NUCLEAR MAGNETIC RESONANCE DETERMINATIONS OF PERMEATION COEFFICIENTS FOR MALEIC ACID IN PHOSPHOLIPID VESICLES

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ABSTRACT Lipid bilayer permeation coefficients for the neutral maleic acid molecule and the maleate monoanion have been determined by proton magnetic resonance techniques. Phosphatidylcholine-cholesterol (2:1) unilamellar vesicles were prepared having an initial maleate anion concentration gradient stabilized by coupling to an impermeant potassium counterion. The coupling was released by addition of valinomycin, and the time evolution of external pH, internal pH, and maleate concentration followed using nuclear magnetic resonance areas and chemical shifts. Transport rate equations were numerically integrated to fit the data, yielding best fit permeation coefficients of 4×10^{-9} and 4×10^{-5} cm/s for maleate monoanion and maleic acid, respectively.

INTRODUCTION

The inherent permeability of lipid bilayer membranes to small hydrophilic solutes has been of fundamental interest in molecular biophysics for many years. Recently, however, interest has heightened in view of the fact that for molecules that dissociate, differences in permeabilities of equilibrating forms may play a very direct role in the concentration of solutes in small vesicular structures of both physiological (1) and pharmacological importance (2). It has been suggested, for example, that preferential permeation of the neutral form of catecholamines leads to their accumulation in chromaffin granules in response to a lowering of intravesicular pH (1, 3). It has also been demonstrated that carboxylic acids can be concentrated in artificial lipid vesicles by shifting the external equilibrium in favor of the more permeable, fully protonated acid by lowering external pH (4).

The quantitative study of dissociable solute permeation in small vesicular structures is not an easy task. Both solute and hydrogen ion concentrations should be monitored inside and outside the vesicle. Monitoring internal concentrations is made difficult by the small size of vesicular structures, and the high surface to volume ratios make very rapid transport measurements necessary under many circumstances.

Nuclear magnetic resonance (NMR) has a number of potential advantages as a method of monitoring transport in vesicular and cellular systems. Most important is its applicability under physiological and near physiological conditions. Also, resonances arising from solutes of interest can display discrete chemical shifts for internal and external environments making possible simultaneous observation of the vesicle interior and exterior without the necessity of physical separation. The area of an NMR, when the spectrum is accumulated under suitable

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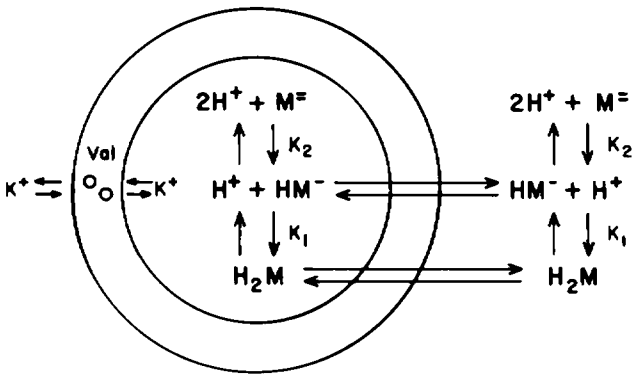

FIGURE 1 Diagram of transport across a vesicle membrane. Arrows indicate species considered to be transportable. Symbols are defined as follows: HM^-, maleate monoanion; $M^=$, maleate dianion; H_2M, maleic acid; Val, valinomycin.

conditions, bears a quantitative 1:1 relationship with numbers of solute molecules. And, resonance position can reflect a second environmental factor of interest, such as pH.

We have made preliminary use of some of these properties in the study of pH-driven accumulation of fumaric acid in unilamellar phospholipid vesicles containing maleic acid buffer. These metabolically important dicarboxylic acids provide a nearly ideal illustrative system, because they have a single, nonexchangeable olefinic proton resonance which reflects pH via its chemical shift over the pH range of 2 to 7. Examination of position and areas for internal and external peaks as a function of time, after lowering external pH, confirmed the strong coupling of carboxylate and proton transport via the selective permeation of the neutral acid molecule (4). The extent of coupling and persistence of a new pseudoequilibrium in which neutral forms of maleic and fumaric acids are at equilibrium in the presence of large total maleate or fumarate gradients would at first suggest negligible permeability for the anionic forms. In fact, persistence may reflect either anion or cation impermeability because of the tendency to maintain electroneutrality during transport.

We wish to present here NMR experiments on a potassium maleate system in which potassium transport has been enhanced by addition of the potassium ionophore, valinomycin, to the point where potassium ion transport cannot be rate limiting. Under these circumstances, we hope to quantitate permeability coefficients for both neutral and monoanion forms of maleic acid. A schematic diagram for the system to be studied is presented in Fig. 1.

MATERIALS AND METHODS

The following material describes a typical maleate transport experiment. Reagent grade maleic acid (Eastman Kodak, Rochester, N.Y.) and KOD were dissolved in 99.8 mol/100 mol D_2O (Bio-Rad Laboratories, Richmond, Calif.) to prepare a 0.20 M maleate stock solution at pH 6.7. The solution was made 1.0 mM in sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS), purchased from Merck Sharpe & Dohme (St. Louis, Mo.) to provide an NMR chemical shift standard. Egg yolk phosphatidylcholine (PC) was isolated from hen eggs following the method of Singleton et al. (5). PC and cholesterol (C) from Fisher Scientific Co. (Fair Lawn, N.J.) were dissolved in $CHCl_3$ in a 2:1 mol ratio and the resulting solution evaporated to dryness on a vacuum line. The dry PC-C mixture was stored at −10°C under nitrogen until needed.

Vesicles having a high internal maleate concentration were prepared by sonicating a 10% (wt/vol) dispersion of the PC-C mixture in the maleate stock solution under a nitrogen atmosphere with a Branson model E bath sonicator (Branson Sonic Power Co., Danbury, Conn.) at 40°C ± 5°C until a relatively clear preparation was obtained (about 3 h). These vesicles have been previously characterized as being unilamellar spherical structures having diameters of ≃320 Å (6). A maleate concentration gradient was established across the membrane by replacing the outside solution with a buffer having a low maleate concentration with a gel permeation column. Transport of maleate monoanion can be best studied if the following conditions are met initially: (*a*) vesicles are not under osmotic stress, (*b*) neutral maleic acid is already at equilibrium across the membrane, (*c*) no transmembrane potassium gradient exists (to minimize the generation of an electrical potential upon valinomycin addition), and (*d*) the outside total maleic acid concentration is such that the area of the outside resonance is observable but smaller than the area of the inside resonance. These conditions can be met by suitable selection of the elution buffer. Because the internal volume will be only 3% of the total volume after elution, an elution buffer low in maleic acid is needed to insure a small outside resonance. To balance neutral acid concentrations, the external pH must be lowered. The acid dissociation constant expressions can be written in terms of pH, neutral acid concentration, and total maleate concentration. Equating neutral acid concentration inside and outside yields the precise value for the pH of the elution buffer that will insure balance. A buffer at 4.9 was used. The elution buffer was made 0.185 M in K_2SO_4 to match the internal osmolarity and potassium concentration. This deuterated buffer, which also contained 1 mM DSS as chemical shift reference, was then used to elute an ≃ 2-ml vesicle sample, as prepared above, from a Sephadex G-50 (Pharmacia Fine Chemicals, Piscataway, N.J.) 0.9 × 5 cm column.

Valinomycin (Calbiochem, San Diego, Calif.) was dissolved in aqueous ethanol at a concentration of 5.4×10^{-4}M and added to vesicle suspensions at zero time of each experiment. Uncouplers in the form of either picric acid or 2,4-dinitrophenol (2,4-DNP) were added in some experiments. When used, they were added before valinomycin, picric acid as a 2.8×10^{-3} M solution in the elution buffer and 2,4-DNP as a 1.1×10^{-4} M solution in 95% ethanol.

Spectra of samples prepared in the above manner were obtained on a Bruker HX 270 spectrometer (Bruker Instruments, Inc., Billerica, Mass.) operating in the pulse Fourier transform mode with quadrature detection at 270 M Hz. Approximately 24 scans at 2-s intervals using 8 K data points, a 90° pulse, and a 3,000 Hz sweep width were required for adequate signal to noise. All spectra were run at 20°C.

In a typical experiment ≃ 2 μl of valinomycin solution was added to 400 μl of a vesicle suspension in an NMR tube, and the contents of the tube vigorously shaken. The first spectrum could be taken within 1 min and successive spectra accumulated in either automated or manual modes. The changes in resonance position could be determined to ± 0.003 ppm. The changes in areas of inside and outside resonances were analyzed by cutting and weighing peaks. Relative fractional internal areas determined in this manner were reproducible within 6%.

RESULTS

Fig. 2 presents the low field portions of the proton NMR spectrum of a vesicle suspension prepared to have 0.2 M maleate at pH 6.7 inside and 1 mM maleate at pH 4.9 outside. The internal and external maleate resonances are initially at 5.98 and 6.22 ppm relative to DSS, respectively. The relationship between chemical shift and pH for maleic acid is well established (4, 7). The resonance shifts 0.3 ppm upfield on dissociation of monoanion to dianion. The position of the outside resonance at zero time is characteristic of pH 5.5. It has been ascertained that the mere presence of vesicles does not alter the chemical shift vs. pH relationship for molecules in the external solution. The apparent change from preparation conditions would be consistent with incomplete exchange of buffers on our column. This is probably not critical because both buffers are at equilibrium with respect to neutral acid and total osmolarity. The resonance position of the inside peak is actually at the limit of the

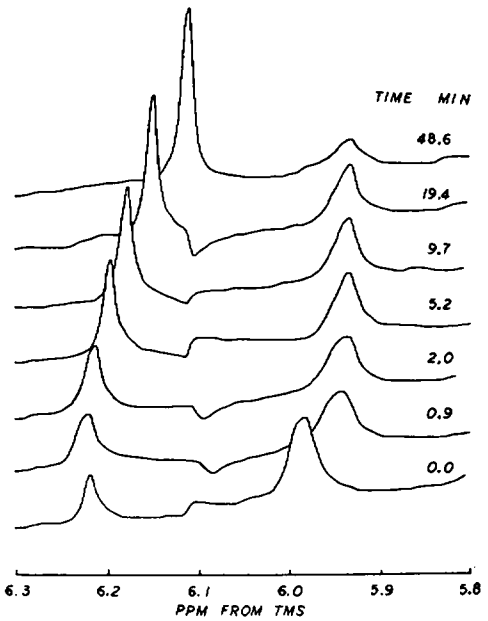

FIGURE 2

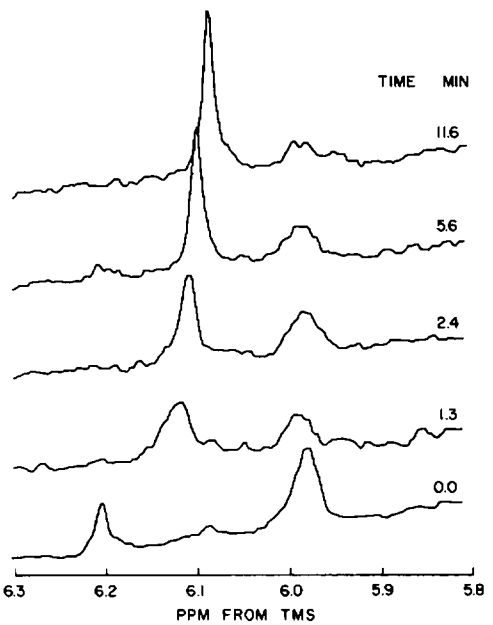

FIGURE 3

FIGURE 2 270 M Hz NMR spectra of potassium maleate on the inside and outside of unilamellar vesicles as a function of time after valinomycin addition. Initial internal and external maleate concentrations are 0.2 and 0.002 M, respectively. Valinomycin was added to 3×10^{-6} M. The temperature was 20°C.

FIGURE 3 270 M Hz NMR spectra of potassium maleate on the inside and outside of unilamellar vesicles as a function of time after picric acid and valinomycin addition. Conditions are the same as for Fig. 2 except that valinomycin was added to 4×10^{-6} M and picric acid to 1×10^{-3} M.

titration curve and would indicate a very high pH (7.2) if treated in a manner similar to the external solution peak. Shifts in some subsequent spectra are beyond the limits of that curve. Therefore, pH cannot be read from this resonance position without regard to the internal vesicle environment. There are several examples of a 0.03–0.05 ppm upfield shift of resonances arising from the inside of a vesicle: choline methyl of the phosphatidylcholine itself and the *N*-methyl of tetramethylammonium chloride, for example. If a pH 6.9 maleate vesicle preparation is examined after sonication without substitution of a low concentration maleate buffer on the outside, the high ratio of areas for external and internal peaks would prevent resolution of two resonances even if separated by 0.05 ppm. However, addition of a small amount of $Pr(NO_3)_3$ to the solution shifts the external maleate resonance, leaving an inside resonance behind which is, in fact, 0.04 ± 0.02 ppm upfield from that of the original position of the outside resonance. The origin of this shift difference is not well understood, but its nonselectivity is reminiscent of an anisotropic susceptibility of the vesicle itself. 0.05 ppm, a number that keeps all internal shifts on the titration curve determined for bulk solutions, has therefore been added to all interior resonance shifts before using them to ascertain interior pH. For the sample in Fig. 2, the pH determined in this way is 6.6, in good agreement with preparation conditions. The relative area of the two resonances is 3.1:1.0. spin lattice relaxation times (T_1s) for the interior and exterior resonances have been measured for

analogous systems and found not to differ significantly. Under these circumstances, peak area is directly related to numbers of molecules. If the interior concentration is 0.20 M and the external concentration is 0.001 M, the exterior to interior volume ratio would have to be 65:1. Area measurements on inside and outside maleate peaks before column exchange of buffer show a ratio of only 32:1, and calculations, assuming a 320 Å diameter, 50 Å bilayer thickness, 60 $Å^2$/lipid, and a lipid concentration of 60 mM, predict a 37:1 volume ratio. It seems likely that the external maleate concentration is higher than 0.001. This is again consistent with imperfect buffer replacement on the column. An external to internal volume ratio of 33:1 and an external concentration of 0.002 M will be used in data analysis. The fact that relative areas and peak positions do not deviate from values measured immediately after column exchange over a period of 2 h indicates a high stability for the system with low permeability to species other than the fully protonated acid.

The successive spectra in Fig. 2 illustrate the effect of adding valinomycin to increase K^+ permeability and thereby release the K^+ — maleate electrostatic coupling of transport. Addition of valinomycin to a concentration of 3×10^{-6} M causes dramatic variations within short times indicating that low K^+ and not low maleate anion permeation rates initially prevented complete equilibration. It is noteworthy that changes in maleate resonance positions (upfield shift) indicate that, initially, pH rises both inside and outside the vesicle. During this same time period, the outside resonance grows in intensity at the expense of the inside resonance indicating loss of internal maleate. We presume this is the result primarily of monoanion and not dianion permeation as the minus two charge on the dianion should make its passage through the hydrophobic portion of the bilayer much less likely. The direction of the pH change is not perplexing if one realizes that maleic acid is a diacid whose monoanion can act as either acid or base, depending on initial pH. Inside the vesicle, the monoanion at pH 6.6 is primarily an acid so its loss will raise the pH. Outside the vesicle, monoanion is less important as an acidic species, and flow of neutral molecule into the vesicle, once equilibrium is upset, is responsible for the rise in external pH.

If one wishes to use the above data to quantitate maleate monoanion permeation, one must be sure that enough valinomycin has been added so that maleate and not K^+ transport is, in fact, rate limiting. Calculations based on the work of Laprade et al. (8) indicate that with our vesicle preparations at 3×10^{-6} M valinomycin, an approximate half time for permeation of K^+ should be 1.0 s and hence not rate limiting. We nevertheless ran experiments, such as that in Fig. 2, at two different valinomycin concentrations. The results of taking an initial rate from the first three points in each case are presented in Table I. If K^+ transport is indeed not

TABLE I
EFFECT OF IONOPHORE AND UNCOUPLER LEVELS ON MALEATE TRANSPORT

Valinomycin	K^+	Uncoupler	Initial flux*
M	*M*		*mol/cm² s*
2.6×10^{-6}	0.36		1.3×10^{-13}
2.3×10^{-5}	0.40		2.2×10^{-13}
1.8×10^{-6}	0.36	9.3×10^{-4} M picric acid	2.2×10^{-13}
2.7×10^{-6}	0.36	1.1×10^{-6} M 2,4-DNP	3.0×10^{-13}

*Average error is $+0.4 \times 10^{-13}$ as determined from maximum and minimum possible slope in each set.

limiting, maleate leakage should be the same in both cases. A small increase in rate with added valinomycin is noted, but it is important to note that dependence on valinomycin is far from linear. This suggests that K^+ transport, although having an effect, is not absolutely limiting.

One can confirm that valinomycin K^+ permeation is not completely limiting by examining the effect of adding a small amount of a second highly permeable anion. 2,4-DNP and picric acid, often used as uncouplers of oxidative phosphorylation, are believed to have highly permeable anions (9). They can carry protons into vesicles by entering in the protonated form, diffusing out as anions, and repeating the process. In our system, if K^+ flow were limiting, 2,4-DNP or picric acid could provide protons for the loss of a neutral maleic acid molecule from the inside, but flow of 2,4-DNP or picrate anions out would simply replace an equivalent number of maleate transports. Once steady-state uncoupler concentrations were established, this would lead to no net increase in rate of maleate loss. If K^+ were not limiting, both maleate and uncoupler anions would flow, leading to an acceleration of maleate loss.

An experiment in which picric acid has been added is depicted in Fig. 3. Note that internal pH no longer rises, a fact consistent with the presence of an additional proton-transporting species. The degree of pH rise inhibition is a complex function of dissociation constants as well as permeation coefficients for both uncoupler and maleate species. The degree of inhibition need not be large and in fact is not very large for 2,4-DNP. However, both picric acid and 2,4-DNP accelerate maleate leakage. This is clear on comparison of Figs. 2 and 3 for picric acid. Results for both uncouplers are summarized in Table I.

Because of the effect of uncouplers, the small effect of increased valinomycin concentration, and the high calculated K^+ permeation rates, any residual effect of the presence of valinomycin on maleate transport coefficients is likely to be of an indirect origin.

DISCUSSION

Ideally, one would like to describe the previous experiments quantitatively in terms of permeation coefficients for the dominant species being transported. Permeation coefficients are operationally defined as the proportionality constant between a flux and a concentration difference for a particular solute. Anion permeation coefficients are in principle most easily extracted from initial rates. Our samples are set up with neutral molecule in equilibrium, and, because we can assume dianion permeability to be much less than monoanion permeability, initial rates will be primarily sensitive to monoanion permeation. We can express the initial rate of change in total maleate concentration inside as follows:

$$\frac{d(H_2M + HM^- + M^=)_i}{dt} = P_{HM^-}\frac{A}{V_i}\Delta(HM^-), \qquad (1)$$

where H_2M, HM^-, and $M^=$ refer to neutral acid, monoanion, and dianion, respectively, A is the vesicle membrane area, V_i is the internal volume, and P_{HM^-} is the monoanion permeation coefficient.

Obviously, our data extend well beyond the validity of the initial rate equation. In particular we have mentioned the importance of neutral molecule permeation in effecting a change in outside pH once beyond time zero. We, therefore, modify the above equation by adding a term that results from neutral acid permeation, Eq. 2. We also can express the

change in internal pH as a sum of terms coming from dissociation of neutral acid and association of dianion, Eq. 3. These may be combined with

$$\frac{d(H_2M + HM^- + M^=)_i}{dt} = P_{H_2M}\frac{A}{V_i}\Delta(H_2M) + P_{HM^-}\frac{A}{V_i}\Delta(HM^-) \quad (2)$$

$$\frac{d(H^+)}{dt}i = \frac{-d(H_2M)_i}{dt} + \frac{d(M^=)_i}{dt} + P_{H_2M}\frac{A}{V_i}\Delta(H_2M), \quad (3)$$

corresponding equations for the vesicle exterior and two equilibrium constant expressions to obtain a set of four coupled, nonlinear differential equations describing the time-course of variation in internal pH, external pH, and total maleate internal or external concentration.

It is important to remember that at long times an electrostatic potential not yet included in the equations will develop and will contribute to overall flow. In the limit of rapid equilibration of potassium ion by valinomycin transport, the electrostatic potential can be expressed as in Eq. 4.

$$RT\ell n[(K^+)_o/(K^+)_i]. \quad (4)$$

The electrostatic potential modifies transport of MH^- by a factor given in Eq. 5.

$$1 - \ell n[(K^+)_i/(K^+)_o]/\ell n[(MH^-)_o/(MH^-)_i]. \quad (5)$$

The potassium concentration at any point in time can be calculated from initial conditions by insisting that electroneutrality be maintained in internal and external solutions. Note that this correction factor is one at time zero where external and internal potassium concentrations are equal and would remain close to one if potassium were in a large excess.

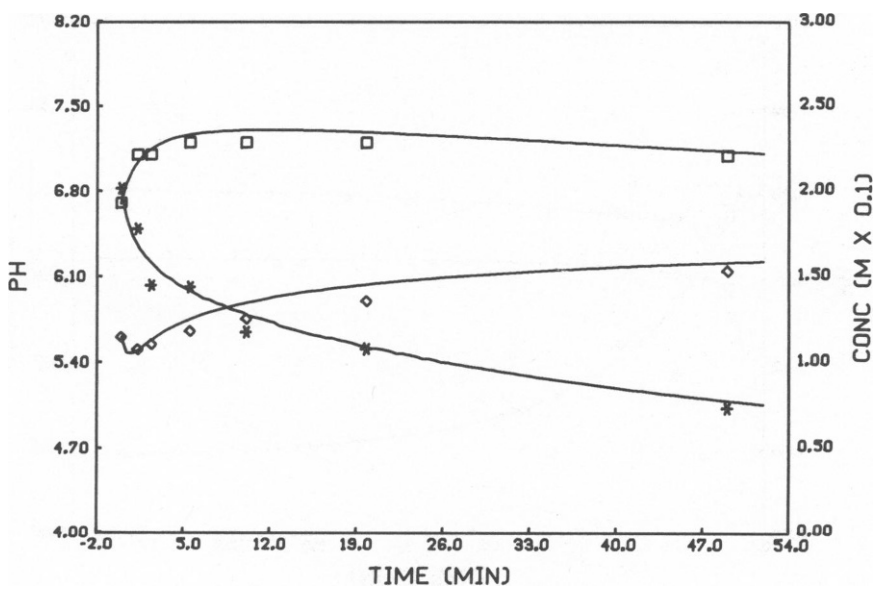

FIGURE 4 Time evolution of pH and internal maleate concentration (conc) in a unilamellar vesicle preparation; □, internal pH; ◇, external pH; *, internal maleate concentration. Conditions are as described in Fig. 2.

The set of four coupled differential equations modified for electrostatic potential contributions was solved by using a Runge-Kutta integration routine employing initial pH, initial concentrations, and an external to internal volume ratio as discussed in Results. The first and second dissociation constants for maleic acid were taken to be 1.2×10^{-2} and 5.4×10^{-7} M (10). The bilayer area per liter of solution was estimated at 10^8 cm^2. The best fit curves for data of Fig. 2 are presented in Fig. 4. The permeation coefficients used are 4×10^{-5} cm/s for H_2M and 4×10^{-9} cm/s for HM^-. Changes of $\pm 50\%$ in either of these produce visually unacceptable fits to the data. Data could be improved by operating at lower initial pHs because insensitivity to pH above 7.2 makes intermediate points on the internal pH curve of Fig. 4 questionable.

The effects of uncoupler as detected in Fig. 3 can be analyzed by modifying Eq. 3 to include transport of protons by the protonated uncoupler:

$$\frac{d(H^+)}{dt} i = \frac{-d(H_2M)_i}{dt} + \frac{d(M^=)}{dt} i + P_{H_2M} \frac{A}{V_i} \Delta(H_2M) + P_{HU} \frac{A}{V_i} \Delta(HU) \qquad (6)$$

and by introducing an equation to account for uncoupler redistribution

$$\frac{d(U^- + HU)_i}{dt} = P_{HU} \frac{A}{V_i} \Delta(HU) + P_{U^-} \frac{A}{V_i} \Delta(U^-)\{1 - \ln[(K^+)_i/(K^+)_o]/ \ln[(U^-)_o/(U^-)_i]\}, \qquad (7)$$

where U^- is the uncoupler anion, HU is the neutral uncoupler, and P_{U^-} and P_{HU} are their respective permeation coefficients.

The fit to data for picric acid is presented in Fig. 5. The maleate permeation coefficients

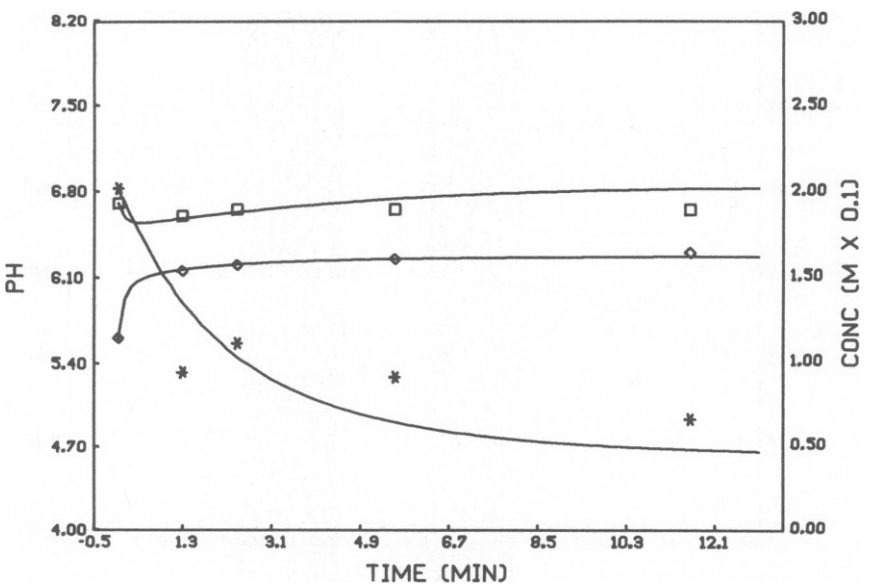

FIGURE 5 Time evolution of pH and internal maleate concentration (conc) in a unilamellar vesicle preparation containing picric acid as an uncoupler. Conditions are as described in Fig. 3, and symbols are as described in Fig. 4.

used in Fig. 4 have been used, along with uncoupler permeation coefficients of 2×10^{-1} and 2×10^{-5}, for neutral molecule and anion, respectively.

The permeation coefficients determined for maleate and maleic acid, given their precision, are realistic in comparison with data for permeation of other small molecules. The neutral molecule coefficient is, in fact, similar to that given for succinimide (1×10^{-5} cm/s) which has a slightly lower molecular weight and slightly lower hydrogen bonding potential (11). The monoanion coefficient is smaller, as it should be for a charged molecule. It is, however, large compared with chloride (10^{-10} cm/s) (12). The large value may be the result of the charge delocalization of maleate or it may be the result of some anion transport facilitation by the presence of the positively charged valinomycin-K^+ complex in the membrane.

The quality of data in the presence of uncoupler does not warrant attaching any quantitative significance to the uncoupler permeation coefficients determined. The experiment is in fact rather insensitive to uncoupler anion permeation coefficients because of the pH at which the experiment is done. A more detailed study will be presented in a subsequent paper. The ability to approximate the time-course of maleate leakage and pH change using maleate coefficients determined in Fig. 4, however, adds credence to the evaluation of the maleate permeation coefficients.

Although one would like to think of a permeation coefficient as characterizing the interaction only between solute and membrane, in nonequilibrium situations it always depends to some extent on interactions between membrane and all species being transported. The importance of spectator ion effects in transport through membranes has been emphasized in recent articles (13). In our system, therefore, interaction between K^+ flux and maleate anion flux is an important consideration for permeation coefficients of the anions. Although we attempt to uncouple these fluxes by adding valinomycin, we must to a certain extent view derived anion coefficients as characterizing a membrane having a particular level of valinomycin-K^+ complex. Reproducibility of membrane preparations must also be kept in mind. The experiments presented are perhaps most valuable as an illustration of the potential for application of magnetic resonance techniques to the study of permeation through model bilayer systems and extension to studies of whole cells.

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REFERENCES

1. JOHNSON, R. G., and A. SCARPA. 1976. Internal pH of isolated chromaffin vesicles. *J. Biol. Chem.* **251**:2189.
2. GREGORIADIS, G. 1976. The carrier potential of liposomes in biology and medicine. *N. Engl. J. Med.* **295**:765.
3. NICHOLS, J. W., and D. W. DEAMER. 1976. Catecholamine uptake and concentration by liposomes maintaining pH gradients. *Biochim. Biophys. Acta.* **455**:269.
4. CRAMER, J. A., and J. H. PRESTEGARD. 1977. NMR studies of pH-induced transport of carboxylic acids across phospholipid vesicle membranes. *Biochem. Biophys. Res. Commun.* **75**:295.
5. SINGLETON, W. S., M. S. GRAY, M. L. BROWN, and J. L. WHITE. 1965. Chromatographically homogeneous lecithin from egg phospholipids. *J. Am. Oil Chem. Soc.* **42**:53.

6. GENT, M. P. N., and J. H. PRESTEGARD. 1974. Cholesterol-phosphatidylcholine interactions in vesicle systems. Implication of vesicle size and proton magnetic resonance line-width changes. *Biochemistry.* **13**:4027.
7. PRATT, L., and B. B. SMITH. 1967. Proton resonance spectra of some unsaturated carboxylic acids. *Trans. Faraday Soc.* **63**:2858.
8. LAPRADE, R., S. CIANI, G. EISENMAN, and G. SZABO. 1975. The kinetics of carrier-mediated ion permeation in lipid bilayers and its theoretical interpretation. *In* Membranes. G. Eisenman, editor. Marcel Dekker, Inc., New York, **3**:180.
9. NEUMCKE, B., and E. BAMBERG. 1975. The action of uncouplers on lipid bilayer membranes. *In* Membranes. G. Eisenman, Marcel Dekker, Inc., New York. **3**:215.
10. KORTUM, G., W. VOGEL and K. ANDRUSSOW. 1960. Dissociation constants of organic acids in aqueous solution. *Pure Appl. Chem.* **1**:267.
11. STEIN, W. D. 1967. The Movement of Molecules Across Cell Membranes. Academic Press Inc., New York. 74.
12. STRYER, L. 1975. Biochemistry. W. H. Freeman & Company, San Francisco, 240.
13. KOZAK, J. J. 1975. Spectator ion effect on the passage of ions through membranes. *Proc. Natl. Acad. Sci. U.S.A.* **72**:683.