Temperature Dependence of Membrane Ion Conductance Analyzed by Using the Amphiphilic Anion 5/6-Carboxyfluorescein

John Bramhall,* Jan Hofmann, Ron DeGuzman, Silvia Montestruque, and Richard Schell

Department of Microbiology and Immunology and The Jonsson Comprehensive Cancer Center, School of Medicine, University of California, Los Angeles, California 90024

Received February 17, 1987; Revised Manuscript Received May 8, 1987

ABSTRACT: The rate of efflux of trapped 5/6-carboxyfluorescein from sealed lipid vesicles showed a marked dependence on (a) temperature, (b) phospholipid acyl chain composition, and (c) the nature of co-trapped counterions. When the dye was salted with sodium, at pH >7, the rate of dye permeation showed a discrete maximum at the melting point of the lipid bilayer (T_c); in the case of membranes composed of dipalmitoylphosphatidylcholine, this discontinuity extended over a very broad temperature range, being detectable at least 10 °C above and below T_c. The peak in dye permeation rate was superimposed on a permeation profile that showed a simple exponential relationship to temperature. Studies with a homologous series of saturated lecithin bilayers revealed a consistent pattern of behavior: a logarithmic dependence of dye permeation rate on temperature with a superimposed discontinuity at T_c. For thin membranes (12–14-carbon acyl chains), the discontinuity was severe, exerting an influence over a very broad temperature range and leading to extremely high overall dye leakage rates. As the acyl chains were lengthened, the discontinuity became less pronounced, almost disappearing at a chain length of 20 carbons. In sharp contrast to these results, dye salted with *N*-methylglucamine [or with tris(hydroxymethyl)aminomethane] showed no efflux maximum at T_c, and base-line leakage rates were generally slower. When dye was salted with ammonium, efflux was too rapid to monitor, even at temperatures well below T_c. The results indicate that the rate of release of electrically charged dyes, such as 5/6-carboxyfluorescein, from sealed lipid vesicles can be tightly coupled to the counterion leakage rate and hence can provide an accurate and convenient assay of relative ion flux across phospholipid bilayers.

5/6-Carboxyfluorescein is an intensely water-soluble dye widely used as a marker for the enclosed aqueous spaces of sealed membrane vesicles. Weinstein et al. (1977) demonstrated how this particular dye can be trapped within the lumen of sonicated phospholipid vesicles at concentrations high enough to cause "self-quenching", rendering the trapped material nonfluorescent. When dye leaks out of the vesicles, it is diluted into the support medium, is no longer self-quenched, and assumes a brilliant green fluorescence. Thus, by monitoring the rate of increase in fluorescence intensity of the system, an accurate determination of the rate of dye permeation can be made.

Under appropriate conditions, vesicles show exceptionally slow rates of dye release. We had noted, for example, that the half-time for leakage of 50 mM 5/6-carboxyfluorescein from DPPC vesicles was several months when they were stored at 4 °C; even though the vesicles aggregated and settled to the bottom of their container, they retained the dull red color characteristic of quenched dye. We also noted, however, that if this same container was held in the hand for a few seconds the contents assumed the brilliant green fluorescence indicative of an irretrievable loss of concentration quenching caused by dye release to the external medium. This suggested that the factors retarding translocation of dye across the vesicle boundary membranes are highly temperature dependent.

Trapped ^{22}Na ions leak out of lipid vesicles exceptionally slowly under most experimental conditions (Papahadjopoulos et al., 1973; El-Mashak & Tsong, 1985); dipalmitoylphosphatidylcholine (DPPC)[1] bilayers, for example, present a very effective barrier to sodium ion permeation at 30 °C. This contrasts sharply with the relatively rapid release of trapped 5/6-carboxyfluorescein under similar conditions (Bramhall, 1984). The paradox here is that this particular dye is anionic at neutral pH and is normally trapped in the form of its sodium salt. Clearly, if an electrically charged dye leaks out of a sealed vesicle at a rate greater than that of any counterion movement, then a transmembrane diffusion potential (ΔE_m) will result from the differential mobilities of ions within the membrane phase. Once established, this diffusion potential should restrict the free permeation of any significant quantity of trapped dye, which should, as a consequence, be released from the vesicle at a rate strongly influenced by the permeation rate of the most mobile counterion. Under such circumstances, the permeation properties, and in particular the temperature dependence of dye permeation (leakage) kinetics, would be reflections of counterion movements (e.g., of sodium permeation) rather than an indication of dye behavior per se; the only way for dye to escape this type of counterion coupling would be cross the membrane as an electrically neutral, undissociated acid. Indeed, previous work from this laboratory has demonstrated directly that the leakage of charged amphiphiles (including 5/6-carboxyfluorescein)

* Address correspondence to this author at M-006 (MS-I), School of Medicine, UCSD, La Jolla, CA 92093.

[1] Abbreviations: DLPC, dilauroylphosphatidylcholine; DMPC, dimyristoylphosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; DSPC, distearoylphosphatidylcholine; DAPC, diarachidoylphosphatidylcholine; Tris, tris(hydroxymethyl)aminomethane; diS-C_3-(5), 3,3′-dipropylthiodicarbocyanine iodide; T_c, lipid gel–liquid-crystalline phase transition temperature; TLC, thin-layer chromatography; T-jump, temperature jump; ΔE_m, transmembrane electrical potential; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; kDa, kilodalton(s).

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from sealed phospholipid vesicles can become tightly coupled to the copermeation of electrolytes, and we have suggested that this principle might be used to monitor ion permeation itself (Bramhall, 1984). Thus, measurements of dye leakage from lipid vesicles, which can be made very rapidly and with great sensitivity in the case of 5/6-carboxyfluorescein, could be expected to provide an accurate reflection of ion flux across phospholipid bilayers under a variety of experimental conditions.

In this report, we demonstrate that 5/6-carboxyfluorescein does indeed generate a diffusion potential as it leaks from sealed vesicles. We identify the ring substituents which have the most effect in regulating the relative permeation rate of the dye, and we describe the temperature dependence of dye permeation across homogeneous lecithin bilayers composed of lipids with varying acyl chain length, in the presence of potential counterions with differing electronic configuration.

Materials and Methods

Chemicals. The fluorescent dyes, fluorescein, 2,7-dichlorofluorescein, and 2,7-dichloro-5/6-carboxyfluorescein, were obtained from Molecular Probes Inc. (Junction City, OR) and purified by thin-layer chromatography on 500-μm-thick silica gel G plates using a developing solvent composed of benzene/ethyl acetate/ligroin/acetic acid (100:14:10:1). The dyes migrated with R_f values of 0.29, 0.35, and 0.14/0.18,[2] respectively. 3,3′-Dipropylthiodicarbocyanine iodide [diS-C_3-(5); Molecular Probes Inc.] was used without further purification. 5/6-Carboxyfluorescein, supplied by Eastman (Rochester, NY), was purified before use by recrystallization and hydrophobic adsorption chromatography on Sepharose LH20 as described by Ralston et al. (1981). Pure 5- and 6-carboxyfluorescein moved to R_f values of 0.08 and 0.13 in this chromatographic system.[2] The purity of phospholipids (DMPC, DPPC, DSPC, DAPC) obtained from Sigma (St. Louis, MO) was verified by analytical thin-layer chromatography on silica gel G using a developing solvent of chloroform/methanol/glacial acetic acid/water (90:40:12:2 v/v). Lipids were dissolved in chloroform/methanol (2:1 v/v), aliquoted into glass tubes, dried under nitrogen at 40 °C, and placed under high vacuum for 18–20 h at 20 °C, in order to remove residual chloroform, before being stored under nitrogen at −25 °C.

Preparation of Lipid Vesicles. Small unilamellar lipid vesicles were prepared by sonication of lipid suspensions in aqueous buffer under an atmosphere of nitrogen; sonication was performed by using a Ultrasonics Model W200R cell disrupter (Heat Systems, Plainview, NY) equipped with a microtip running at 30% of maximum power and with 95% duty cycle. Typically, 5 mg of DPPC was sonicated in 0.5 mL of buffer (containing materials to be trapped). During sonication, the temperature of the lipid suspension was maintained at least 5 °C above the lipid's melting point (T_c + 5 °C, e.g., at 46 °C in the case of DPPC). The vesicle preparation was centrifuged (100000g, 30 min) to remove structures other than small vesicles, then cooled to at least 20 °C below the lipid melting point (T_c − 20 °C), and separated from untrapped solutes by gel permeation chromatography using a Bio-Rad P-10 gel (0.5 × 17 cm column) equilibrated with isotonic buffer. The vesicles eluted with the void volume; free solutes were retarded. Buffer osmolarity was determined with a vapor pressure osmometer (Wescor Model 5100C).

Quantitative Studies. Equilibrium measurements of dye and ^{22}Na leakage were performed by using plastic multiwell microdialysis chambers with a 200-μL cavity on each side of a semipermeable membrane (M_r 12 000 cutoff), as previously described (Bramhall, 1984). A suspension of phospholipid vesicles was placed in one compartment, and an equal volume of isotonic buffer was placed in the second. The cells were rotated at approximately 5 rpm for 18 h at the experimental temperature. Solutes liberated from the vesicle lumen distribute equally between the two dialysis chambers with an equilibrium half-time of approximately 40 min; accordingly, proportional release of ^{22}Na from vesicles was determined from the ratio of the radioactivity of the vesicle-free compartment (Q_a) to the total radioactivity of the two compartments (Q_t) according to eq 1.

$$\text{proportional release} = 2Q_s/Q_t \quad (1)$$

Equilibrium binding experiments were performed with the same apparatus. Phospholipid vesicles prepared in 50 mM NaCl were placed in one chamber; 50 mM sodium phosphate buffer, containing 10 μM fluorescent dye, was placed in the opposite chamber. The cells were rotated as described above, and the percentage binding of dye was determined from the ratio of fluorescence intensities in equal-volume samples removed from the vesicle-free (F_a) and vesicle-containing (F_v) compartments. The samples were diluted (at least 10-fold) with 50 mM Na_2HPO_4 prior to measurement of fluorescence intensity in order to ensure that sample fluorescence was unaffected by variations in sample pH. The quantity of membrane-bound dye (F_m) was calculated from eq 2.

$$F_m = F_v - F_a \quad (2)$$

The membrane/buffer partition coefficient, k_b (binding coefficient), was calculated from eq 3 as the slope of the line

$$k_b = (F_m/F_a)/(V_a/V_m) \quad (3)$$

obtained by plotting F_m/F_a against V_m/V_a, where V_m and V_a are the volumes of the membrane and aqueous phases, respectively; V_m was calculated by assuming a partial specific volume for DPPC of 1 mL/g.

1-Octanol/water partition coefficients (k_p) were determined for fluorescein derivatives by fluorescence assay after extraction of aqueous solutions of dye, at various pH values, with 1-octanol. After extraction, the organic phase was made basic by the addition of a small quantity of triethanolamine, thus ensuring that fluorescence would not be affected by variations in sample pH.

Determination of Dye Diffusion Potentials. For the determination of relative ionic permeabilities, DPPC vesicles containing 10 mM potassium gluconate, 90 mM choline gluconate, and 10 mM Tris/HEPES (pH 7.4) were suspended in isotonic buffer to a final lipid concentration of 0.4 mg/mL. The transmembrane electrical potential (ΔE_m) was monitored with the potential-sensitive dye 3,3′-dipropylthiodicarbocyanine iodide [diS-C_3-(5)] by measuring the changes in light absorbance associated with changes in ΔE_m (Wright et al., 1981; Schell et al., 1983). Absorbancies were monitored at two wavelengths (655 and 685 nm), and for each observation, ΔE_m values were calculated from standard responses to imposed K^+–valinomycin diffusion potentials of known magnitude. The relationship between absorbance and membrane potential is known to be linear over the range −63 to +63 mV (Gunther et al., 1984). Relative ion permeabilities were calculated from the observed values of ΔE_m obtained with known concentra-

[2] The two R_f values reflect the separation of 5- and 6-substituted fluorescein derivatives. All the studies of carboxyfluorescein described in this report were performed using unresolved mixtures of the 5- and 6-isomers.

tions of permeant ion by using a generalized constant field equation for monovalent and divalent ions (Sergio Cianni, personal communication). Relative permeabilities were converted to absolute values by reference to standard values for potassium permeability under the given conditions (temperature, pH, and ionic strength).

Dye Permeation Kinetics. Sealed phospholipid vesicles containing trapped dye, and suspended in cold isotonic buffer, were rapidly diluted (1:200) into well-stirred isotonic buffer equilibrated to the experimental temperature. Typically, 10 μL of a vesicle suspension at a lipid concentration of 1 mg/mL was added to 2 mL of buffer. The fluorescence emission intensity of the sample was recorded continuously subsequent to this dilution (zero time). The dye leakage rate (k_{rel}) was calculated from eq 4 as the slope of the line obtained by

$$k_{rel} = \ln (F_\infty - F_t)/t \quad (4)$$

plotting log $(F_\infty - F_t)$ against time (t) where F_∞ is the fluorescence intensity of the sample after complete liberation of trapped dye following detergent-induced lysis of vesicles and F_t is the fluorescence intensity at time t after initial dilution/temperature shift.

Optical Measurements. Absorbance measurements were made by using an Aminco DW-2 double-beam spectrophotometer with a thermostated cell compartment; fluorescence and light-scattering measurements were performed on a Fluorolog II spectrometer (Spex Industries, Metuchen, NJ) equipped with temperature control accessories and a magnetic stirrer. The monochromator band-pass was set at 1.8 nm (0.5-mm slit width) for both excitation and emission side optics; thus isolating dye fluorescence emission from scattered light. Sample temperatures were monitored with a platinum resistance thermometer housed in the optical cuvette. Thermally induced lipid phase transitions were detected by monitoring the Rayleigh scattering intensity of vesicle suspensions as a function of temperature. Linear temperature gradients were generated with a digital temperature programmer (Neslab Inc., Portsmouth, NH).

Results

Temperature Dependence of Dye Release Kinetics. The experimental system used for this work consisted of sealed, small unilamellar lipid vesicles of defined composition, suspended in isotonic buffers at defined temperature. The vesicles contained a high concentration of the sodium salt of the acidic dye 5/6-carboxyfluorescein. Release of dye from the vesicles leads to an increase in the fluorescence intensity of the system (Weinstein et al., 1977), a parameter which could thus be used to measure dye release kinetics. Figure 1 shows the temperature dependence of the rate of leakage of 5/6-carboxyfluorescein from vesicles composed of synthetic DPPC. The vesicles were suspended in a sodium pyrophosphate buffer whose anion composition consisted predominantly of multiply charged species unlikely to diffuse across the bilayer to the vesicle lumen. Inside the vesicle was a high concentration of dye, tending to leak out with its chemical potential gradient; also trapped within the vesicle was sodium, salted with the anionic forms of the dye. Initial release kinetics followed a simple exponential function from which a rate constant was derived (eq 4). The measurements revealed a sharp peak in ion permeation rate at T_c, the melting point of the DPPC bilayers (41 °C). This peak was superimposed on behavior at temperatures remote from T_c (both above and below) in which ion permeation rates appeared to be exponentially proportional to temperature. The sharp maximum in dye release at T_c differs markedly from the behavior of several

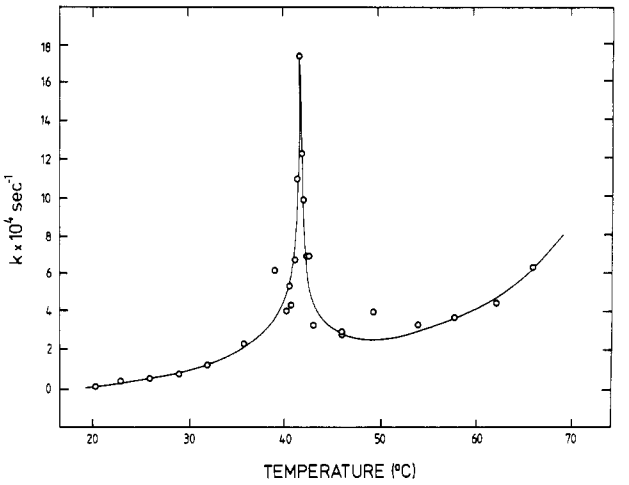

FIGURE 1: Temperature dependence of dye permeation. The rate constant for permeation of 5/6-carboxyfluorescein across DPPC bilayers was determined over a range of temperatures using sodium as a counterion to the anionic dye. Unilamellar DPPC vesicles, containing 50 mM sodium 5/6-carboxyfluorescein (pH 7.4, 206 mOsm) and stored at 15 °C, were suspended, at zero time, in 100 mM sodium pyrophosphate/citrate buffer (pH 7.0, 207 mOsm), equilibrated at defined temperatures in the sample compartment of a fluorescence spectrometer. Final lipid concentration was 10^{-5} M. The initial rate of fluorescence intensity increase (excitation and emission wavelengths of 490 and 516 nm, respectively) was determined at each experimental temperature and is shown here in the form of a direct plot. The sharp maximum in the rate of ion leakage occurs at 41.4 °C, coinciding with the temperature of the major gel–fluid phase transition of DPPC.

electrically neutral amphiphilic dyes (Jähnig & Bramhall, 1982; Bramhall, 1984) and shows strong similarity to the results originally presented by Papahadjopoulos et al. (1973) for the self-diffusion of sodium across lipid bilayers.

Effects of Membrane Thickness. In Figure 2, dye permeation rates, measured over a broad range of temperatures, were determined for several members of a homologous series of phosphatidylcholines. The results have been plotted as Arrhenius functions and are presented in a reduced temperature plot which superimposes T_c for each of the lipid species. This means that for each point on the abscissa every lipid is at the same physical state relative to T_c. Clearly, acyl chain length dramatically influenced dye permeability, but the series of saturated lecithin bilayers displayed a remarkably consistent pattern of behavior. All the lipids showed a direct (exponential) dependence of 5/6-carboxyfluorescein permeation rate on temperature with a superimposed discontinuity at T_c. This discontinuity exerted an influence over a relatively broad temperature range and led to extremely high dye permeation rates across thin membranes. With increasing acyl chain length, the discontinuity became less pronounced, disappearing at an acyl chain length of 20 carbon atoms. For the series of saturated lecithins studied, there was a strong inverse dependence of dye permeation rate on acyl chain length, which became apparent when all measurements were made at a common temperature relative to T_c. For lipid bilayers in the gel phase, 10 °C below T_c, the dye permeation rate constants for 14:0, 16:0, 18:0, and 20:0 phosphatidylcholine were 608, 104, 41, and 33 μs^{-1}, respectively.

Very Rapid Dye Release from DMPC Vesicles. Figure 2 is incomplete with respect to DMPC ($C_{14:0}$) simply because at temperatures within 5 °C of T_c, dye leakage rates became much too rapid to measure with our equipment; in fact, DLPC ($C_{12:0}$) could not be used at all in these experiments because these very thin bilayers showed excessive ion permeation rates at all accessible temperatures. Reactions with half-times less

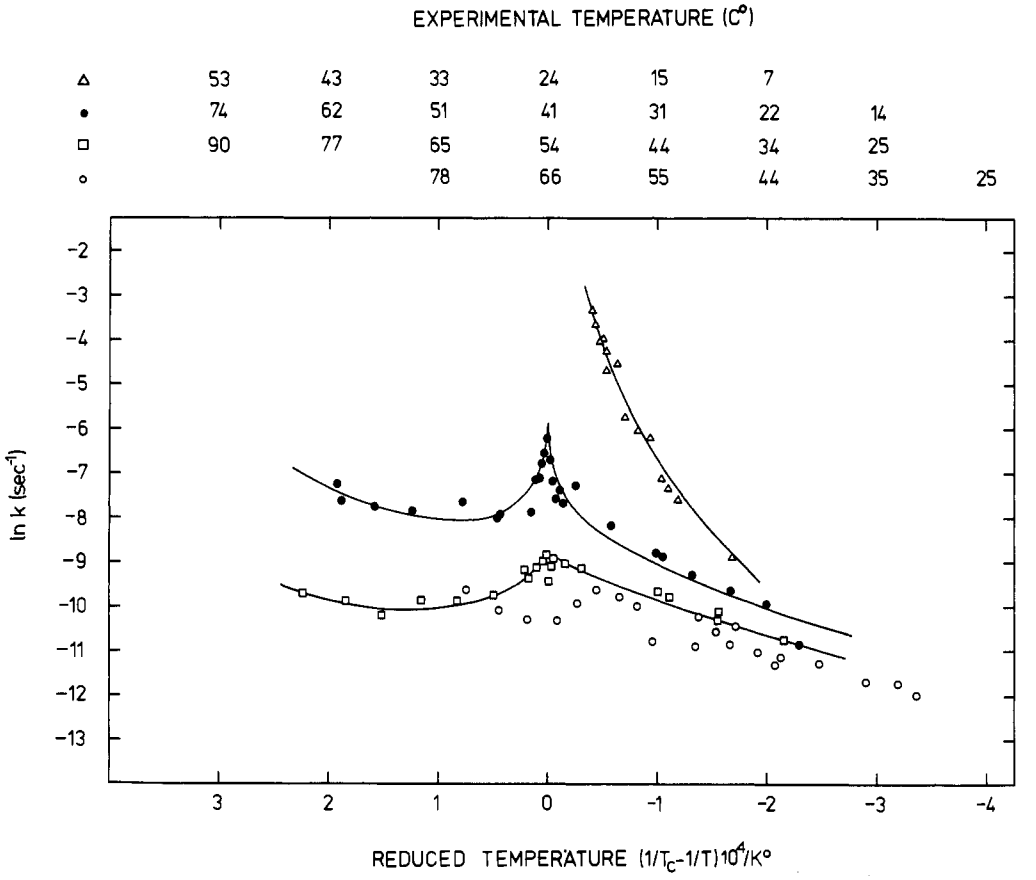

FIGURE 2: Acyl chain composition has a profound influence on dye permeation. Phospholipid vesicles containing 50 mM sodium 5/6-carboxyfluorescein in 100 mM sodium pyrophosphate/citrate buffer (pH 7.8) were resuspended at zero time in 150 mM sodium pyrophosphate/citrate buffer (pH 7.8) at defined temperature. Final lipid concentration was approximately 10^{-5} M. Initial rates of dye leakage (monitored by increase in sample fluorescence intensity) were measured over a range of temperatures and with a variety of phospholipid compositions. The results are presented here in the form of Arrhenius functions plotted on a reduced temperature abscissa. Data for five different phospholipids are presented: (△) DMPC (T_c = 24 °C); (●) DPPC (T_c = 41 °C); (□) DSPC (T_c = 54 °C); (○) DAPC (T_c = 66 °C). The upper abscissa shows a range of experimental temperatures coinciding with the reduced temperatures of the lower abscissa, permitting an easy comparison of the range of experimental temperatures used for each lipid in this study. Note that zero on the lower abscissa corresponds to T_c for each lipid listed on the upper abscissa.

than 2 s are too rapid to monitor with confidence without equipment specifically designed for measurement of rapid responses to instantaneous changes in temperature. To generate the data shown in Figure 2, we employed an extremely simple temperature jump (T-jump) method in which small samples of lipid vesicles containing trapped dye (stored at T_c − 20 °C) were rapidly injected into much larger volumes of buffer preequilibrated to the experimental temperature. Using this approach, we were able to achieve accurate T-jumps in excess of 40 °C within the 1-s mixing time of our system. When measurements were made at temperatures above T_c for the experimental lipid, the vesicle samples were very rapidly (<1 s) shifted through T_c. The only practical difficulties encountered were with DMPC where, because of the exceptionally fast rates of dye release displayed by DMPC at T_c, significant leakage of dye occurred before the experimental temperature was attained. However, Figure 3 shows that by integrating the extent of dye release during the 5 s immediately following a T-jump, it was possible to attain a reasonably accurate profile of the temperature dependence of dye release close to T_c, even though precise kinetic data were unobtainable. At temperatures well below and well above T_c, integrated dye release was directly proportional to temperature; close to T_c, all dye was released within the 5-s integration interval.

Clearly, with the T-jump method used (injection of a small volume of vesicles, at 4 °C, into a large volume of buffer at an elevated temperature), vesicles very rapidly pass through their phase transition when experimental temperatures are

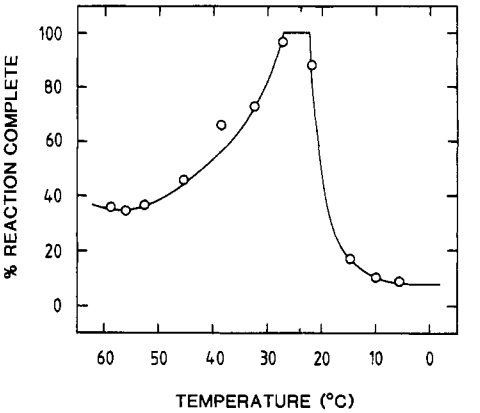

FIGURE 3: Rapid kinetic studies with DMPC. DMPC vesicles containing 50 mM sodium 5/6-carboxyfluorescein in 100 mM sodium pyrophosphate/citrate buffer (pH 7.8), stored at 4 °C, were resuspended, at zero time, in 150 mM sodium pyrophosphate/citrate buffer (pH 7.8) equilibrated to the experimental temperature. Typically, 10-μL vesicles were added to 2 mL of stirred buffer, giving a final lipid concentration of approximately 10^{-5} M. The proportional release of dye occurring over the first 5 s following this rapid temperature jump from 4 °C to the designated experimental temperature was monitored by integrating the coincident increase in fluorescence intensity.

greater than T_c. This is an imperfect way of measuring precise release kinetics, but it is doubtful that measurements could be made with any greater precision by using current-pulse T-jump equipment. Even with our simple method for meas-

Table I: Relationship between Dye Release and Counterion Identity[a]

counterion	temp (°C) 20	32	45
sodium	5.2	1.6	0.5
ammonium	<0.1	ND	ND
Tris	27.5	12.1	3.8
N-methylglucamine	35.4	11.4	4.7

[a] DPPC vesicles containing 50 mM 5/6-carboxyfluorescein adjusted to pH 7.4 with base (NH_4OH, NaOH, Tris, or *N*-methylglucamine) were dialyzed against isotonic sodium phosphate buffer (pH 7.4) for 22 h, as described under Materials and Methods. Dialysis was performed at three different temperatures, and samples were taken for fluorescence assay at approximately 2-h intervals. The table shows the half-times (in hours) for dye release, calculated from the initial release kinetics measured in the presence of four different counterions. The half-time for equilibration of free dye between microdialysis chambers was approximately 40 min. ND, not determined.

uring approximate release kinetics, it is striking how similar the DMPC response profile is to that exhibited by DPPC (illustrated in Figure 2). Our conclusions from these observations are that DMPC and DPPC are qualitatively similar with respect to the temperature dependence of their permeability to dye and that in every case studied the temperature dependence of 5/6-carboxyfluorescein release shows strong similarities to the process of sodium ion permeation.

Influences of Counterions on Leakage of 5/6-Carboxyfluorescein. If, indeed, dye release is influenced by counterion permeation, then it could be predicted that dye leakage rates would vary according to the identity of the counterions present in any given experimental system. We have already demonstrated that 5/6-carboxyfluorescein leakage rates can be increased, dramatically, by the presence of membrane-permeant anions *outside* the sealed vesicles containing the dye (Bramhall, 1984); Table I shows that the same was true for membrane-permeant cations trapped *inside*, with the dye. Conventionally, 5/6-carboxyfluorescein is brought into aqueous solution by titration with an appropriate base, typically NaOH, before being trapped within lipid vesicles. When, however, NH_4OH was used to titrate the dye, the resulting solution was effectively incapable of being trapped because the dye leaked out of DPPC vesicles within the time necessary for removal of untrapped dye on a desalting column (see Materials and Methods). It is difficult to see how NaOH and NH_4OH could exert such dramatically different influences on the spontaneous leakage of electrically neutral, undissociated dye, and the clear implication is that NH_4^+ (as opposed to NH_3) readily permeated DPPC bilayers, even at low temperatures, to allow the rapid, coupled release of 5/6-carboxyfluorescein anions.[3] Conversely, Table II shows that when the dye was neutralized with large, polar bases such as tris(hydroxymethyl)aminomethane or *N*-methylglucamine before being trapped, there was a significant decrease in the subsequent dye leakage rates at all temperatures measured, again implying that the protonated forms of these bases diffused very slowly through

[3] An alternative explanation for the behavior in the presence of NH_4^+ counterions was considered, that NH_3 leaked out of the sealed vesicles with its concentration gradient, leading to a lowering of the internal pH, in turn leading to an increase in the relative proportion of electrically neutral dye molecules. However, when DPPC vesicles containing 50 mM 5/6-carboxyfluorescein (titrated to pH 7.4 with NH_4OH) and 15 mM Na_2SO_4 were passed down desalting columns equilibrated *either* with 77 mM sodium phosphate, pH 7.4, containing 15 mM Na_2SO_4 *or* with 94 mM ammonium phosphate, pH 7.4, the same rapid leakage of dye was seen in both cases. Under the latter conditions, no concentration gradient of NH_3 existed across the vesicle boundary bilayer, and we therefore assume that dye release could not have been stimulated by pH changes created by spontaneous, independent translocation of ammonia.

Table II: Relative Permeabilities of Anions across DPPC Bilayers[a]

ion	P_i/P_K	SD	*n*
Cho^+	0.10	0.03	15
Glu^-	0.10	0.02	15
Cl^-	0.95	0.06	10
F^-	1.46	0.23	10
Br^-	2.25	0.18	10
dye^-	61800	2400	21

[a] DPPC vesicles containing 10 mM potassium gluconate, 90 mM choline gluconate, and 10 mM Tris–HEPES (pH 7.4) were suspended in 100 mM solutions of the potassium salts of several ions (in the case of 5/6-carboxyfluorescein, the dye concentration was 50 μM in 100 mM potassium gluconate). Anion permeability (P_i) relative to potassium–valinomycin permeability (P_K) was determined from the constant field equation, as described under Materials and Methods. Membrane potentials were measured by using diS-C_3-(5). Abbreviations: Cho^+, choline; Glu^-, gluconate; Cl^-, chloride; F^-, fluoride; Br^-, bromide; dye^-, 5/6-carboxyfluorescein; SD, standard deviation; *n*, number of determinations. In all cases, the external solution was isosmotic to the internal (trapped) buffer and was of the same pH. All measurements were made at 45 °C.

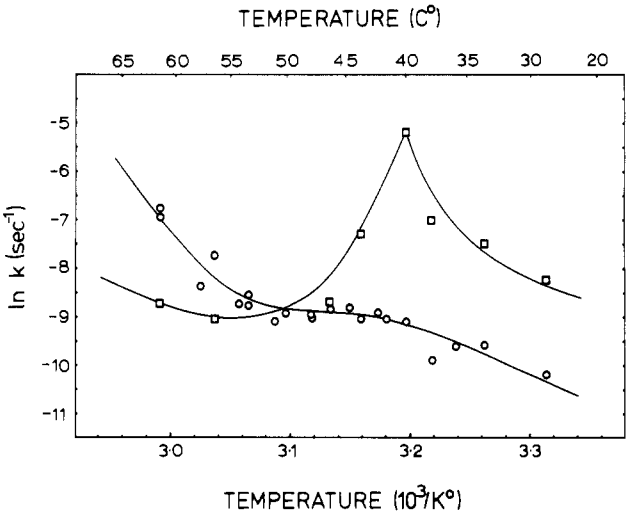

FIGURE 4: Influence of counterions on temperature dependence of dye permeation. DPPC vesicles containing 50 mM 5/6-carboxyfluorescein (adjusted to pH 7.4 with either sodium hydroxide or *N*-methylglucamine) were resuspended (zero time) in 70 mM sodium phosphate, pH 7.4, equilibrated to the defined temperature within the sample compartment of a fluorescence spectrometer. Measurement of dye leakage rates was made over a range of temperatures by monitoring initial rates of increase in fluorescence emission intensity, plotted here against experimental temperature in the form of an Arrhenius function. (□) Sodium salt; (○) *N*-methylglucamine salt.

DPPC bilayers and suggesting that dye leakage rates might conveniently be used to compare relative permeation rates of the charged forms of the various bases with which it might be salted.

Figure 4 shows a direct comparison of the temperature dependence of 5/6-carboxyfluorescein leakage from sealed DPPC vesicles in the presence of two different co-trapped counterions, *N*-methylglucamine and sodium. Under otherwise identical experimental conditions, "sodium" vesicles and "*N*-methylglucamine" vesicles exhibited a strikingly different temperature profile for dye leakage. In the absence of sodium, the dramatic peak in dye efflux rates at T_c was abolished, giving strong support to the suggestion that counterion identity can exert considerable influence on the efflux of trapped 5/6-carboxyfluorescein.

Figure 5 shows the effects of varying the ratio of Na^+ and $Tris^+$ used to neutralize 5/6-carboxyfluorescein. When $Tris^+$ was the only internal cation, dye efflux was relatively slow when measured at 35 °C (half-time for dye release approxi-

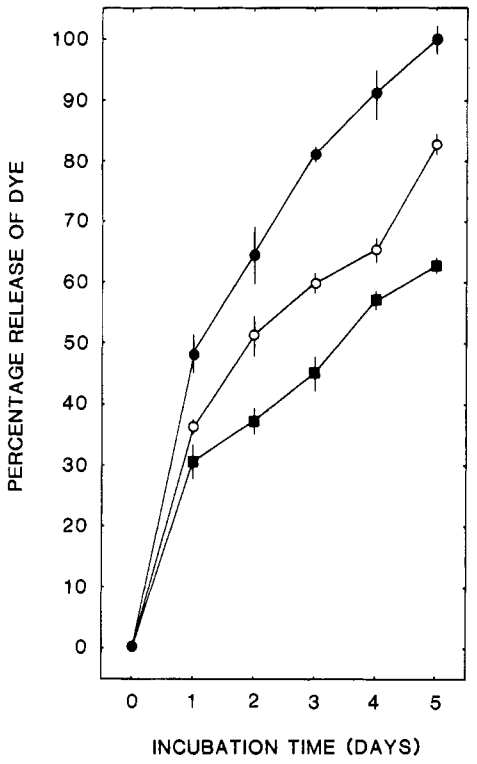

FIGURE 5: Influence of co-trapped sodium on dye efflux rates. DPPC vesicles containing 50 mM 5/6-carboxyfluorescein, adjusted to pH 7.4 with Tris base, together with 50 mM Tris-HCl (■), 45 mM Tris-HCl/5 mM sodium chloride (○), or 50 mM sodium chloride (●) were dialyzed against isotonic sodium phosphate buffer (pH 7.4) for 5 days, as described under Materials and Methods. The compositions of the three different trapped salt/dye solutions resulted in trapped dye:sodium ratios of 1:0, 1:0.1, and 1:1, respectively. Dialysis was performed at 35 °C, and samples were taken for fluorescence assay at daily intervals. The figure shows the proportion (percent of total) of dye release measured in the presence of the three different concentrations of co-trapped sodium. The half-time for equilibration of free dye between microdialysis chambers was approximately 40 min; total releasable dye was determined in the presence of 0.05% NP40 detergent.

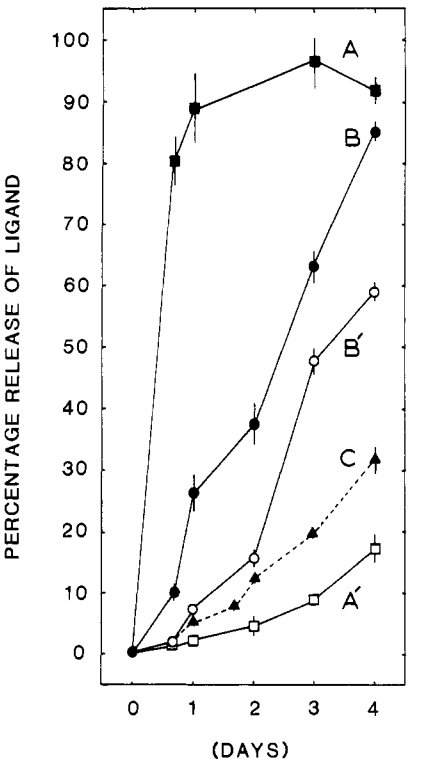

FIGURE 6: Stoichiometry between dye release and permeation of sodium ions. DPPC vesicles containing 50 mM dye (fluorescein or 5/6-carboxyfluorescein adjusted to pH 7.4 with Tris base) together with 50 mM ^{22}NaCl were dialyzed against isotonic sodium phosphate buffer, at 32 °C, as described for Figure 5. At appropriate time intervals, samples were withdrawn and assayed for fluorescence intensity (excitation and emission wavelengths of 490 and 516 nm, respectively) and ^{22}Na content. Total releasable dye and ^{22}Na were determined in the presence of 0.05% NP40 detergent. In the figure, release of dye (A) and ^{22}Na (A′) from fluorescein-loaded vesicles is plotted together with release of dye (B) and ^{22}Na (B′) from 5/6-carboxyfluorescein-loaded vesicles against time of dialysis. The release of ^{22}Na from vesicles containing and suspended in 50 mM 5/6-carboxyfluorescein (C) is also plotted against time.

mately 4 days), a rate assumed to be a reflection of slow $Tris^+$ flux across the membrane barrier. When Na^+ was added to the internal buffer, in 1:1 molar stoichiometry with dye, the rate of dye release was sharply increased (half-time approximately 1 day), and when this dye/sodium stoichiometry was reduced, there was a corresponding reduction in dye efflux rates.

Influences of Dye on Counterion Permeation. Dye efflux was clearly influenced by counterion permeation, but we wondered whether the converse was also true; was counterion efflux influenced by dye permeation? This is a particularly important point in view of the contrast between our measured rapid rates of dye efflux, alleged to be coupled to Na^+ permeation, and the published accounts of slow Na^+ exchange diffusion kinetics across lecithin bilayers, measured in the absence of dye. Figure 6 shows that when fluorescein was dissolved in Tris–phosphate buffer in the presence of $^{22}Na^+$ ions (1:1 molar stoichiometry between sodium ion and dye), there was no correlation between the proportion of trapped dye released within a given period of time and the corresponding proportion of $^{22}Na^+$ released. The half-time for dye leakage was approximately 10 h at 32 °C, and essentially all the fluorescein was released within 2 days (Figure 6A), whereas the half-time for leakage of co-trapped $^{22}Na^+$ was on the order of 1 month, and at the 2-day time point, 96% of the trapped $^{22}Na^+$ still remained inside the vesicles (Figure 6A′). In contrast, when the same experiment was performed with 5/6-carboxyfluorescein (again using the same molar ratio of dye to $^{22}Na^+$), not only did dye release occur very much more slowly (half-time for leakage 2.5 days; Figure 6B) but also the co-trapped $^{22}Na^+$ now effluxed at a dramatically increased rate (half-time approximately 3 days; Figure 6B′). The same phenomena were seen at several experimental temperatures above and below T_c.

We eliminated the possibility that 5/6-carboxyfluorescein perturbed the DPPC bilayer structure, making the membranes leaky to sodium ions, by demonstrating (Figure 6C) that $^{22}Na^+$ leakage rates were influenced to only a slight extent by the presence of 50 mM 5/6-carboxyfluorescein on *both* sides of the boundary membrane. Because the accelerated leakage of sodium was only seen when there was a concentration gradient of the dye, we concluded that the leakage of 5/6-carboxyfluorescein involved permeation of anionic forms of the dye, thus creating a dye diffusion potential across the membrane which decreased the energy barrier to sodium counterion permeation. In contrast, it appeared that the leakage of fluorescein itself involved the migration of electrically neutral dye molecules across membrane barriers, generating no appreciable membrane potential and thus escaping from regulation by, and of, counterion flux.

Direct Demonstration of E_m Generated by 5/6-Carboxyfluorescein Leakage. The electrical potential difference existing across the membranes of pure DPPC vesicles was measured, directly, by using the potential-sensitive dye diS-

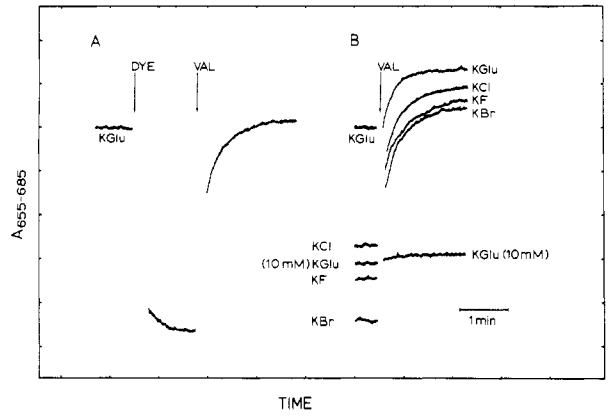

FIGURE 7: Anion diffusion potentials. DPPC vesicles, loaded with 10 mM potassium gluconate, 90 mM choline gluconate, and 10 mM Tris/HEPES (pH 7.4), were suspended in 100 mM solutions of several anions (salted with potassium). Upon the addition of valinomycin (4 μg/mL), the existing membrane potential (ΔE_m), defined by the relative membrane permeation coefficients of the ions present, was converted to the potassium–valinomycin diffusion potential, E_K (by virtue of the extremely high permeation coefficient of the ionophore/cation complex). In the absence of mobile counterions, e.g., when the external solution contained potassium gluconate, $\Delta E_m = E_K$. In the presence of permeant anions, $\Delta E_m \neq E_K$, as E_K is short-circuited by the mobile anion. ΔE_m was monitored by measuring the difference in absorbancies at 655 and 685 nm. The disparity between ΔE_m and E_K in the presence of different anions is proportional to the differences in permeability of DPPC bilayers to potassium and to the anion in question. Final concentration of diS-C_3-(5) was 1.5 μM; lipid concentration was 0.3 mg/mL. All measurements were made at 45 °C.

C_3-(5). In common with other dyes of this type, diS-C_3-(5) shows changes in its absorption profile and in its fluorescence properties which are dependent on the concentration of dye within the membrane phase. Under appropriate experimental conditions, the precise relationship between membrane dye concentration and membrane potential ensures that the magnitude of ΔE_m can be determined either by fluorescence or by light absorbance measurements. For a variety of reasons, detection of changes in fluorescence emission intensity is usually more sensitive and less likely to be complicated by turbidity changes in samples containing sealed membrane systems. However, in the experiments reported here, it was impossible to record fluorescence signals from diS-C_3-(5) in the presence of 5/6-carboxyfluorescein because of the spectral overlap between the two dyes. Fortunately, the fluorescein derivative is optically transparent at wavelengths above 600 nm, permitting the accurate measurement of diS-C_3-(5) absorbance changes at 655 and 685 nm (Figure 7). Because of the fact that the observed optical signal was measured as the difference between intensities at two wavelengths, interference attributable to sample tubidity and other factors was essentially eliminated. Absorbance measurements were calibrated at 45 °C by using imposed potassium–valinomycin potentials under conditions where K^+ was thus the major mobile ion; under such conditions, eq 5 is applicable where

$$E_K = E_m = 63 \log ([K]_i/[K]_o) \quad (5)$$

$[K]_i$ and $[K]_o$ are the internal and external potassium ion concentrations, respectively. In the presence of permeant anions, $E_m \neq E_K$ by a factor which is a function of the anion's ability to short-circuit the potassium diffusion potential, i.e., to cross the membrane barrier.

Table II illustrates the relative permeabilities of DPPC bilayers toward several ions measured under standard conditions. Using potassium permeability as a reference, it was observed that 5/6-carboxyfluorescein permeates DPPC bilayers more readily than potassium ions by a factor of at least 10^4 at pH 7.4. Furthermore, dye permeation influences the equilibrium value of the potassium–valinomycin diffusion potential and thus must be electrogenic; i.e., 5/6-carboxyfluorescein permeates as an anion.

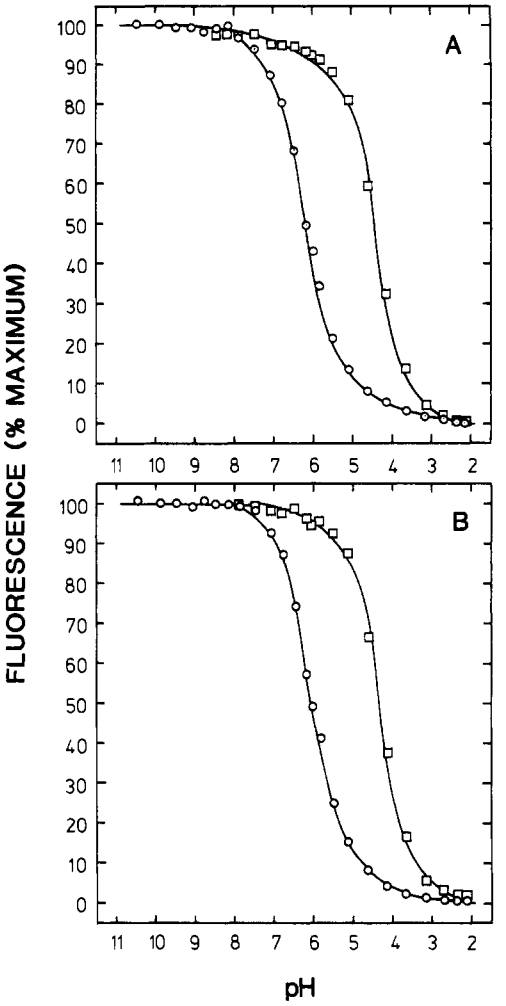

FIGURE 8: pH dependence of emission intensity of fluorescein dyes. Fluorescein derivatives were dissolved in 50 mM sodium phosphate buffers (variable pH) to give a final dye concentration of 10^{-7} M. Fluorescence emission intensity was measured at the wavelength of maximum emission (516 nm for fluorescein and 526 nm for halogenated derivatives); excitation was at 490 nm for fluorescein and 505 nm for halogenated derivatives. (A) 5/6-Carboxyfluorescein (○); 2,7-dichloro-5/6-carboxyfluorescein (□). (B) Fluorescein (○); 2,7-dichlorofluorescein (□).

pH-Dependent Ionization of Fluorescein Derivatives. Dilute aqueous solutions of fluorescein demonstrated a brilliant green fluorescence when irradiated at 490 nm; the emission maximum is at 520 nm. This fluorescence was seen only in alkaline solutions. As the pH of the dye solution was lowered below pH 7, the fluorescence intensity progressively decreased until, at pH 3, the solution was essentially nonfluorescent. Figure 8 illustrates this behavior for fluorescein and three derivatives. For both fluorescein and 5/6-carboxyfluorescein, the midpoint of the titration curve occurred at approximately pH 6.3, corresponding to the pK_a for the dissociable phenolic hydroxyl group of the xanthoyl ring system (Figure 9); this decrease in fluorescence intensity is primarily attributable to the dramatic decrease in the fluorescence quantum yield which occurs upon protonation but is also accompanied by changes in the absorption properties of the molecules (Thomas et al., 1979). Clearly, the addition of a carboxyl group at position 5/6 had little effect on the dissociation of the phenolic hydroxyl.

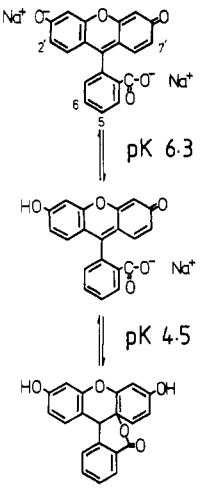

FIGURE 9: Dissociation states of fluorescein.

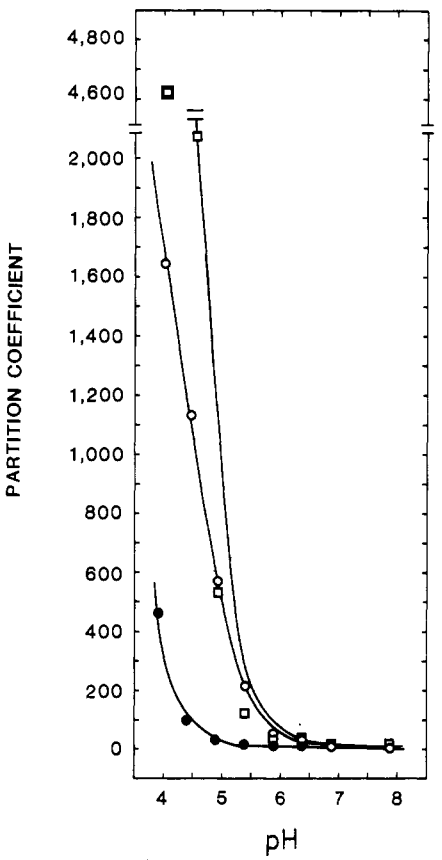

FIGURE 10: pH dependence of dye partitioning into hydrocarbon. 1-Octanol/water partition coefficients (k_p) were determined for fluorescein derivatives by fluorescence assay after extraction of aqueous solutions of dye, at various pH values, with 1-octanol. After extraction, the organic phase was made basic by the addition of a small quantity of triethanolamine, thus ensuring that fluorescence would not be affected by variations in sample pH. (●) 5/6-Carboxyfluorescein; (○) fluorescein; (□) 2′,7′-dichlorofluorescein.

Halogenation of the xanthoyl ring, however, yielding the 2′,7′-dichloro derivatives of fluorescein and 5/6-carboxyfluorescein, substantially alters the dissociation properties of the hydroxyl group, shifting the pK_a for dissociation (and hence the midpoint of the fluorescence titration curve) to approximately 4.5.

Partitioning of Dyes between Aqueous and Hydrophobic Phases and Binding to Membranes. *n*-Decane and 1-octanol are both solvents that have been used to mimic the low dielectric environment of the central domain of lipid bilayers. Figure 10 illustrates the pH dependence of octanol/water

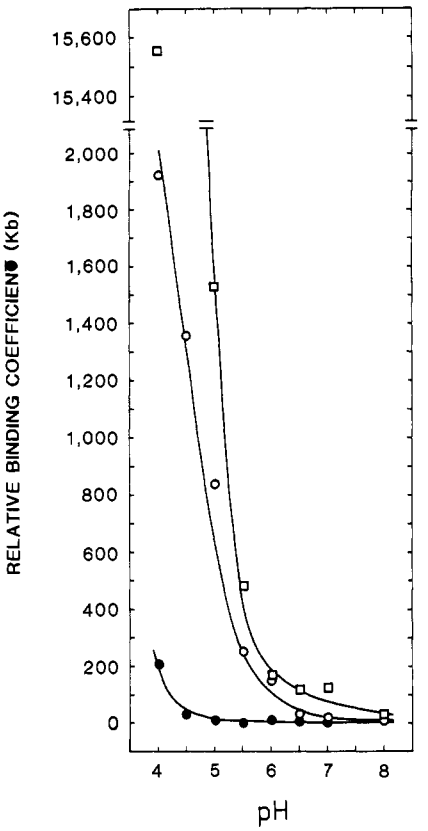

FIGURE 11: pH dependence of dye binding to membranes. Phospholipid vesicles prepared in 50 mM NaCl were placed in one chamber of a microdialysis cell; 50 mM sodium phosphate buffer, containing 10 μM fluorescent dye, was placed in the opposite chamber; the opposing chambers were separated by a 12-kDa cutoff dialysis membrane. The cells were rotated at approximately 30 rpm at 20 °C, and percentage binding of dye was determined from the ratio of fluorescence intensities in equal-volume samples from the vesicle-free (F_a) and vesicle-containing (F_v) compartments. The samples were diluted (at least 10-fold) with an aqueous solution of Na_2HPO_4 prior to measurement of fluorescence intensity in order to ensure that sample fluorescence was unaffected by variations in sample pH. The quantity of membrane-bound dye (F_m) was calculated from eq 2 (Materials and Methods). The membrane/buffer partition coefficient, k_b (binding coefficient), was calculated as the slope of the line obtained by plotting F_m/F_a against V_m/V_a, where V_m and V_a were the volumes of the membrane and aqueous phases, respectively; V_m was calculated by assuming a partial specific volume for DPPC of 1 mL/g. (●) 5/6-Carboxyfluorescein; (○) fluorescein; (□) 2′,7′-dichlorofluorescein.

partitioning for the fluorescein derivatives used in this study. Halogenation of the xanthoyl ring resulted in an increased solubility in 1-octanol, but the partitioning properties of the dyes were clearly dominated by the degree of dissociation, and lactone state, of carboxylate group(s). This same pattern was repeated in the degree to which fluorescein dyes partitioned into phospholipid bilayers (Figure 11). The dramatic reduction of solubility in octanol and phospholipid caused by the addition of a carboxylate moiety to fluorescein helps to explain why the 5/6 acid-substituted dye can be retained within sealed lipid vesicles under conditions that permit the rapid release of the unsubstituted fluorescein.

Ionization of the dye's alcohol function appeared to exert little influence on the degree to which 5/6-carboxyfluorescein associated with DPPC bilayers; however, the equilibrium dialysis techniques used to quantitate membrane-bound dye were incapable of resolving a membrane binding coefficient less than 1 ($K_b < 1$; eq 3). With pure, homogeneous phospholipid vesicles, the position and sharpness of the bilayer major gel–liquid-crystalline phase transition can give information about the presence of membrane adulterants or im-

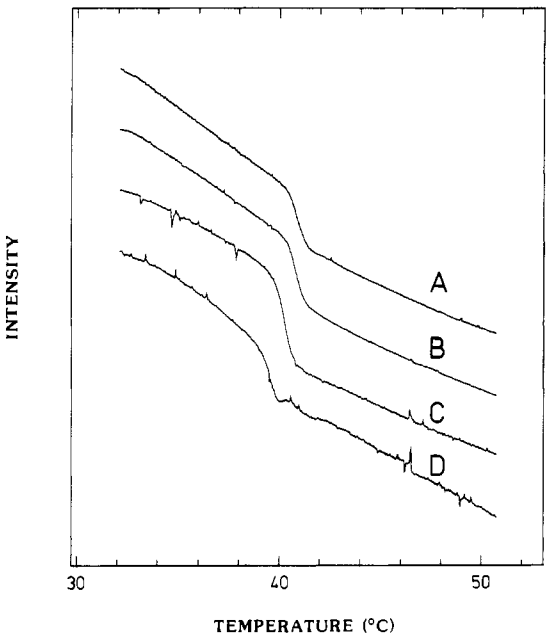

FIGURE 12: Bilayer perturbation by 5/6-carboxyfluorescein. Phospholipid vesicles, loaded with dye, were resuspended in buffer, also containing dye, to a final lipid concentration of approximately 2.5 mM and then placed in the sample compartment of a fluorescence spectrometer adjusted for detection of Rayleigh light scatter, perpendicular to the exciting radiation, at 600 nm. The light-scattering property of each sample was recorded as the sample temperature was increased from T_c − 10 °C to T_c + 10 °C at a heating rate of 0.2 °C/min. Buffer pH values for samples A, B, C, and D were 8.2, 7.8, 6.9, and 6.1, respectively.

purities. For example, if cholesterol is added to lecithin vesicles at a molar ratio of greater than 3–4 mol %, there is a noticeable broadening of the phase transition (Caruthers & Melchior, 1983). Figure 12 shows that at pH >7 even in the presence of 50 mM 5/6-carboxyfluorescein the lipid melting point occurred at 41 °C (the characteristic T_c for pure DPPC vesicles) and was relatively sharp (half-width of approximately 1 °C). This result indicated that high concentrations of the dye had little effect on bilayer organization above neutral pH and that $K_b < 1$.[4] As the pH was lowered, there was a progressive increase in the degree of membrane disruption as more and more dye became membrane bound, and at pH <6.5, the melting point of DPPC vesicles was considerably broadened and shifted to lower temperatures. The clear implication is that, provided the internal pH is 7.4 or above, the presence of high concentrations of trapped 5/6-carboxyfluorescein is unlikely to exert a substantial perturbing influence on the phospholipid packing in the boundary membrane of unilamellar DPPC vesicles.

Discussion

It is apparent that flux of 5/6-carboxyfluorescein across sealed membrane systems can, under appropriate conditions, become entrained to or regulated by coincident cation symport or anion antiport. Because, in general, membrane ion conductance is low for homogeneous lipid bilayers, this type of regulation would explain why a relatively hydrophobic dye can be trapped in lipid vesicles in spite of its intrinsic readiness to diffuse across the membrane barrier. The additional observation that different counterions result in different temperature profiles for dye efflux rates leads us to conclude that the pattern of behavior seen in the presence of sodium counterions reflects the temperature dependence for sodium ion permeation. Indeed, the behavior exhibited by the dye is qualitatively very similar to the known temperature dependence of sodium ion permeation across saturated lecithin bilayers, as determined by a number of different techniques including equilibrium dialysis, dilatometry, and isotope exchange (Papahadjopoulos et al., 1973; Blok et al., 1983; El-Mashak & Tsong, 1985).

Quantitatively, the rates of dye release appear to be much greater than those anticipated for sodium ion efflux, although we have made no attempt to calculate permeability coefficients or permeation rate constants from our data; in this report, we are primarily concerned with relative, rather than absolute, ion permeation rates. However, in our experimental system, there is a large transmembrane diffusion potential, created from the chemical potential gradient of anionic dye, which must increase the electrochemical potential of trapped cations and may even modify the membrane permeability properties (El-Mashak & Tsong, 1985), leading to rates of sodium permeation much greater than those seen under simple exchange–diffusion conditions (Figure 6). Nevertheless, the qualitative similarities between the permeation behavior of sodium ions in the presence and absence of large dye diffusion potentials suggest that the nature of the principal barrier to ion permeation is not apparently influenced by the existence of ΔE_m, although the actual height of the barrier may be lowered significantly, and that sodium ion permeation occurs by the same general mechanism whether it is driven by an electrochemical gradient, by a chemical potential, or by thermal energy at equilibrium.

Because of our observation that fluorescein appears to escape counterion coupling (presumably because it is able to permeate as a neutral solute), we have concluded that the 5/6-carboxyl substitution is responsible for the permeation of 5/6-carboxyfluorescein as a univalent anion, leading to dramatically lower dye leakage rates, and regulation by counterion flux. Certainly, the partitioning and membrane binding studies reveal that protonation of the fluorescein alcohol function seems to be of minor importance, relative to the number and state of dissociation of the dye carboxyl groups, in determining the ease with which fluorescein derivatives may penetrate hydrophobic barriers (Figures 8–11).

Clearly, because of the fact that 5/6-carboxyfluorescein can assume an electrically neutral configuration when fully protonated, there is the possibility (becoming more probable as the pH is lowered) of neutral dye permeation across membrane barriers, thus escaping regulation by counterion flux. Under the experimental conditions reported here, the concentration of neutral dye molecules within the vesicle lumen was generally less than the total concentration of dye by a factor of 10^9. The fact that each vesicle contained, on the average, fewer than 10^6 dye molecules makes neutral dye permeation an extremely improbable event, and the observed pH dependence of dye release kinetics is presumed to be a consequence of the pH dependence of the relative concentration of monovalent dye anion in aqueous solutions of 5/6-carboxyfluorescein. The same logic probably applies to dyes such as phenol red (Bra-

[4] Because 5/6-carboxyfluorescein is amphiphilic, it seems reasonable to assume that membrane-bound dye is located at the membrane/water interface rather than being dissolved in the hydrophobic central domain of the bilayer; in this respect, the dye would resemble cholesterol. For the generation of the Rayleigh scatter profiles displayed in Figure 5, small DPPC vesicles (2 mg) were suspended in aqueous buffer (2 mL) containing 50 mM 5/6-carboxyfluorescein. If the membrane/buffer partition coefficient for the dye was unity (K_b = 1), then the concentration of dye in the membrane phase would also be 50 mM, implying a dye:phospholipid molar ratio of approximately 4%, sufficient to cause an appreciable shift in T_c.

ganza et al., 1983) and perhaps even to calcein (Kendall & MacDonald, 1982), which are also readily retained within lipid vesicles. The intrinsic temperature-dependence profile for permeation of 5,6-carboxyfluorescein itself (in the absence of any counterion restriction) remains to be determined.

Initial rates of dye leakage do not appear to be influenced by the osmotic gradients created when solute is lost from the lumen of sealed vesicles. We had originally considered the possibility that water loss from a vesicle, consequential to the loss of dye and counterion, could lead to shrinkage of the vesicle and distortion of the bilayer structure, even though, in general, small vesicles do not act as osmometers (Johnson & Buttress, 1973). DPPC vesicles may be a singular exception (Lichtenberg et al., 1981); however, the presence of an highly osmotic buffer (850 mOsm sucrose) on either side of the boundary bilayer of DPPC vesicles failed to influence initial dye leakage rates to any significant extent, and rapid loss of trapped fluorescein (as opposed to 5/6-carboxyfluorescein) appeared to exert very little influence on the coincident efflux of ^{22}Na ions (Figure 6), again suggesting that osmotic forces play little role in the processes under discussion here.

The data presented in Figure 1 suggest that dye permeation rates steadily increase with temperature but that there is a pronounced discontinuity, superimposed on this behavior, which results in a transient peak of dye flux coinciding with the melting point of the bilayer. The molecular explanation for such permeation discontinuities at T_c is still under debate [for a review, see Deamer and Bramhall (1986)]. In Figure 1, the discontinuity is centered at T_c (41 °C for DPPC), but it extended over a very broad temperature range, being detectable for at least 10 °C above and below T_c. Attributing increased dye/ion conductance at T_c to the consequences of lateral phase separation (Papahadjopoulos et al., 1973; Marsh et al., 1976; Kanehisa & Tsong, 1978) fails to account, in a satisfactory manner, for this very broad temperature range (in excess of 20 °C) over which conductance "anomalies" can be detected.

Conversely, interpreting the peak in permeation rate at T_c as the result of violent variations in the lateral compressibility of the bilayer head-group domain at T_c (Doniach, 1978; Nagle & Scott, 1978; Mitaku et al., 1978; Harkness & White, 1978) would not explain why 5/6-carboxyfluorescein should experience greater difficulty than fluorescein in penetrating the bilayer head-group region when the effective Stokes radii of the two solutes are so similar. The fact that these radii are greater than that of a hydrated sodium ion also argues against simple oscillations in head-group spacing being the rate-determining barrier and arbiter of selectivity for ion permeation (El-Mashak & Tsong, 1985).

It is generally accepted (although by no means obligatory) that ligands, with the singular exception of protons (Bramhall, 1987), permeating across phospholipid bilayers must become, at some point, immersed in a low dielectric medium; indeed, the close relationship between permeation rates and oil–water partition coefficients, which holds for several different groups of permeant solutes, was instrumental in the formulation of the currently accepted models of membrane structure. The emphatic dependence of dye permeation rates on acyl chain composition firmly places the hydrophobic domain of the acyl chains themselves as the primary barrier to counterion penetration. Although it is tempting to relate this dependence to the effects of membrane "dielectric thickness" (Vodyanoy & Hall, 1984), there is substantial experimental evidence to suggest that the mean distance separating opposing glycerol moieties does not change much in response to alterations in acyl chain length (Cornell & Separovic, 1983). Furthermore, when bilayer thickness *is* changed substantially, for example by passing through the lipid phase transition, the effects of this change are not reflected in the corresponding rates of ion permeation. As shown in Figure 1, the dye leakage rates across DPPC bilayers are essentially the same at 5 °C above T_c as they are at 5 °C below T_c even though membrane thickness decreases by at least 50% upon passing above the phase transition temperature (Watts et al., 1978). Thus, not only does the underlying exponential relationship between temperature and permeation rate appear to be independent of bilayer phase state but also ion conductance seems to correlate rather poorly with the actual (geometric) thickness of the bilayer, although, for fully saturated systems, there certainly is a strong inverse relationship between the number of carbon atoms in a fatty acid chain and the degree of permeability to ions such as sodium. This raises the possibility that limits to counterion permeation rates, rather than being related to the energy acquired by a charged particle by virtue of its distance from the membrane/water interface (Neumcke & Läuger, 1969), might instead be dominated by the necessity for the ion to traverse a tortuous path defined by the acyl chains. The suggestion that solute permeation occurs through regions of free space within the bilayer created as a result of β-coupled configurations (kinks) that move along the acyl chains (Träuble, 1971) may provide a reasonable mechanical explanation for slower ion penetration of bilayers composed of lipids with long acyl chains.

Registry No. DLPC, 18285-71-7; DMPC, 13699-48-4; DPPC, 63-89-8; DSPC, 4539-70-2; DAPC, 71259-34-2; Tris, 77-86-1; Cho^+, 62-49-7; Glu^-, 526-95-4; Na, 7440-23-5; NH_3, 14798-03-9; Cl^-, 16887-00-6; F^-, 16984-48-8; Br^-, 24959-67-9; 5/6-carboxyfluorescein, 72088-94-9; *N*-methylglucamine, 6284-40-8.

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Influence of Immune Complexes on Macrophage Membrane Fluidity: A Nanosecond Fluorescence Anisotropy Study†

Howard R. Petty,* Charles D. Niebylski, and Joseph W. Francis
Department of Biological Sciences, Wayne State University, Detroit, Michigan 48202
Received October 24, 1986; Revised Manuscript Received May 29, 1987

ABSTRACT: Time-resolved fluorescence anisotropy (TRFA) and steady-state anisotropy measurements and fluorescence intensification microscopic observations were made on RAW264 macrophages labeled with 1,6-diphenyl-1,3,5-hexatriene (DPH) or 1-[4-(trimethylammonio)phenyl]-6-phenyl-1,3,5-hexatriene (TMA-DPH). Microscopic analysis revealed that the fluorescent probe DPH was found in association with plasma membranes and small vesicles. Macrophages treated with immune complexes could not be distinguished from untreated cells, indicating that the same membrane compartments were labeled. The probe TMA-DPH was exclusively localized to the plasma membrane. Steady-state anisotropy measurements indicated that in vitro culture conditions did not significantly affect membrane fluidity. TRFA measurements were conducted to determine the physical properties of macrophage membranes during immune recognition and endocytosis. Data were analyzed by iterative deconvolution to yield ϕ, the rotational correlation time, and r_∞, the limiting anisotropy. These parameters may be interpreted as the "fluidity" and order parameter of the membrane environment, respectively. Typical values for untreated macrophages were ϕ = 7.8 ns and r_∞ = 0.12. Binding and endocytosis of immune complexes prepared in 4-fold antigen excess increase these values to ϕ = 22.1 ns and r_∞ = 0.15. However, receptor-independent phagocytosis of latex beads decreases these values to ϕ = 2.2 ns and r_∞ = 0.10. Addition of catalase before, but not after, immune complex incubation with cells diminishes the effect upon membrane structure, suggesting that H_2O_2 participates in fluidity changes. Pretreatment of macrophages with the membrane-impermeable sulfhydryl blocker *p*-(chloromercuri)benzenesulfonic acid also diminished these effects. Cell-free and immune complex free supernatants of immune complex treated macrophages can decrease the fluidity of resting macrophages. Arachidonic acid at 10 μM increased membrane fluidity while prostaglandin E2 at 10 nM decreased fluidity. Our results indicate an important role of oxidizing agents, sulfhydryl residues, and soluble factors in controlling macrophage membrane fluidity during immune recognition. These factors may account for as much as 98% of the change induced by immune complexes.

The macrophage cell surface is a crucial interface linking molecular immune recognition to the triggering of effector functions. Antibody-dependent recognition triggers a broad spectrum of cellular responses including phagocytosis, cytolysis, release of lysosomal enzymes and mediators such as prostaglandin E2, and release of reactive oxygen metabolites. The molecular membrane events surrounding these functional changes are just beginning to be understood. Murine macrophages possess at least two distinct cell surface receptors for the Fc domain of IgG; FcR2a[1] is specific for IgG2a while FcR2b binds IgG2b (Unkeless, 1977). In the case of IgG2b recognition, the CH2 region of the Fc domain interacts with the FcR2b (Diamond et al., 1985). The IgG2b receptor is a M_r 50 000 glycoprotein (Mellman & Unkeless, 1980). Upon

†This work has been supported by NSF Grant PCM-8207838, by NSF Biological Instrumentation Grant PCM-8313893, and by a grant from the Children's Leukemia Foundation of Michigan.

[1] Abbreviations: DPH, 1,6-diphenyl-1,3,5-hexatriene; TMA-DPH, 1-[4-(trimethylammonio)phenyl]-6-phenyl-1,3,5-hexatriene; TRFA, time-resolved fluorescence anisotropy; db-cAMP, N^6,2′-*O*-dibutyryladenosine cyclic 3′,5′-phosphate; FcR2a, Fc receptor for IgG2a; FcR2b, Fc receptor for IgG2b; PCMBSA, *p*-(chloromercuri)benzenesulfonic acid; HBSS, Hank's balanced salt solution; PBS, phosphate-buffered saline; EDTA, ethylenediaminetetraacetic acid; BSA, bovine serum albumin; DMPC, dimyristoylphosphatidylcholine.

0006-2960/87/0426-6340$01.50/0