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Estimation of Transmembrane pH Gradients from Phase Equilibria of Spin-Labeled Amines†

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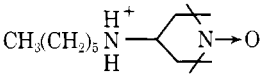
ABSTRACT: Spin-labeled secondary amines have been used to measure transmembrane proton gradients in sonicated liposomes. The electron paramagnetic resonance spectra of these probes show changes in the ratio of membrane associated to free aqueous probe as a function of transmembrane pH gradient. As the pH gradient is increased, inside acidic, the amount of membrane associated probe increases. The results are accounted for by a simple thermodynamic theory.

Gradients of pH across biological membranes are associated with many energy transducing and transport phenomena. As a result, methods which provide a simple and direct means of measuring gradients of H^+ activity have been actively sought.

Estimations of transmembrane pH gradients (ΔpH) are currently obtained by measuring the equilibrium distribution of radioactive weak acids or bases (Addanki et al., 1968; Rottenberg et al., 1972) or by the use of fluorescent amines such as 9-aminoacridine (Deamer et al., 1972). In the latter case, a pH-dependent uptake of the dye is accompanied by a quenching of fluorescence, and a quantitation of ΔpH is achieved by empirical calibration methods.

In this paper, we report on a new approach which has been taken to obtain rapid estimates of ΔpH in sonicated liposomes. By utilizing electron paramagnetic resonance (EPR)[1] spectroscopy, the membrane aqueous solution partition coefficient of spin label I is obtained. It will be shown that this equilibrium



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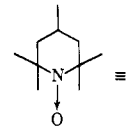
is sensitive to transmembrane pH gradients in a manner predicted by a simple thermodynamic theory.

Experimental Section

Materials. Phosphatidylcholine (PC) was prepared from fresh hen eggs according to the procedure of Singleton et al. (1965). The purified phospholipid in chloroform was stored at −20 °C under an argon atmosphere at a concentration of 75 mg/mL. *N,N,N*-Trimethyl-*N*-tempoylammonium bromide was a gift of Carole Hamilton.

Synthesis of N-Hexyl-N-tempoylamine[2] (*I*). 4-Amino-2,2,6,6-tetramethylpiperidinyl-1-oxy (0.50 g; 2.92 mmol) and



≡ tempoyl radical

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[1] Abbreviations used: EPR, electron paramagnetic resonance; PC, phosphatidylcholine; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; TLC, thin-layer chromatography.

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n-hexyl bromide (0.33 g; 1.99 mmol) were dissolved in 20 mL of methanol containing 0.4 g (1.99 mmol) of anhydrous potassium carbonate. The mixture was refluxed for 4 h and cooled, and the solid KBr and excess K_2CO_3 were removed by filtration. The methanol was removed under reduced pressure and the resulting oil was fractionated by column chromatography on silica gel (18 × 2.4 cm) eluting with hexane:acetone, 1:1 (v/v). A small amount of *N,N*-dihexyl-*N*-tempoylamine eluted first, followed by the major product I. The solvent was removed under reduced pressure to yield 0.250 g of pure I as a viscous red oil. Analysis gave: C, 70.3; H, 12.1; N, 10.8. The calculated composition for $C_{12}H_{31}N_2O$ is: C, 70.5; H, 12.2; N, 11.0.

Synthesis of N-Cyclohexyl-N-hexylamine (II). Cyclohexylamine (10.0 g; 0.101 mol) was added to 11.5 g of 1-bromohexane (0.070 mol) and 14 g of potassium carbonate in 60 mL of dry methanol. The mixture was stirred under reflux for 16 h, then cooled, and filtered. The methanol was removed from the filtrate under reduced pressure and the desired secondary amine was obtained from the crude mixture by vacuum distillation. *N*-Cyclohexyl-*N*-hexylamine distilled at 63 °C at 0.40 Torr. Analysis of the clear liquid gave: C, 78.8; H, 13.6; N, 7.7. The calculated composition for $C_{12}H_{25}N$ is: C, 78.6; H, 13.7; N, 7.6.

Preparation of Sonicated Vesicles. Aliquots of PC in chloroform were removed from the supply of stock lipid. The solvent was removed under a flow of nitrogen and the lipid was further dried under vacuum for 30 min. The lipid was suspended in the appropriate buffer (see below) and sonicated in an ice bath under nitrogen flow at a power of 35 W (Sonifier Heat Systems-Ultrasonics, Inc., Plainview, N.Y.) until sonicates became nearly clear (usually 20 min, depending upon sample size). The sonicated vesicles were then centrifuged at 31 000*g* for 30 min to remove small amounts of unsonicated lipid and titanium dust from the sonicator tip.

Vesicles which were used to measure the binding constant of the charged form of spin label I in the absence of a pH gradient were sonicated in 250 mM sodium phosphate buffer, pH 6.8, at a lipid concentration of approximately 15% w/v. An exact determination of the final lipid concentration (after sonication and centrifugation) was obtained by phosphate analysis as previously described (Cafiso & Hubbell, 1978). In order to obtain the final desired vesicle concentrations, the vesicle suspension was diluted with 250 mM sodium phosphate, pH 6.8.

Vesicles used to measure the binding constant of I as a function of bulk pH in the absence of a pH gradient were sonicated in buffer solution of 100 mM glycine plus 100 mM sodium phosphate at pH values of 6.0, 7.0, 8.0, 9.0, 10.0, and 12.0 at identical vesicle concentrations (approximately 1% w/v). To test for the presence of lyso-PC in sonicates prepared at basic pH values, aliquots of sonicated samples were lyophilized. The dried lipid was dissolved in chloroform and spotted on silica gel TLC plates which were developed in chloroform:methanol:acetic acid:H_2O (50:25:8:4) and compared with standards of PC and lyso-PC.

For spin label reduction experiments, both $FeSO_4$ and glutathione were added to lipid suspensions (5% w/v) in 100 mM sodium citrate, pH 6.8, to a final concentration of 100 mM before sonication. After sonication, the external $FeSO_4$ and glutathione were removed by gel filtration on a column of Bio-Gel A-0.5m (8 × 180 mm) which was equilibrated with 100 mM citrate and 100 mM Na_2SO_4, pH 6.8. For pH dependent reduction experiments, the column was equilibrated with citrate buffers at pH values of 2.25, 2.65, 3.00, 3.35, and 3.70. Complete removal of the external reducing agent was demonstrated by the lack of reduction of the impermeable spin label cation *N,N,N*-trimethyl-*N*-tempoylammonium bromide.

For vesicles across which pH gradients were to be established, PC at 5% w/v was sonicated in 250 mM sodium phosphate buffer, pH 6.04, or 250 mM sodium phosphate, pH 7.5. The former buffer was used to prepare vesicles with an acidic interior, while the latter was used to prepare vesicles with a basic interior volume.

Generation of Transmembrane pH Gradients (ΔpH). pH gradients were established across sonicated vesicles by gel filtration through a column of Bio-Gel A-0.5 m (180 × 8 mm, void volume approximately 2.5 mL) at 0 °C which was equilibrated with the desired buffer. ΔpH is defined as pH(inside vesicle) − pH(outside vesicle) and ΔpH values of 0, −0.43, −0.96, −1.46, and −2.02 were obtained by passing vesicles through columns equilibrated with sodium phosphate buffers at pH values of 6.04, 6.47, 7.00, 7.50, and 8.06, respectively. Vesicles were stored at 0 °C until used (never longer than 3 h). Buffer exchange was confirmed by measuring the pH of the vesicle fraction. The vesicle fraction was detected by light scattering and ran in the void volume of the column. A similar procedure was followed to obtain vesicles with a basic interior volume.

Prior to recording spectra, the samples were warmed to room temperature and spin label I was added from a stock solution in ethanol (2×10^{-3} M) to a final concentration of 20 μM.

EPR Measurements and Analysis of Spectra. EPR measurements both at room temperature and at 0 °C were made on a Varian E-109 spectrometer as previously described and the ratios of bound-to-free spin were determined from the spectra as previously described (Cafiso & Hubbell, 1978).

Spin Reduction Experiments. Vesicles containing trapped glutathione and $FeSO_4$ were mixed with spin label to a final concentration of 5×10^{-5} M at 0 °C. The sample was placed in a temperature controlled cavity at 0 °C and the spectral changes were recorded as a function of time. For the pH-dependent experiments, the vesicles were diluted to a final concentration of approximately 1% w/v before the spin label was added.

Results

Permeability of Phospholipid Vesicles to Spin-Labeled Amines. Mixtures of Fe^{2+} salts and glutathione are excellent reducing agents for nitroxide radicals. The membrane impermeability of these agents makes it possible to prepare vesicles with a reducing interior as described in the experimental section. When spin label I is added to vesicles which contain internalized Fe^{2+} and glutathione (at 0 °C and pH 6.8), reduction of I occurs within seconds. However, if the membrane impermeable spin label, *N,N,N*-trimethyl-*N*-tempoylammonium bromide is added to the same vesicle preparation, initial reduction rates have a half-life greater than 6 h. Since the impermeable nitroxide is reduced very slowly, the vesicles must be relatively free of external reducing agent. The above results suggest that spin label I readily permeates phospholipid vesicles, even at 0 °C.

Binding of Spin Label I to Phospholipid Bilayers. Figure 1A shows the EPR spectra of spin label I in the presence and absence of phospholipid vesicles, pH 6.8, ΔpH 0. In the presence of vesicles, the spectrum of I is a superposition of a narrow and broad spectrum and is similar to the spectra reported for spin-labeled phosphoniums (Cafiso & Hubbell, 1978) and quaternary ammonium analogues of I (Castle & Hubbell, 1976) in the presence of phospholipid vesicles. The broad signal arises from spin which is membrane bound and the narrow,

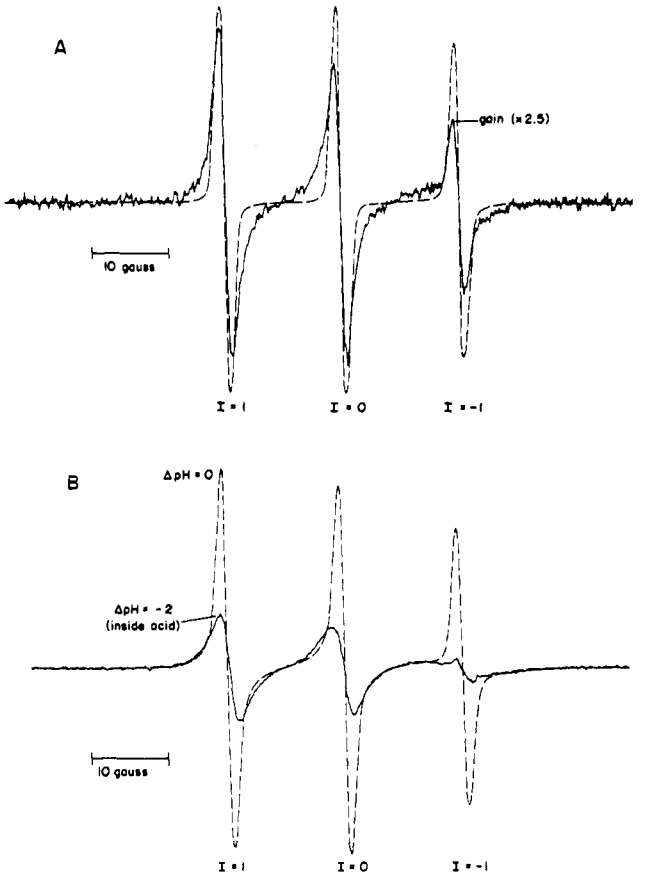

FIGURE 1: (A) EPR spectra of samples containing 2×10^{-5} molar spin label (I) in aqueous solution (- - -) and in the presence of PC vesicles, $\simeq$15% w/v (—). In each case the samples contained 250 mM sodium phosphate, pH 6.8. The gain of the spectrometer in the presence of vesicles was set to 2.5 times that for the label in aqueous solution alone. (B) EPR spectra of 2×10^{-5} molar I in the presence of 2% w/v PC vesicles with (—) and without (- - -) a pH gradient. The vesicles were prepared as described in the text.

sharp spectrum is a result of the free aqueous spin label. These two populations are referred to as "bound" and "free" spin, respectively. In Figure 1B, two spectra of spin label I in vesicles of the same concentration, with and without a pH gradient, are compared. A dramatic increase in the bound population of I is observed in the presence of a pH gradient (ΔpH −2). If the pH of the vesicle suspension is varied in such a way that ΔpH = 0, only very small changes are seen in the binding equilibria (see below). Thus it appears that the partitioning of I in the vesicles is a function of ΔpH.

Thermodynamic Analysis of the Binding Equilibrium. Spin label I is present in solution in both charged and neutral forms, and both these forms are in equilibrium between membrane and aqueous phases. In the scheme shown in Figure 2, the charged form of I is assumed to bind at the membrane interfaces in a manner analogous to the binding of the quaternary analogues of I previously described (Castle & Hubbell, 1976). We will show below that the membranes are impermeable to this species. In the following discussion we identify four discrete regions of space accessible to the charged form of label I: V_{m_o}, V_{m_i}, V_o and V_i. V_{m_o} and V_{m_i} are defined as the effective volumes per vesicle of the phases at the membrane outer and inner surfaces occupied by the charged form of label I and giving rise to an EPR spectrum characteristic of a bound spin.[3] At equi-

[3] This is essentially the Guggenheim convention for thermodynamic description of surface phases (Aveyand & Haydon, 1973).

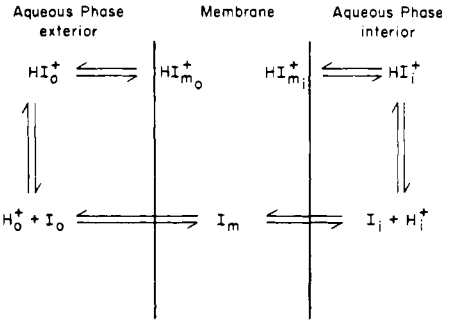

FIGURE 2: Distribution and multiple equilibria exhibited by spin label I and similar amines in phospholipid vesicles. The protonated forms are designated by HI^+ and the unprotonated forms by I. The subscripts i, o, m_i, m_o, and m specify the location of the label in the inner aqueous compartment of the vesicle (i), the outer aqueous solution (o), the membrane interior surface (m_i), the membrane exterior surface (m_o), or the membrane interior (m).

librium the moles of charged I in volumes V_{m_o} and V_{m_i} will be designated $N_{m_o}{}^+$ and $N_{m_i}{}^+$. The volumes V_o and V_i refer to the volumes per vesicle of the outer and inner aqueous phases, respectively; the moles of charged I in these volumes will be designated $N_i{}^+$ and $N_o{}^+$.

The neutral form of I is assumed to partition into the bilayer interior, but no assumptions will be made regarding the detailed distribution within this phase. The total number of moles of neutral I in the membrane will be designated N_m, while the number of moles of this species in the aqueous volumes V_o and V_i will be referred to as N_o and N_i. The chemical potentials for I in the membrane and aqueous phases (charged or neutral) are taken as

$$\mu_m(r) = \mu_m^\circ(r) + RT \ln A_m(r) \qquad \text{(membrane phase)}$$

$$\mu = \mu^\circ + RT \ln A \qquad \text{(aqueous phase)}$$

where μ° is the chemical potential of the label in the Henry's Law standard state, R and T are the universal gas constant and absolute temperature, respectively, and $A_m(r)$ and A are the activities of I in the membrane and aqueous phases, respectively. The chemical potential and activity of I in the membrane phase is assumed to be a function of r, the position in the membrane with respect to the center of the spherical vesicle. In general, this is expected to be the case since the properties of the membrane phase are not uniform along its thickness. As indicated above, the $\mu_m(r)$ for the charged form of I is taken as infinite for all values of r within the membrane except in the neighborhood of $r = r_o$ and r_i, the outer and inner vesicle radii, respectively. No assumptions need be made regarding the form of $\mu_m(r)$ for the neutral species. In dilute solutions of spin label in all phases, we approximate the activity ratios as $A_j/A_k \approx X_j/X_k \approx N_jV_k/N_kV_j$, where X_j and X_k are the mole fractions and N_j and N_k are the number of moles of I in the phases of volume V_j and V_k per vesicle. Imposing the equilibrium constraint that the chemical potential be the same in all phases, it is readily shown for the neutral form of I that

$$\frac{N_m}{N_o} = \frac{1}{V_o}\int K(r) 4\pi r^2 dr \quad \text{and} \quad \frac{N_m}{N_i} = \frac{1}{V_i}\int K(r) 4\pi r^2 dr \qquad (1)$$

where $4\pi r^2 dr$ is a spherical volume element between r and $r + dr$ in the membrane and $K(r) = e^{-(\mu_m^\circ(r) - \mu^\circ)}/RT$ is the partition coefficient for the neutral form of I between the aqueous phases and this volume element in the membrane. The integration extends across the bilayer thickness. It should be noted that the detailed form of $K(r)$ need not be known since the values of the integrals in eq 1 will be obtained experimentally.

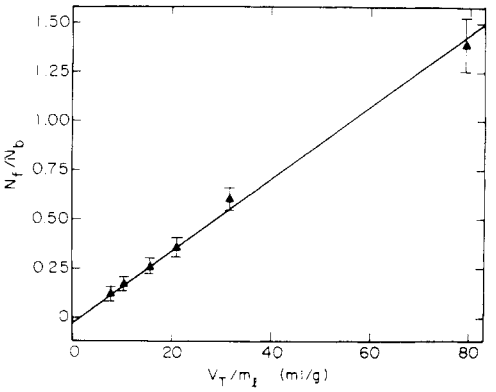

FIGURE 3: Equilibrium binding of spin label I to phospholipid vesicles in the absence of a pH gradient. The ratio N_f/N_b (free-to-bound label) is plotted vs. the total solution volume per mass lipid, V_T/M_l. Different vesicle concentrations all contain 250 mM sodium phosphate, pH 6.8. The slope of this line is 0.0178 g/mL.

For the charged form of I in dilute solutions

$$K_1 \simeq \frac{N_{m_o}{}^+}{N_o{}^+}\frac{V_o}{V_{m_o}} \text{ and } K_2 \simeq \frac{N_{m_i}{}^+}{N_i{}^+}\frac{V_i}{V_{m_i}} \tag{2}$$

where K_1 and K_2 are the equilibrium binding constants for the charged form of I to the outer and inner membrane surfaces, respectively.

The proton dissociation equilibria for I in both the inner and outer aqueous volumes are assumed to have the same equilibrium constant, K_a, defined as

$$K_a \simeq \frac{(H^+)(I)}{(HI^+)} = \frac{N_i(H_i{}^+)}{N_i{}^+} = \frac{N_o(H_o{}^+)}{N_o{}^+} \tag{3}$$

Equations 1, 2, and 3 may then be combined to obtain the ratio of total moles of bound (i.e., membrane associated) to total moles of free spin as a function of $H_i{}^+$ and $H_o{}^+$:

$$\frac{N_b}{N_f} = \frac{N_{m_o}{}^+ + N_{m_i}{}^+ + N_m}{N_o{}^+ + N_i{}^+ + N_o + N_i} = \frac{\frac{1}{V_i}\int K(r)4\pi r^2 dr + K_1\frac{V_{m_o}}{V_i}\frac{H_o{}^+}{K_a} + K_2\frac{V_{m_i}}{V_i}\frac{H_i{}^+}{K_a}}{\frac{V_o}{V_i}\left\{\frac{H_o{}^+}{K_a}+1\right\} + \frac{H_i{}^+}{K_a} + 1} \tag{4}$$

It will be shown below that $(H_i{}^+)$, $(H_o{}^+) \gg K_a$ and

$$K_a\frac{1}{V_i}\int K(r)4\pi r^2 dr \ll \left(K_1\frac{V_{m_o}}{V_i}H_o{}^+ + K_2\frac{V_{m_i}}{V_i}H_i{}^+\right) \tag{5}$$

for all conditions under which ΔpH was studied. Under these conditions, eq 4 may be simplified to

$$\frac{N_b}{N_f} \simeq \frac{K_1\frac{V_{m_o}}{V_i}H_o{}^+ + K_2\frac{V_{m_i}}{V_i}H_i{}^+}{\frac{V_o}{V_i}H_o{}^+ + H_i{}^+} \tag{6}$$

The vesicles used in our experiments are compositionally symmetric, and we shall assume that $K \equiv K_1 \approx K_2$. Further justification for this assumption will be discussed below. With this simplification, eq 6 reduces to:

$$\frac{N_b}{N_f} = \frac{K\frac{V_{m_i}}{V_i}\left(1 + \frac{V_{m_o}}{V_{m_i}}10^{\Delta pH}\right)}{1 + \frac{V_o}{V_i}10^{\Delta pH}} \tag{7}$$

where $\Delta pH = pH_i - pH_o$.

Our objective in arriving at eq 7 is to provide a reasonable theoretical model with which to compare the experimentally determined values of N_b/N_f as a function of ΔpH obtained from the EPR spectra. To do this, values for V_{m_o}/V_{m_i}, V_o/V_i, and KV_{m_i}/V_i are required. In addition, estimates of K_a must be made to insure the validity of the approximation 5 made in arriving at eq 7.

Values of the variable V_o/V_i and the constant $V_{m_o}/V_{m_i} = 2.3$ for sonicated egg PC vesicles were obtained as previously described (Cafiso & Hubbell, 1978). Values of KV_{m_i}/V_i and K_a are obtained from a study of the binding of I to vesicles as a function of vesicle concentration at fixed pH (at ΔpH 0) and as a function of pH (at ΔpH 0) at fixed vesicle concentration, respectively.

Binding as a Function of Vesicle Concentration at Fixed pH, ΔpH 0. For the case where $H_i{}^+ = H_o{}^+$, eq 7 reduces to

$$\frac{N_b}{N_f} = \frac{K\frac{V_{m_i}}{V_i}\left(1 + \frac{V_{m_o}}{V_{m_i}}\right)}{1 + \frac{V_o}{V_i}} \tag{8}$$

which is simply the expression for the equilibrium partition of I between two phases of volumes $(V_o + V_i)$ and $(V_{m_o} + V_{m_i})$. The volume ratio V_o/V_i can be expressed in terms of intensive quantities as:

$$\frac{V_o}{V_i} = \frac{V_T}{m_l}\left(\frac{1}{\bar{V}_i}\right) - \left\{\frac{\bar{V}_l}{\bar{V}_i} + 1\right\} \tag{9}$$

where V_T is the total solution volume, m_l is the mass of lipid in the vesicles, $\bar{V}_l$ is the volume of hydrated lipid bilayer per gram lipid, and $\bar{V}_i$ is the internal volume per gram lipid. $\bar{V}_l$ and $\bar{V}_i$ for the vesicles will be taken as 0.9985 and 0.5 mL/g as reported previously (Cafiso & Hubbell, 1978). Combining eq 8 and 9, we obtain

$$\frac{N_f}{N_b} = \frac{1}{\beta\bar{V}_i}\left\{\frac{V_T}{M_l} - \bar{V}_l\right\} \text{ where } \beta = K\frac{V_{m_i}}{V_i}\left\{1 + \frac{V_{m_o}}{V_{m_i}}\right\} \tag{10}$$

Equation 10 shows that a plot of the experimental values N_f/N_b vs. V_T/m_l will be linear with a slope $1/\bar{V}_i\beta$. Figure 3 shows data plotted in this fashion at various vesicle concentrations, and ΔpH 0. The slope for this binding curve is 0.018 g/mL giving a value of 34 for KV_{m_i}/V_i. This value will be used below to predict values of N_b/N_f as a function of ΔpH.

Binding as a Function of pH where ΔpH = 0. If the vesicle concentration is held constant and pH is varied (with ΔpH = 0), the ratio N_b/N_f will be determined by eq 4 with $H^+ = H_i{}^+ = H_o{}^+$. By measuring the ratio N_b/N_f at pH values above and below the pK_a, we can estimate K_a and $1/V_i\int K(r)4\pi r^2 dr$ from a least-squares fitting procedure. Figure 4 shows experimental data for N_b/N_f vs. pH for ΔpH = 0 and the best fit to the data obtained with eq 4 (with $H_i{}^+ = H_o{}^+$) using K_a and $1/V_i\int K(r)4\pi r^2 dr$ as adjustable parameters. From this fit we estimate $K_a = 1.26 \times 10^{-10}$ mol/L and $1/V_i\int K(r)4\pi r^2 dr = 1237$. Thin-layer chromatography on the samples which were held at pH 12 for the duration of the measurement showed very little production of lyso-PC (less than 1%). These results indicate that the inequalities expressed in eq 5 are valid for the pH ranges investigated.

ΔpH-Dependent Binding of I to Phospholipid Vesicles. To test the ΔpH dependence of eq 7, we have measured values of N_b/N_f as a function of ΔpH. These are shown in Figure 5 as points, while the solid line represents N_b/N_f as predicted by eq 7 with $KV_{m_i}/V_i = 34$ and $V_{m_o}/V_{m_i} = 2.3$. The parameter V_o/V_i may be varied at will by changing the vesicle concentration, and Figure 6 shows the excellent agreement between

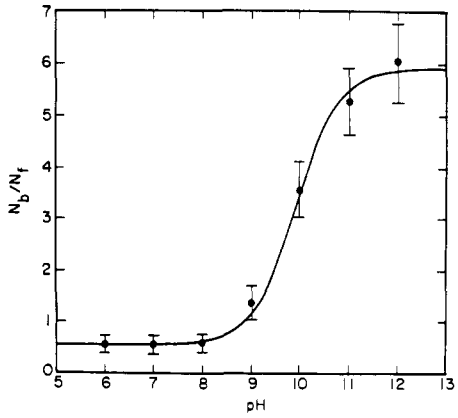

FIGURE 4: A plot of N_b/N_f vs. pH where $\Delta pH = 0$. Points (●) are experimentally determined values of N_b/N_f for I in the presence of PC vesicles (≃1% w/v) at various pH values. The solid line (—) is the best fit of eq 4 to these points using $K_a = 1.26 \times 10^{-10}$ and $1/V_i \int K(r) 4\pi r^2 dr = 1237$.

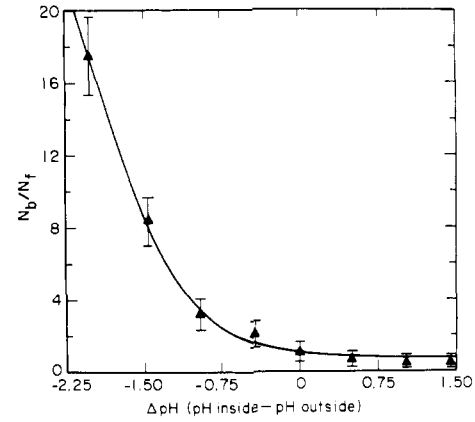

FIGURE 5: A plot of N_b/N_f vs. ΔpH showing both calculated and experimentally determined values. The points (▲) are ratios determined experimentally from 2×10^{-5} molar I in PC vesicles (≃2% w/v) at several values of ΔpH. The solid line (—) is the theoretical dependence of N_b/N_f vs. ΔpH, eq 6, calculated for $V_o/V_i = 106$ and $KV_{m_i}/V_i = 34$; see text.

the experimental and predicted values of N_b/N_f for two values of V_o/V_i, both different from that of Figure 5. It should be noted that vesicles with pH gradients were prepared at 0 °C and brought to room temperature just prior to ΔpH measurement. Immediately after warming to room temperature, no transmembrane potential ($\Delta\psi$) could be detected by phosphonium spin labels which have been shown to respond to $\Delta\psi$ (Cafiso & Hubbell, 1978). However, if the vesicles were allowed to stand at room temperature, a transmembrane potential did develop, presumably due to movement of H^+ down its concentration gradient.[4] After a long time at room temperature, $\Delta\psi$ reached a limiting value of $2.303RT/ZF(\Delta pH)$. The same potential could be established immediately by the addition of CCCP to the vesicle preparation. The transmembrane flow of the small number of H^+ ions needed to reach electrochemical equilibrium is not expected to significantly deplete the pH gradient in the presence of a high buffer concentration and impermeant anions. This is apparently the case, since ΔpH as measured by spin label I is time independent during development of $\Delta\psi$. The rate of development of $\Delta\psi$ is not affected by the addition of 0.1 mM *N*-cyclohexyl-*N*-hexylamine, a structural analogue of I.

Relative Permeabilities of the Charged and Neutral Forms of I. The model for the equilibrium states of the vesicle-spin label system shown in Figure 2 presumes that the bilayers are permeable to the neutral form of I and impermeable to the charged form. Estimates for the relative permeabilities of the charged and neutral forms of I have been obtained by measuring the reduction kinetics of I in the presence of vesicles containing internalized reducing agent. If the charged and neutral forms of I have significantly different permeabilities, the rate of spin-label transport across the vesicle membranes should be pH dependent. Under conditions where the rate of spin reduction is fast compared with the transport kinetics, and in the absence of transmembrane potentials, it can be shown that the rate of *total* spin transport across the membrane is just

[4] Full transmembrane equilibrium potentials were generally developed within 1–2 h at room temperature. In principle, the proton permeability can be determined directly from the time dependence of $\Delta\psi$. However, permeabilities estimated from $(\partial\Delta\psi/\partial t)$ varied from preparation to preparation. It is likely that this variability results from low-level, variable amounts of free fatty acids in the phospholipids which act as proton carriers in the system, and the permeabilities thus do not reflect intrinsic H^+ permeabilities.

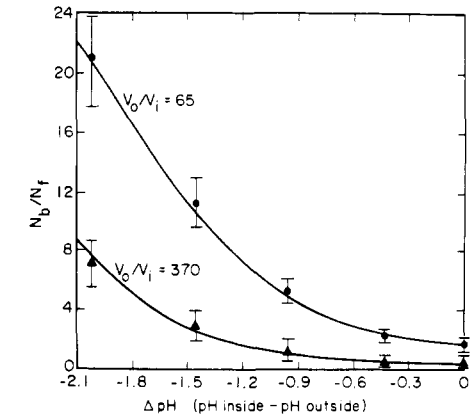

FIGURE 6: A plot of N_b/N_f vs. ΔpH for two values of V_o/V_i. The points (● and ▲) are experimentally determined values of N_b/N_f and the solid lines (—) represent the dependence of N_b/N_f upon ΔpH as determined by eq 6, $KV_{m_i}/V_i = 34$.

$$-\frac{dN_T}{dt} = k'N_T$$

where N_T is the total moles of unreduced spin at any time and

$$k' = \frac{(H^+)Kk_1 + k_2K_a\frac{1}{V_m}\int K(r)4\pi r^2 dr}{(H^+)K + \frac{V_o}{V_{m_o}}[(H^+) + K_a] + K_a\frac{1}{V_m}\int K(r)4\pi r^2 dr} \quad (11)$$

where k_1 and k_2 are the rate constants for transport of the charged and neutral species, respectively, K_a is the proton dissociation constant of I and K is the binding constant of the charged form of I to the membrane.

Figure 7A shows the rate of decay of total spin label I as a function of pH in the presence of vesicles containing internalized Fe^{2+}–glutathione as a reducing agent. Only acidic pH was studied, because reduction was too rapid to follow when the external pH was more alkaline than ≈4. In all cases, the decay follows first-order kinetics. Figure 7B shows a plot of the logarithm of the experimental first order rate constants, k' (determined from Figure 7A), as a function of pH along with plots of log k' vs. pH predicted by eq 11 for various values of k_1 and k_2.

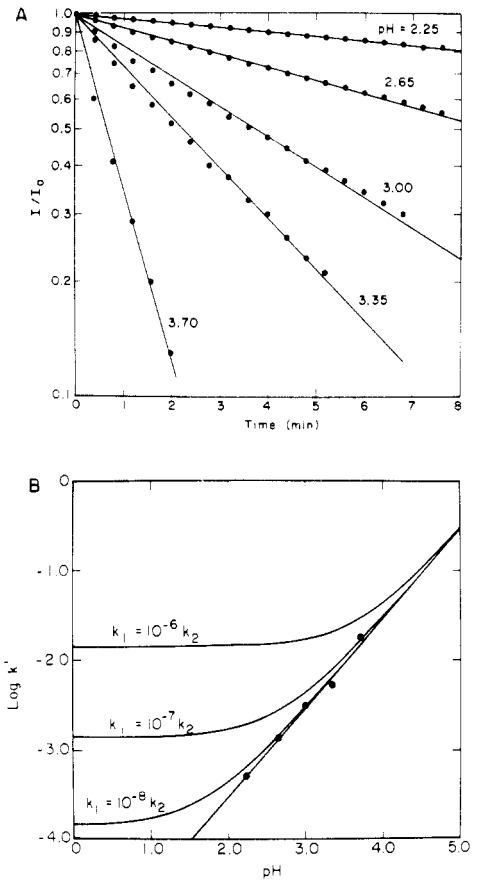

FIGURE 7: (A) Reduction kinetics for spin label I plotted for several values of pH. The logarithm of (I/I_o), the fraction of initial spin remaining at any time, is plotted vs. time. The spin label is mixed at $t = 0$ with vesicles ($\simeq$1% w/v) which contain a reducing interior (Fe^{2+} and glutathione, see text). (B) The logarithms of the first-order rate constants, obtained from A, are plotted (●) vs. pH. The solid curves (—) are calculated from eq 11 for several different values of k_1; k_2 is equal to 4.8×10^4 s^{-1} as determined from the slope of the line through the experimental data. These curves were calculated for $V_o/V_{m_o} = 680$, $1/V_m \int K(r) 4\pi r^2 dr = 458$, and $K_a = 1.26 \times 10^{-10}$.

Equation 11 predicts a linear dependence of k' on pH for $k_1 = 0$. The deviation from a linear dependence shown at low pH values is predicted by eq 11 only for nonzero values of k_1. The experimental data fit well on a curve predicted by eq 11[5] for $k_1 = 0$ and $k_2 = 4.8 \times 10^4$ s^{-1}. To within experimental error, the data presented in Figure 7B indicate that k_1 must be at least eight orders of magnitude smaller than k_2. Thus for all practical purposes the charged form is impermeable.

Discussion

The changes in the EPR spectrum of spin label I with ΔpH, shown in Figures 5 and 6, agree remarkably well with the simple theory presented. It is possible to accurately predict values of ΔpH from the spectra of I given that certain intensive properties of the vesicles are known.

The model presented in Figure 2 and expressed by eq 7 explicitly assumes impermeability of the charged form of I. If the charged form were permeable, the spin label would act as a proton carrier. Equation 7 would still apply in this situation, but, at equilibrium, ΔpH would be coupled to a transmembrane electric potential and the equilibrium ΔpH would be the same as the initial ΔpH only for strongly buffered solutions. This would considerably reduce the usefulness of I as a probe of ΔpH. There are two key experiments which indicate the impermeability of the ammonium ion of I. First, the pH dependence of the influx of I into vesicles as measured by spin reduction with internalized reducing agents gives an upper limit for the permeability of the ammonium ion which is eight orders of magnitude less than the neutral form. Second, a high concentration of *N*-cyclohexyl-*N*-hexylamine, a structural analogue of I, does not change the rate of production of $\Delta\psi$ in vesicles with a pH gradient. This indicates that the alkyl secondary amines are not efficient proton carriers and consequently must have relatively impermeable charged forms. Furthermore, a quaternary analogue of I has been shown to permeate egg PC vesicles very slowly, with a half-life on the order of 9 h at 25 °C (Castle & Hubbell, 1976). These results provide strong support for our contention that PC vesicles are very impermeable to the charged form of I.

K_1 and K_2 were assumed to be equal in the derivation of eq 7. This is a reasonable approximation for compositionally symmetric membranes and the excellent agreement between the predictions of eq 7 and the experimental data suggests that the different lipid packing densities on the inner and outer monolayers do not drastically affect the binding equilibria of I. This conclusion was reached previously for the binding of phosphonium spin labels to PC vesicles (Cafiso & Hubbell, 1978). In biological membranes, however, K_1 may be different from K_2 due to differences in composition and differences in surface charge between the interior and exterior of the bilayer.

The changes in the partitioning of label I in response to changes in ΔpH are very similar to those found for phosphonium spin labels in response to changes in transmembrane potential (Cafiso & Hubbell, 1978). Even though the thermodynamic potentials determining the inside-outside equilibrium for these two classes of probes are different, the changes in membrane aqueous partition with ΔpH or $\Delta\psi$ occur for the same reason. In both cases, the ratio of bound/free probe is larger when the probe becomes internalized because of the larger ratio of membrane surface to aqueous volume on the vesicle interior compared with the vesicle exterior. As a result, the formalism used to quantitate the two phenomena is very similar. In particular, the sensitivity of the two methods depends on the same experimental parameters: K, the partition coefficient of the probe, and the vesicle concentration expressed as V_o/V_i (eq 9). In the present case, we define the sensitivity, S, as

$$S = \frac{\partial(N_b/N_f)}{\partial(\Delta pH)}$$

$$= K \frac{V_{m_i}}{V_i} (2.303) 10^{\Delta pH} \frac{V_{m_i}/V_{m_o} - V_o/V_i}{(1 + V_o/V_i 10^{\Delta pH})^2} \quad (12)$$

which was obtained by differentiating eq 7. For any particular vesicle geometry and concentration, eq 12 indicates that the sensitivity increases linearly with K. This parameter is easily varied by increasing the alkyl chain length, but a practical limit is reached when the amplitude of the free signal becomes too small to be reliably measured. For any value of K the sensitivity is maximal at a value of (V_o/V_i) such that

$$\frac{\partial S}{\partial(V_o/V_i)} = 0$$

Solution of this equation gives

[5] To estimate $1/V_m \int K(r) 4\pi r^2 dr$ and V_o/V_{m_o} in eq 11, it is necessary to calculate the ratios V_i/V_m and V_i/V_{m_i} and V_m/V_{m_o} according to the model presented previously (Cafiso & Hubbell, 1978). V_i/V_m, the ratio of the interior aqueous vesicle volume to membrane volume, has a value of $\simeq$0.37 so that $1/V_m \int K(r) 4\pi r^2 dr = (V_i/V_m)(1/V_i) \int K(r) 4\pi r^2 dr = 458$. V_i/V_{m_i} is $\simeq 8$ and $V_o/V_{m_o} \simeq 680$.

$$(V_o/V_i)_{max} = 10^{-\Delta pH} + 2(V_{m_i}/V_{m_o}) \quad (13)$$

where $(V_o/V_i)_{max}$ is the value for maximum sensitivity. Apparently, the choice of vesicle concentration for maximum sensitivity depends on the range of ΔpH to be estimated. This is evident from the plots in Figure 6 where S is just the slope of the curve at any point.

The technique described in this report for estimating transmembrane pH gradients is similar in principle to that described by Deamer et al. (1972) using the fluorescent amine 9-aminoacridine. The charged form of the acridine is believed to be impermeable and concentration gradients of acridine develop across membranes in the presence of pH gradients for the same reasons that gradients of I develop under similar conditions. However, the experimental detection of the gradient is fundamentally different in the two approaches. For the acridine, quenching of fluorescence is used to monitor the internal dye concentration while equilibrium membrane binding is used to monitor internal concentrations of I. The spin label approach thus does not rely on probe–probe interactions and in principle may be used with very low concentrations of I, on the order of a few molecules per vesicle. In addition, this technique should prove to be advantageous in the study of transmembrane pH gradients in vesicles containing light sensitive proteins such as rhodopsin.

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Isolation of an Active Variable-Domain Fragment from a Homogeneous Rabbit Antibody Heavy Chain. Physiochemical and Immunological Properties†

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ABSTRACT: A fragment corresponding to most of the variable domain of the rabbit heavy chain (V_H) was obtained by tryptic digestion of the mildly reduced and aminoethylated heavy chain from rabbit antibody 3T72. The domain size peptide was purified by gel filtration and shown to extend between residues 11 (Leu) and 122 (Lys) of the heavy chain by sequence analysis. The molecular size of the fragment (approximately 11 000) was determined by gel filtration under denaturing conditions. Under nondenaturing conditions (20 mM sodium acetate, pH 5.5, 0.1 M NaCl), however, the fragment exists as a mixture of monomeric and dimeric species. The variable-domain fragment retains the allotypic determinants of the heavy chain (a_1), as shown by double diffusion on agar plates and radioimmunoassay. Upon recombination of the heavy-chain variable-domain fragment with its homologous light chain, partial recovery of specific binding activity toward the SIII polysaccharide antigen was demonstrated. The method reported here is reproducible (with yields varying between 40 and 60%) and may provide a general method for obtaining the variable region of the heavy chain for antigen binding and allotypic and amino acid sequence studies.

Considerable evidence now supports the concept initially proposed by Edelman (1970) that antibodies are comprised of compact globular domains of approximately 110–120 amino acid residues in length. These independently folded homology regions are interconnected by extended portions of the polypeptide chain. The globular domains have been shown to be highly resistant to endopeptidase attack, while the interdomain connecting peptides are amenable, under controlled conditions, to hydrolysis by various endopeptidases (Gall and D'Eustachio, 1972).

Inbar et al. (1972) reported the isolation of a fragment that comprises the variable domains of both H[1] and L chains from

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[1] Abbreviations used: PBS, 10 mM phosphate buffer, pH 7.4, 0.15 M NaCl; H, heavy; L, light; DEAE, diethylaminoethyl; Tris-HCl, 2-amino-2-hydroxymethyl-1,3-propanediol hydrochloride; EDTA, (ethylenedinitrilo)tetraacetic acid; TPCK, (1-tosylamido-2-phenyl)ethyl chloromethyl ketone; Pth, phenylthiohydantoin; DTT, dithiothreitol.

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