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A fluorescence quenching technique for the measurement of paramagnetic ion concentrations at the membrane/water interface. Intrinsic and X537A-mediated cobalt fluxes across lipid bilayer membranes

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We have characterized the quenching of *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)phosphatidylethanolamine by Co^{2+} in egg phosphatidylcholine (PC) lipid bilayer vesicles. The quenching constant obtained is 59 M^{-1}. We demonstrate one use of this fluorescence quenching technique by measuring intrinsic and X537A-mediated transmembrane Co^{2+} fluxes in large unilamellar PC vesicles. The intrinsic rate constant for Co^{2+} flux we measure is $3 \cdot 10^{-6}$ s^{-1}. We confirm that the neutral Co – $(X537A)_2$ complex is the main component of the X537A-mediated cobalt flux. Since this method measures the concentration of Co^{2+} at the site of the fluorophore, it is generally applicable to the measurement of paramagnetic ion concentrations in the region of the membrane/water interface.

Introduction

Studies of interactions between ions and biological or model membrane systems often are complicated by the presence of electrical charges at the membrane surface. These charges, which arise from constituent membrane molecules and membrane-adsorbed ionic species, produce an electrostatic surface potential that attracts counterions and repels coions. As a consequence, the aqueous concentration of ions immediately adjacent to the membrane/solution interface is different from that in the bulk aqueous phase. Techniques which determine only bulk aqueous ion concentrations (e.g., ion selective electrodes, conductivity, equilibrium dialysis, etc.), cannot directly measure the concentration of ions at the surface of membranes. However, a variety of other physical techniques have been used to estimate the surface concentration of ions. These include ^{31}P-NMR [1,2], electrophoretic mobility of vesicles [1,3], fluorescent probes [3], electron spin resonance of Mn^{2+} [4], conductance changes in black lipid membranes [5], and surface potential changes on lipid monolayers [6].

We introduce here a fluorescence quenching method to measure the concentration of paramagnetic ions near the membrane-solution interface. The ability to detect single photons, the high sensitivity of fluorophores to quenching, and the time domain of fluorescence phenomena (about 10^{-8} s) have made fluorescence quenching measurements powerful techniques for the study of molecular interactions, dynamics and structure

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Abbreviation NBD-PE, *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)phosphatidylethanolamine

[7–11]. A simple Stern-Volmer relationship (see Eqn. 2) can be used to describe the dependence of a membrane-bound fluorophore's emission on the concentration of quencher.

Many paramagnetic substances act as quenchers of fluorescence. The paramagnetic cobaltous ion is an efficient quencher of N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)phosphatidylethanolamine (NBD-PE) in lipid bilayer membranes. The NBD fluorophore of this phospholipid is covalently linked to the headgroup and thus is located at the membrane surface.

We investigated the Co^{2+} induced fluorescence quenching of small amounts of NBD-PE incorporated as a probe in large unilamellar egg phosphatidylcholine (PC) vesicles. To characterize the fluorescence quenching technique under controlled conditions for changes in cobalt concentration, we studied the X537A-mediated transmembrane flux of Co^{2+} in PC vesicles containing NBD-PE. We observed that the ionophore transports most of the cobaltous ions as the neutral $Co \sim (X537A)_2$ complex. This concurs with the X537A transport of Ca^{2+} and Mn^{2+} as investigated by a variety of other techniques [13–15]. Moreover, we were able to determine quantitatively the intrinsic permeability of large unilamellar PC vesicles to cobalt ($1 \cdot 10^{-11}$ cm/s).

Materials and Methods

Egg phosphatidylcholine (PC) and N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)phosphatidylethanolamine (NBD-PE) were purchased from Avanti Biochemicals (Birmingham, AL). Ethylene glycol bis(β-aminoethyl ether)-*N*,*N'*-tetra-acetic acid (EGTA) was purchased from Sigma (St. Louis, MO). Sepharose 4B-Cl was purchased from Pharmacia Fine Chemicals (Piscataway, NJ). Spectrapor-2 dialysis tubing (12000–14000 molecular weight cutoff, 6.4 mm cylinder diameter) was purchased from A.H. Thomas Co. (Philadelphia, PA). Lasalocid (X537A, lot No. 1496-24ARx) was obtained from Hoffmann-La Roche, Inc. (Nutley, NJ). 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (Hepes) buffer and octyl β-D-glucopyranoside (octyl glucoside) were purchased from Calbiochem (La Jolla, CA). To remove fluorescent contaminants, octyl glucoside was recrystallized by dissolving in hot acetone and precipitating by the addition of petroleum ether. Water was filtered and deionized to 18 M$\Omega \cdot$ cm quality in a Super-Q System (Millipore Corp., Bedford, MA). All solvents were spectroscopic grade (Aldrich, Milwaukee, WI). All other chemicals were reagent grade.

Preparation of vesicles. Large unilamellar vesicles were prepared by the method of Mimms et al. [16] with modifications. Aliquots of stock solutions of PC (5–10 μmoles) and NBD-PE (0.1% of PC) in chloroform were combined with octyl glucoside at a mole ratio of 1 : 15 (PC/octyl glucoside), and placed in a small glass test tube. The chloroform was evaporated under a stream of nitrogen and residual solvent was removed under high vacuum for at least 4 h. The dried lipid/detergent mixture was dispersed in 0.5 ml of buffer (100 mM NaCl, 5 mM Hepes, 3 mM NaN_3, at pH 7.4) which, for the efflux studies, also contained 10 mM $CoCl_2$. The lipid/detergent solution was dialyzed in a temperature controlled, constant flow, 25 ml capacity dialysis chamber at 25°C. Two liters of buffer were pumped through the chamber at the rate of 100 ml/h, with continuous mixing by a magnetic stir bar. This dialysis yielded large unilamellar vesicles which were passed through a Sepharose 4B-CL gel filtration column (2 cm^2 by 20 cm) pre-equilibrated with Co^{2+}-free buffer at room temperature. Vesicles were stored under N_2 at 4°C and were used within 24 h.

Fluorescence measurements. Steady-state fluorescence was monitored continuously with a photon counting Fluorolog spectrofluorometer (Spex Ind., Metuchen, NJ). The excitation wavelength was 455 nm with a slit width of 2.5 nm. The emission wavelength was 540 nm with a slit width of 10 nm. Dark counts were in the range of 2 to 6 photon counts per second. The fluorescence intensity was monitored on a chart recorder and simultaneously converted to 12-bit digital form with a DT2108-A analog to digital converter (Data Translation, Inc., Marlborough, MA) in an IBM Personal Computer (IBM-PC, International Business Machines, Inc., White Plains, NY). All data analyses were performed on the computer. The fluorescence samples (3.3 ml) were contained in teflon stoppered, 1 cm by 1 cm path length quartz cuvettes and were stirred continuously with a

Spinfin teflon coated magnetic stirrer (Bel-Arts, Pequannock, NJ). The cuvette holder was thermostated to 25°C. X537A was added to the samples in 11-μl aliquots from stock solutions in ethanol. Addition of the equivalent amount of only ethanol had no observable effect on any of the results.

Other methods. Phosphate analyses of all vesicle preparations were done by the procedure of Rouser et al. [17]. The concentrations of $CoCl_2$, $MgCl_2$ and $NiCl_2$ stock solutions were determined by conductivity. The concentrations of X537A ethanolic stock solutions were determined by absorbance at 310 nm ($\epsilon_{310} = 4.1 \cdot 10^3\ M^{-1} \cdot cm^{-1}$).

Data Analyses

The quenching of NBD-PE by Co^{2+} can be generally described by [11,12]:

$$\frac{F_0}{F} = (1 + K_D[Co^{2+}])(1 + K_S[Co^{2+}]) \quad (1)$$

where F_0 and F are the observed fluorescence intensities in the absence and in the presence of Co^{2+}, respectively. K_D is the dynamic quenching constant describing the bimolecular collisional interaction between a fluorophore in the excited state and the quencher, and K_S is the static quenching constant characterizing fluorescence quenching resulting from complexation between the unexcited fluorophore and quencher [11,12]. However, in the range of 0 to 40 mM bulk aqueous phase concentrations of cobalt used in this study, quenching of NBD-PE was found to be linearly dependent on $[Co^{2+}]$. Therefore, we used the linearized form of Eqn. 1:

$$\frac{F_0}{F} = 1 + K[Co^{2+}] \quad (2)$$

where $K = K_D + K_S$.

Eqn. 2 applies to vesicles in which all the fluorophore molecules are equally accessible to the paramagnetic cobaltous ion. The basis of our flux studies is the selective quenching by Co^{2+} of the fluorophores on one of the lipid bilayer's constituent monolayers. In the absence of cobalt, the observed fluorescence of the labeled vesicles is the sum of the fluorescence intensities from each of the monolayers. Initially, only the fluorophores in one monolayer of the lipid bilayer are exposed to Co^{2+}. Applying Eqn. 2 to the fluorescence from this fraction yields the following relation:

$$\frac{F_0}{F_0 - F} = \frac{1}{f_a} + \frac{1}{f_a K[Co^{2+}]} \quad (3)$$

where f_a is the fraction of total fluorophore accessible to quencher. Since our unilamellar vesicles are large, averaging about 2300 Å in diameter [16], the inner and outer monolayers have nearly identical areas. Our results are consistent with a surface density of NBD-PE that is the same for both monolayers, so that $f_a = 0.5$.

$[Co^{2+}]$ in Eqns. 1 to 3 is the concentration of cobaltous ions at the location of the NBD fluorophore. It is less than the concentration of Co^{2+} in the bulk aqueous phase. This is a result of the positive electrostatic potential at the surface arising from the adsorption of the Co^{2+} to PC [18]. The theory of the diffuse double layer describes well the electrical potential adjacent to the membrane, $\psi(x)$, as a function of distance, x, from the charged membrane/water interface [19,20]. The Boltzmann equation, in turn, gives the cobalt concentration at any distance from the membrane/water interface:

$$[Co^{2+}](x) = [Co^{2+}](\infty) \cdot e^{-2F\psi(x)/RT} \quad (4)$$

where $[Co^{2+}](\infty)$ is the cobalt concentration in the bulk aqueous phase, F is the Faraday constant, R is the gas constant and T is the absolute temperature. To relate the bulk aqueous cobalt concentration to the concentration of Co^{2+} at the fluorophore, we have used the diffuse double layer theory, Eqn. 4 and 1.8 M^{-1} for the 1:1 intrinsic association constant for the adsorption of Co^{2+} to PC membranes [18]. From molecular models, we have empirically estimated that the NBD moieties are located about 3 Å from the plane of the phosphate groups of PC, where Co^{2+} presumably adsorbs [1].

In our experiments the total inner volume of all vesicles is only about 0.1% of the external volume. Thus, in the efflux and influx experiments, the external solution becomes an infinite sink and source of Co^{2+}, respectively. The simple scheme illustrated in Fig. 1 describes the efflux of Co^{2+}. We assume that the ionophore adsorbed onto the membrane phase is at equilibrium with the iono-

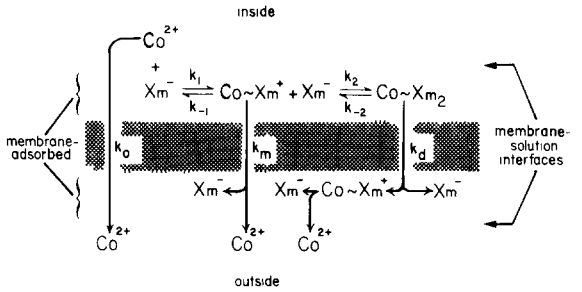

Fig 1 Diagrammatic representation of the efflux of cobalt and cobalt-X537A complexes from lipid vesicles. The shaded areas represent the membrane The complexation reactions between Co^{2+} and the adsorbed ionophore at the inside are assumed to be in equilibrium The dissociations on the outside are not at equilibrium because the extravesicular volume is an infinite sink for Co^{2+} Rate constants are indicated next to each arrow

phore in the aqueous phase and that the complexation reactions between cobalt ions and ionophore molecules at the inside are at equilibrium [21].

Solving the differential equations for the rate of loss of internal cobalt, yields the following expression for the intravesicular cobalt concentration:

$$[Co^{2+}]_i(t) = [Co^{2+}]_i(0) \cdot e^{-(k_0 + k'[X_m^-] + k''[X_m^-]^2)t} \quad (5)$$

where the subscript i refers to the inside of the vesicle, $[X_m^-]$ is the concentration of membrane-adsorbed, uncomplexed ionophore, and we define $k' = k_m(k_1/k_{-1})$ and $k'' = k_d(k_2/k_{-2})(k_1/k_{-1})$, where the rates are those indicated in Fig. 1. Analogously for the influx experiments, the time dependence of the internal cobalt concentration is given by:

$$[Co^{2+}]_i(t) = [Co^{2+}]_o \cdot \left(1 - e^{-(k_0 + k'[X_m^-] + k''[X_m^-]^2)t}\right) \quad (6)$$

where the subscript o refers to the outside of the vesicle.

Eqns. 5 and 6 describe a single-exponential time dependence for both influx and efflux of cobalt, with the same rate constant. We define this observed rate constant as·

$$k_{obs} = k_0 + k'Q[X] + k''Q^2[X]^2 \quad (7)$$

where Q is a constant of proportionality ($[X_m^-] = Q[X]$), and [X] is the total concentration of ionophore (see Discussion).

Resulting

Quenching of NBD-PE by Co^{2+}: steady-state fluorescence

0.2 mM PC large unilamellar vesicles containing 0.1 mol% NBD-PE were exposed to 2.0 mM to 20 mM Co^{2+}, added only external to the vesicles. The steady-state fluorescence was measured at each Co^{2+} concentration. A plot of $F_0/(F_0 - F)$ vs. $1/[Co^{2+}]$, where $[Co^{2+}]$ is the cobaltous ion concentration at the fluorophore (see Data Analyses), is shown in Fig. 2. The line represents the best fit of the data to Eqn. 3 with $1/f_a$ fixed as 2. From the slope we obtained $K = 59\ M^{-1}$. If the adsorption of Co^{2+} to PC was ignored, and the data was plotted as a function of the Co^{2+} concentration in the bulk aqueous phase, then the best fit to the data gave $K = 85\ M^{-1}$ and $f_a = 0.33$. The latter values are inconsistent with the amount of quenching observed for each monolayer in the flux studies. Fig. 3 is a standard Stern-Volmer plot of quenching data from large unilamellar vesicles containing 0.1 mol% NBD-PE whose internal and external compartments had equivalent bulk aqueous phase cobalt concentrations in the range of 2.0 mM to 40 mM. Equillibration of Co^{2+} across the vesicle bilayer was achieved by initially adding cobalt to the vesicles and then incubating them at

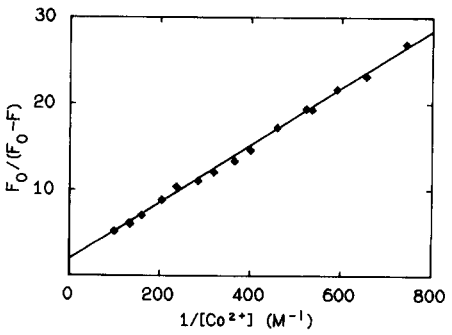

Fig 2 Double reciprocal plot of the fraction of fluorescence quenched as a function of extravesicular Co^{2+} concentration at the position of the NBD moiety, in 0 2 mM PC large unilamellar vesicles containing 0.1 mol% NBD-PE The line represents the least squares fit of the data to Eqn 3, with the value of f_a fixed at 0.5 ($1/f_a$ is the ordinate intercept of the line)

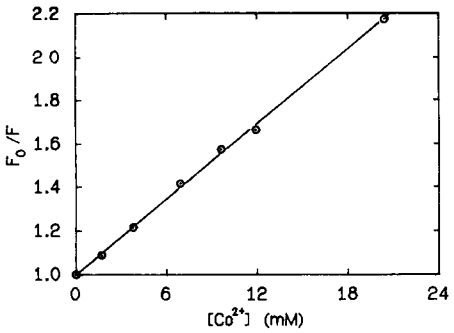

Fig. 3 Fluorescence quenching of 0.1 mol% NBD-PE in 0.2 mM PC large unilamellar vesicles as a function of Co^{2+} concentration at the position of the NBD moiety Co^{2+} is at equal concentrations on both sides of the vesicle bilayer. The line represents the least-squares fit of the data to Eqn. 2

25°C for 6 h in the presence of $5 \cdot 10^{-7}$ M X537A. The line is the best fit of the data to Eqn. 2 and gives $K = 58\ M^{-1}$. Control experiments using equivalent amounts of diamagnetic Mg^{2+} instead of cobaltous ions showed no changes in fluorescence, indicating that mere adsorption of divalent cations to the membrane-water interface does not cause NBD-PE fluorescence quenching. However, Ni^{2+}, another paramagnetic cation, gave results similar to those for Co^{2+} (data not shown).

Intrinsic membrane permeability to Co^{2+}

The fluorescence of 0.2 mM PC large unilamellar vesicles containing 0.1 mol% NBD-PE and loaded with 10 mM Co^{2+} was monitored periodically over 36 h. The leakage of cobalt was detected by the slow increase of the NBD-PE fluorescence (data not shown). The cobalt efflux followed a single-exponential kinetics with a rate constant of $3 \cdot 10^{-6}\ s^{-1}$ (k_0 in Fig. 1). Multiplying this by the volume to area ratio of the vesicles gives a membrane permeability of $1 \cdot 10^{-11}$ cm/s. This value is in reasonable agreement with tracer flux experiments using $^{45}Ca^{2+}$ in PC small, sonicated vesicles which showed a permeability of $2 \cdot 10^{-12}$ cm/s [22]. Vesicles containing a ten fold higher concentration of the NBD-labeled lipid (1.0 mol%) gave the same permeability value, indicating that this background leakage is an intrinsic property of the PC vesicles and is not due to the presence of the fluorescent probe.

X537A-facilitated membrane-permeability to Co^{2+}: efflux experiments

The fluorescence of 0.2 mM PC large unilamellar vesicles with 0.1 mol% NBD-PE and containing 10 mM Co^{2+} was monitored continuously (at 5 to 20 s intervals) before and for an hour or more after the addition of ionophore *. Fig. 4A shows the time dependence of the fluorescence for various concentrations of X537A. As the cobaltous ions leave the vesicles, and quenching of NBD-PE in the inner monolayer is reduced, the fluorescence increases. After the maximum fluorescence had been reached, the addition of 10 mM EGTA (a strong chelator of Co^{2+}) gave no further increase in fluorescence. This indicates that cobalt was diluted below a concentration that would give any detectable quenching. We used Eqns. 3 and 4, and the theory of the diffuse double layer, to convert the fluorescence intensities into intravesicular cobalt concentrations (see Data Analyses). A semilogarithmic plot of the data, normalized to initial conditions, is shown in Fig. 4B. The lines are the best fit of the data to Eqn. 5 with k_{obs} as the free parameter (defined in Eqn. 7). These observed rates are plotted as a function of the total ionophore concentration in Fig. 5. The line is the best fit of the data to Eqn. 7, with $k_0 = 3 \cdot 10^{-6}\ s^{-1}$ (from the intrinsic membrane permeability, see above). The respective values of $k'Q$ and $k''Q^2$ which give the best fit are $1.6 \cdot 10^2\ M^{-1} \cdot s^{-1}$ and $6.2 \cdot 10^8\ M^{-2} \cdot s^{-1}$.

X537A-facilitated membrane-permeability to Co^{2+}: influx experiments

0.2 mM PC large unilamellar vesicles containing 0.1 mol% NBD-PE and pre-equilibrated with ionophore concentrations of 0.5 μM to 4.0 μM, were exposed to 10 mM externally added Co^{2+}. The fluorescence was monitored continuously before and after the addition of cobalt. The fluorescence of NBD-PE on the inner monolayer decreased with time (data not shown). Using Eqns. 3 and 4, and $\psi(x)$ from the diffuse double layer theory, $[Co^{2+}]_i(t)$ was determined. The observed

* The addition of X537A to vesicles causes an instantaneous quenching of NBD-PE. This never exceeded 2% under our experimental conditions. We corrected our data for this small effect.

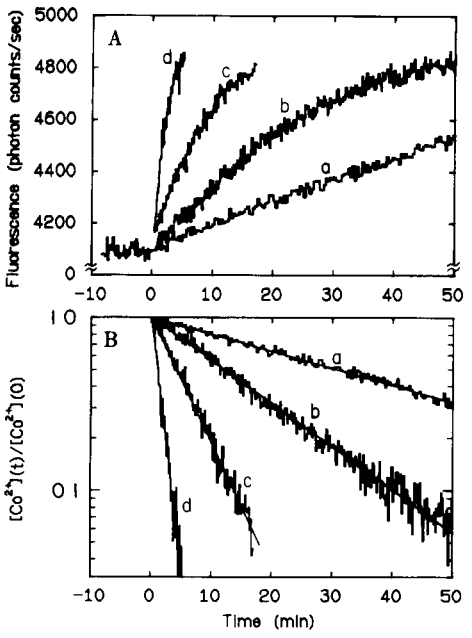

Fig. 4. (A) Time-dependent increase in fluorescence due to X537A-mediated Co^{2+} efflux in 0.2 mM PC large unilamellar vesicles containing 0.1 mol% NBD-PE. Ionophore added at zero-time to total concentrations of 0.5 μM (a), 1.0 μM (b), 2.0 μM (c), and 4.0 μM (d). (B) Same data as (A), converted to intravesicular Co^{2+} concentration, normalized to initial conditions. The lines represent the least-squares fit of the data to Eqns. 5 and 7. The slopes are k_{obs}.

rates were obtained from the slopes of a semilogarithmic plot of the data according to Eqn. 6. The dependence of the rates on [X] was well fit by a second order polynomial (Eqn. 7), with essentially

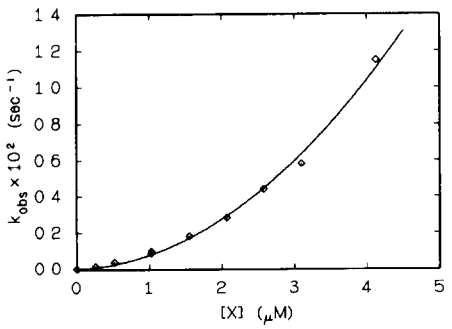

Fig. 5. Observed efflux rates, k_{obs}, as a function of total ionophore concentration. The line represents the least-squares fit of the data to Eqn. 7, with k_0 fixed at $3 \cdot 10^{-6}$ s^{-1} (intrinsic Co^{2+} efflux rate, in the absence of X537A).

the same parameters as in the efflux experiments (see above).

We also examined the dependence of k_{obs} on the cobalt concentration at [X] = 1.0 μM. In the range of 2.0 mM to 20 mM externally added cobalt, k_{obs} was independent of $[Co^{2+}]$, as expected from Eqn. 7.

Discussion

Our results demonstrate that the quenching of a membrane bound NBD fluorophore by cobaltous ions provides an effective method to determine the concentration of those ions in the region of the membrane-solution interface. By having NBD as a fluorescent reporter group covalently linked to the head group of one of the membrane's constituent phospholipids we were able to detect fluorescence emission exclusively in the interface region of the membrane. Concentrations of cobalt in the range of 10^{-4} M to 10^{-2} M could be detected at the site of the NBD moiety of NBD-PE by the decrease in the fluorophore's emission. The value obtained for the quenching constant, K, of 59 M^{-1} represents relatively efficient quenching for a membrane-bound fluorophore with an aqueous phase quencher (c.f., Ref. 25). It suggests that the NBD group is located in the aqueous region of the membrane-water interface where it is readily accessible to Co^{2+}. The distance of the NBD moiety from the plane of the phosphate groups where the membrane's negative charges are located and where Co^{2+} adsorbs is not directly known; but, due to its covalent linkage to the amine of phosphatidylethanolamine, it could not extend out from this plane by more than a few Ångströms. For solving Eqn. 4, we chose 3 Å as the average distance between NBD and the plane of negative charge.

The equivalence between the values of K obtained for fluorescence quenching of NBD-PE when cobalt was present only at the external surface of the vesicle, as in Fig. 2, and when both monolayers were exposed to quencher, as in Fig. 3, indicates that the quenching constants for NBD-PE in the external and internal monolayers of the phospholipid bilayer are, within experimental error, the same. A priori, we expect that the small amount of NBD-PE in the membrane should be

randomly distributed between both monolayers of the phospholipid bilayer such that selective quenching of probe at only one surface of the membrane by cobalt should yield a value for the fraction of probe accessible to quencher that reflects the portion of total lipid present in that monolayer. As is observed in Fig. 2, the data is well fit with a ordinate intercept derived from a value for the fraction accessible of 0.5 which also represents the portion of total lipid present in a single monolayer of a large unilamellar vesicle. From the studies of cobalt flux there was further indication that the surface density of probe is equivalent for both monolayers. We found that the amount of initial fluorescence lost upon addition of cobalt to only the extravesicular medium was nearly identical to the decrease of fluorescence that occurred when cobalt was allowed to equilibrate across the membrane of the same sample through the action of ionophore.

The emission spectrum of NBD-PE overlaps the absorbance spectrum of cobalt (data not shown). At concentrations of cobalt on the order of 10^{-2} M and higher, diminution of NBD-PE emission by trivial energy transfer (i.e. recapture by the Co^{2+} in solution of emitted photons) was seen. Generally, these concentrations are outside the range of biological relevance for divalent cations; but, if they are used, then corrections must be made for this effect. Since there is spectral overlap between NBD emission and cobalt absorbance, Förster energy transfer can also occur [23]. However, the spectral overlap integral is negligible and, consequently, this type of quenching is insignificant.

The intrinsic and the ionophore-mediated cobalt fluxes, as measured by this fluorescence quenching technique, are consistent with the scheme shown in Fig. 1. The intrinsic rate $k_0 = 3 \cdot 10^{-6}$ s^{-1} implies that the half-life of cobalt efflux is 64 h in large unilamellar PC vesicles at 25°C. Furthermore, the dependence of the observed rates on the ionophore concentration is well fit by a second order polynomial, as expected from Eqn. 7. NMR studies of X537A-mediated Mn^{2+} fluxes in small, sonicated PC vesicles [13], tracer experiments of X537A-facilitated $^{45}Ca^{2+}$ fluxes across PC/cholesterol black lipid membranes [14], and voltage-sensitive monazomycin conductance modulation by X537A-mediated Ca^{2+} fluxes in phosphatidylethanolamine/phosphatidylserine black lipid membranes [15], show the same second order dependence on [X] for these divalent cations.

At pH 7.4, the NBD-labeled phosphatidylethanolamine has a single negative charge at the phosphate of the parent lipid. In 0.1 M NaCl, and at NBD-PE concentrations of 1 mol% or less, the contribution of the negative charge on NBD-PE to the membrane surface potential is negligible [19]. The concentration gradients of Co^{2+} and Cl^- will elicit a small diffusion potential which can drive the transmembrane flux of the charged $Co - X_m^+$ species. We can exclude this effect in our experiments because: (a) we observed no change in the rate of cobalt influx as a function of external $CoCl_2$ concentration in the range of 2.0 mM to 20 mM; and (b) conductance and tracer experiments with X537A-mediated calcium ion fluxes in black lipid membranes showed the transport of the charged $Ca - X_m^+$ complex to be negligible compared to that of the neutral $Ca - (Xm)_2$ complex [15].

In all of our experiments the concentrations of Na^+, H^+ and PC were constant. We expect, therefore, that the amount of membrane-adsorbed, anionic form of X537A available to complex with Co^{2+}, is simply a constant fraction, Q, of the total concentration of ionophore (see Data Analyses). Q depends on various equilibrium constants (aqueous association of $X537A^-$ with sodium and protons; heterogeneous association between membrane-adsorbed $X537A^-$ and aqueous sodium and protons; hydrophobic adsorption of $X537A^-$ to the membrane phase). Only some of these values have been reported in the literature [13,21,24], therefore, we analysed our data in terms of the total ionophore concentrations.

Our data shows that fluorescence quenching of a membrane bound fluorophore can be used to measure transmembrane fluxes of paramagnetic ions and that this technique yields results comparable to other methods for measuring ion transport. We have followed transport in the seconds time domain but, because fluorescence occurs in the nanosecond time scale, and can be measured with high sensitivity, stopped flow kinetic methods could be applied to the fluorescence quenching technique.

In contrast to other methods for the direct

determination of ion activity at the membrane surface, this technique has broader applicability in terms of the types of membrane systems that can be studied. Fluorescence quenching can be applied to lipid bilayers in such systems as small, sonicated unilamellar vesicles, multilamellar vesicles, planar lipid bilayer membranes and reconstituted lipid/protein systems. Potentially, the technique can also be used in biological membranes amenable to labelling with the appropriate fluorophore. For biological membranes and any other membrane system containing charged species, a quantitative estimation of surface concentrations for quencher ion can be complicated by a lack of knowledge about the charge distribution at the surface. Nevertheless, relative changes can be measured. Diamagnetic ions such as Ca^{2+} and Mg^{2+} cannot be studied directly with this technique; but, their concentration at the membrane/water interface could be determined by their competitive inhibition of the quenching of the fluorophore by Co^{2+} or Ni^{2+}.

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