cdot\Delta[\text{nig-H}]_i - k_1\cdot\Delta[\text{nig-H}]_e + k_2\cdot\Delta[\text{nig-M}]_i - k_2\cdot\Delta[\text{nig-M}]_e \quad \text{(A-20)}$$

where the subscripts 'i' and 'e' for the nigericin-ion complexes refer to their concentrations in the inner

and outer layers of vesicles. We can express $[nig\text{-}H]_i$ in terms of the total nigericin concentration in the inner layer $[nig]_{0i}(=[nig^-]_i+[nig\text{-}H]_i+[nig\text{-}M]_i)$ using Eqn. A-21.

$$K_H=[nig^-]\cdot[H^+]/[nig\text{-}H];\quad K_M=[nig^-]\cdot[M^+]/[nig\text{-}M] \tag{A-21}$$

$$[nig\text{-}H]_i=[nig]_{0i}\cdot[H^+]/\{K_c+[H^+]\} \tag{A-22}$$

$$K_c\approx K_H+K_H\cdot[M^+]/K_M \tag{A-23}$$

using $[H^+]_i=[H^+]_e=[H^+]$; $[nig]_{0i}=[nig]_{0e}=[nig]_0$ at equilibrium; $\Delta[nig]_{0i}=-\Delta[nig]_{0e}$; Eqns. A-7 and A-22, we can rewrite Eqns. A-19 and A-20.

$$-d\Delta[H^+]_i/dt=a_{11}\cdot\Delta[H^+]_i+a_{12}\cdot\Delta[nig]_{0i} \tag{A-24}$$

$$-d\Delta[nig]_{0i}/dt=a_{21}\cdot\Delta[H^+]_i+a_{22}\cdot\Delta[nig]_{0i} \tag{A-25}$$

$$a_{11}=k_1\cdot[nig]_0\{(\ln 10)\cdot[H^+]/b_i\}\{K_c/(K_c+[H^+])^2\}\times\{1+b_i\cdot V_i/b_e\cdot V_e\}$$

$$a_{12}=2\cdot k_1\cdot\{(\ln 10)\cdot[H^+]/b_i\}\{[H^+]/(K_c+[H^+])\}$$

$$a_{21}=(k_1-k_2)\{[nig]_0\cdot K_c/(K_c+[H^+])^2\}\{1+b_i\cdot V_i/b_e\cdot V_e\}$$

$$a_{22}=2(k_2\cdot K_c+k_1\cdot[H^+])/(K_c+[H^+]) \tag{A-26}$$

Also total nigericin concentration in the lipid medium $[nig]_0$ can be expressed in terms of the total nigericin concentration in the vesicle solutions $[Nig]_0$. Taking the inner radius of vesicles to be 100 Å and outer radius to be 150 Å [6], we can calculate the total volume of the lipid phase in vesicle solutions to be $2.38\cdot V_i$. Using the estimate of $V_e/V_i(=2.25/[lip])$ we can write,

$$[nig]_0=0.95\cdot[Nig]_0/[lip] \tag{A-27}$$

With such equations describing ΔpH decay, the relaxation behaviour is a sum of two exponentials with relaxation times τ_1 and τ_2.

$$\tau_1^{-1}+\tau_2^{-1}=a_{11}+a_{22};\quad \tau_1^{-1}\cdot\tau_2^{-1}=a_{11}\cdot a_{22}-a_{12}a_{21} \tag{A-28}$$

The experimentally observed τ^{-1} for the rate-limiting step of ΔpH decay has to be the slower of the two relaxation rates. τ^{-1} increases with $[nig]_0$, with negligible rate for $[nig]_0=0$. Such a situation can be realised when $a_{22}\gg a_{11}$. In this case, the faster relaxation is associated with the equilibration of nigericin between the two layers of the membrane and has a time constant τ_f.

$$\tau_f^{-1}\approx a_{22} \tag{A-29}$$

The slower relaxation associated with ΔpH decay has a time constant τ.

$$\tau^{-1}=a_{11}-a_{12}a_{21}/a_{22} \tag{A-30}$$

Eqns. A-26–A-30 can be used to obtain a detailed expression for τ^{-1} (see Eqn. 1 in the text).

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