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ELECTROSTATIC FORCES CONTROL THE PENETRATION OF MEMBRANES BY CHARGED SOLUTES

JOHN BRAMHALL

Department of Microbiology and Immunology, UCLA School of Medicine, Los Angeles, CA 90024 (U.S.A.)

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Using fluorescent, anionic dyes such as carboxyfluorescein as model solutes, it is shown that the forces allowing such solutes to be retained within sealed lipid vesicles, against a large concentration gradient, can be primarily electrostatic in nature. At temperatures distant from that of the ordered-fluid lipid phase transition a small number of the anionic dye molecules trapped within lipid vesicles are capable of traversing the lipid bilayer and establishing an electrical diffusion potential across the membrane. Further solute movement can then only occur with the concomitant permeation of ions which restore electrical balance. A significant flux of dye can be triggered by (a) increasing the permeability of the membrane to ions (for example by the addition of ionophores such as gramicidin, or by allowing the lipid to approach a phase transition) or by (b) adding lipophilic counterions such as tetraphenylborate or dinitrophenol to the system.

Introduction

The permeabilities of simple lipid membranes towards ions, sugars, soluble spin labels and polycyclic dyes have all been demonstrated to display a sharp maximum at a temperature coincident with the major gel to liquid-crystalline phase transition of the membrane (T_c). This behavior has been explained, in the case of ions, by theories which postulate that membrane permeability is proportional to the lateral compressibility of the lipid matrix, which is itself maximal at T_c [1–3]. While these theories convincingly describe molecular events which could account for the behavior of small ions they do not adequately explain the critical changes in permeability at T_c which are observed for comparatively large molecules [4,5].

In this report it is demonstrated that the principal factor retarding the rate of permeation of charged amphiphilic solutes, such as some drugs and dyes, across lipid bilayers is not the energy required for the penetration of the hydrophobic core of the membrane but rather the electrostatic field generated across the bilayer as a consequence of the solute's own diffusion potential. Thus, one explanation of the critical behavior of charged solutes such as the common dyes carboxyfluorescein or aniline naphthalenesulphonic acid (ANS) is that the rate of dye permeation becomes dependent on the rate of co-permeation of small counterions.

Materials

5(6)-Carboxyfluorescein supplied by Molecular Probes Inc. (Junction City, OR) was purified by acid precipitation prior to use. A stock solution of 50 mM carboxyfluorescein in distilled water was adjusted to pH 7.6 with NaOH. 8-Anilino-1-naphthalenesulfonate (ANS) was supplied by Serva

Abbreviations: DMPC, dimyristoylphosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; Dns-, dansyl, 5-dimethylaminonaphthalene-1-sulfonyl; ANS, 8-anilino-1-naphthalenesulfonate; CCCP, carbonyl cyanide *m*-chlorophenyl hydrazone.

(Heidelberg, F.R.G.) and was used without further purification. [^{3}H]Lactose (1.2 Ci · mmol^{-1}) was supplied by CEA (Paris, France). Sodium tetraphenylborate and ammonium acetate were supplied by Aldrich (Milwaukee, WI); 2,4-dinitrophenol and carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP) were supplied by Sigma (St. Louis, MO).

Dns-glucosamine was prepared using a modification of the general method of Hartley and Massay [6]. Gucosamine (Schuchardt, Munich) (22 mg, 0.1 mmol) was dissolved in 3.5 ml 0.1 sodium carbonate buffer (pH 10.0). Dansyl chloride (Sigma, St. Louis) (30 mg, 0.11 mmol) was dissolved in 3.5 ml acetone. The two solutions were combined and stirred, in the dark, for 1 h at 20°C. The reaction mixture was evaporated to dryness under reduced pressure and resuspended in 100 ml chloroform/methanol (2:1, v/v). This suspension was filtered through anhydrous sodium sulfate, and the filtrate was concentrated by evaporation under reduced pressure. This material was fractionated by preparative thin-layer chromatography on silica gel using chloroform/methanol (2:1, v/v) as the developing solvent. The pure product moved to R_f 0.53. Dns-choline was prepared from 2-aminoethyltrimethylammonium chloride (Sigma, St. Louis) using the above procedure. The pure product moved to R_f 0.14.

The purity of the phospholipids DMPC and DPPC supplied by Fluka (Buchs, Switzerland) was verified by thin-layer chromatography on silica gel with a developing solvent of chloroform/methanol/glacial acetic acid/water (90:40:12:2, v/v). The buffer used throughout this study was 50 mM sodium pyrophosphate adjusted to pH 7.6 with 1 M citric acid.

Methods

Small unilamellar lipid vesicles were prepared by sonication of lipid suspension in aqueous dye solution under an atmosphere of nitrogen. Typically, 5 mg DMPC were sonicated in 0.5 ml dye solution (50 mM, 5(6)-carboxyfluorescein adjusted to pH 7.6 with NaOH) containing trace quantities of [^{3}H]lactose. Lipid aggregates and titanium debris were removed by centrifugation at 100 000 × *g* for 30 min, and the small vesicles were annealed for 30 min at 30°C. This preparation was slowly cooled to 4°C before being loaded onto a 1.5 × 24 cm Sephadex G-50 column equilibrated with 50 mM citrate/pyrophosphate buffer (pH 7.6) at 4°C. The vesicles, containing trapped dye, eluted with the void volume; free dye was retarded.

Steady-state and slow kinetic fluorescence measurements were performed on a Spex Fluorolog II spectrofluorometer equipped with temperature control accessories and magnetic stirrer. Rapid kinetic measurements were obtained with a conventional stopped-flow apparatus equipped with gas-pressured syringes and temperature-control accessories. The illumination source was a 200 W mercury-xenon compact arc lamp (Hanovia) powered by a stabilized supply unit (Heinzinger TNX 250). Exciting radiation was passed through a heat filter and a dispersal grating monochromator (Bausch & Lomb 33-86-01). Emitted radiation went through an appropriate high-frequency cut-off filter to an end-window photomultiplier tube. The output from the detection photomultiplier was fed to a time-base transient recorder (Physical Data 514A) and then either to an oscilloscope display, or to a data processor (Tektronix 4051) via an appropriate interface (Physical Data IEEE 488). Hard copies of the recorder output were obtained from a copy terminal (Tektronix 4631).

Equilibrium measurements of dye leakage were conducted using plastic multiwell dialysis chambers. Vesicles were prepared as described above, but containing 4 mM carboxyfluorescein in 50 mM citrate/pyrophosphate buffer (pH 7.6), with trace quantities of [^{3}H]lactose. Portions (250 μl) of this vesicle preparation containing dilute dye were added to polypropylene centrifuge tubes (1.2 ml capacity) previously equilibrated to 4°C and containing 10 μl of aqueous or ethanolic stock solutions of test reagents. The mixtures were thoroughly agitated, and maintained at 4°C for 30 min. 100-μl portions (in duplicate) were removed from each sample with a cold glass syringe and injected into plastic multiwell dialysis chambers. Each sample was dialysed against 100 μl 50 mM citrate/pyrophosphate (pH 7.6) buffer for 10 h at 4°C with continuous slow agitation. Following this dialysis, duplicate 25-μl samples were removed from both sample and dialysate chambers, and either assayed for [^{3}H]lactose content by liquid

scintillation counting, or for dye content by fluorescence assay. Percentage release of dye and lactose was calculated for each sample.

Results and Discussion

An extensive study of an homologous series of fluorescent solutes has revealed a major difference between the translocation kinetics of charged and electrically neutral amphiphiles which otherwise have very similar structural characteristics. These marked differences in behavior are exemplified in Fig. 1, which shows permeation rates of amphiphilic fluorescent dyes through bilayers of vesicles made from DMPC. Using a conventional stopped-flow apparatus, an aqueous dispersion of small unilamellar DMPC vesicles was rapidly mixed with a solution of an amphiphilic fluorescent dye, (a) Dns-galactoside or (b) Dns-choline

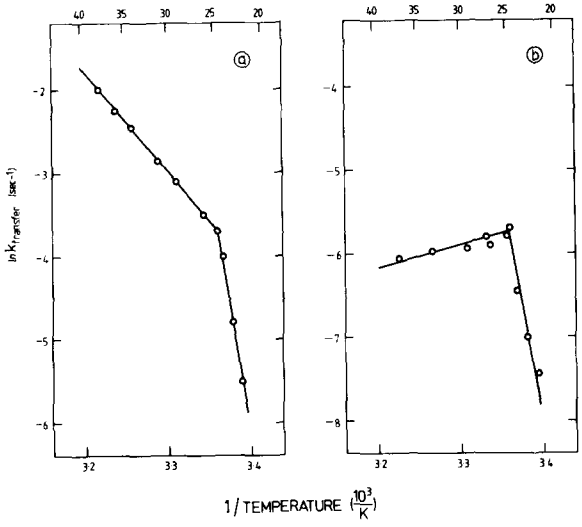

Fig. 1. Permeation of amphiphilic dyes across phosphatidylcholine bilayers. Using a conventional stopped-flow apparatus, an aqueous dispersion of small unilamellar DMPC vesicles was rapidly mixed with a solution of an amphiphilic fluorescent dye (Dns-galactoside, a; or Dns-choline, b). Fluorescence intensity was monitored during mixing (dead time less than 7 ms) and continuously thereafter until equilibrium had been attained. The equilibration half-time varied between 5 s and 90 min according to the nature of the components and conditions. The data was plotted to a first order rate equation to yield a rate constant for translocation/transfer across the bilayer. These permeation rates were evaluated over a range of temperatures, and are presented here in the form of an Arrhenius plot. The molar lipid : dye ratio was 100 : 1.

over a range of temperatures. By a fast diffusion-controlled process the dye molecules attain a partition equilibrium between water and the outer surface of the vesicles. This causes a rapid increase in fluorescence intensity because the efficiency of fluorescence is greater when the dyes are located in an environment of low dielectric constant. In a second step, the inner surface of the vesicle becomes populated with dye molecules. This process involves permeation of the dye through the bilayer, and is slow. The accompanying increase in fluorescence intensity is detected, and the permeation rate is evaluated. The sharp maximum in translocation rate at $T = T_c$, seen with Dns-choline (cationic), is similar to that observed previously with charged spin labels [4] and polycyclic dyes such as ANS [5,7,8]. The neutral dye Dns-galactoside shows no such maximum, and permeation rates increase continuously with temperature. Earlier attempts to rationalize this behavior [9] as being the result of lipid cluster formation at T_c do not explain why dyes having very similar structural dimensions, differing only in their charge configuration, should display such dramatically different permeation behavior. If, however, it is postulated that the rate of permeation of these solutes is limited by the rate of co-permeation of counterions which maintain (or restore) electrical balance across a membrane, then a perfectly satisfactory explanation of the observations can be presented.

Net movement of a small number of molecules of a charged solute, such as ANS, across a lipid layer, in the absence of a parallel movement of counterions, will result in a separation of charges across the membrane and the formation of a trans-membrane potential ($\Delta\psi$). This potential difference across the bilayer, by creating a large electrostatic field across the two faces of the membrane, opposes further net movement of charged solutes. Significant translocation of ANS across vesicle membranes should only occur if (a) the permeability of the membrane towards counterions (e.g., sodium) is increased; or (b) lipophilic counterions, which can permeate freely across the bilayer in exchange for ANS anions, are added to the system. (The geometry and dielectric properties of a small unilamellar vesicle assure that movement of a very small number of charged

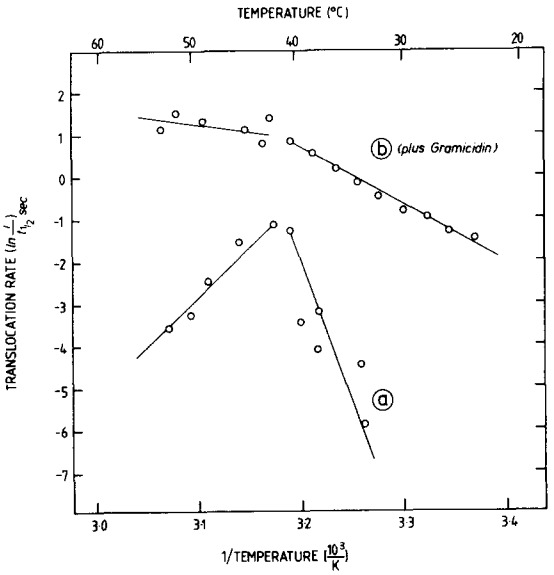

Fig. 2. The effect of gramicidin on the rate of translocation of ANS across phosphatidylcholine bilayer. ANS (final concentration $5 \cdot 10^{-6}$ M) was mixed with a suspension of DPPC vesicles ($5 \cdot 10^{-5}$ M lipid), suspended in buffer, using rapid-mixing, stopped-flow techniques described under Methods and in Ref. 5. The half time for translocation of ANS across the vesicle bilayers was determined at the indicated temperatures, and the data are presented here in the approximate form of an Arrhenius plot. Unlike the translocation kinetics of the dansylated compounds shown in Fig. 1, which very closely approximated first order, ANS translocation showed a more complicated kinetic pattern. Hence, the use of equilibration half-time as an operational parameter of initial translocation rates. In plot b the results were obtained with lipid vesicles that had been pretreated with gramicidin (final concentration $1.3 \cdot 10^{-6}$ M).

molecules across the membrane bilayer will result in an electrostatic field large enough to compensate for the concentration gradient of solute.) The results of two series of experiments designed to test this hypothesis are described below.

In the first series of experiments the anionic dye ANS was rapidly mixed with sealed lipid vesicles over a range of temperatures, and the subsequent movements of the dye across the vesicle membranes were monitored by fluorescence spectroscopy. Fig. 2a illustrates the temperature dependence of the rate of ANS translocation across these vesicle membranes, and confirms that this rate achieves a sharp maximum at a temperature coinciding with the T_c for the lipid. A similar effect was observed in an earlier, careful, study by Tsong using DMPC membranes [5]. Fig. 2b indicates that when the vesicles were pre-incubated with gramicidin (an antibiotic which forms sodium channels in lipid bilayers) not only did the rate of translocation increase at all temperatures, but, significantly there was no longer a rate maximum at T_c. Instead, the translocation kinetics appeared very similar to those observed previously for the electrically neutral dyes such as Dns-galactoside. This change in temperature profile suggests that, in the absence of gramicidin, ANS translocation is rate-limited by the co-permeation of sodium ions; consequently, the rate maximum at T_c for ANS merely reflects the well-characterized temperature dependence of sodium permeation kinetics [10]. Hence, ascribing activation energies, derived from line slopes in which Arrhenius plots, to the processes of dye translocation [5,9] is meaningless.

The pore-former gramicidin wa used in these experiments, rather than a mobile anion or anion carrier, to eliminate the possibility that the increases in permeation rate of ANS could be caused by complex formation with a hydrophobic carrier, such as had been observed previously with valinomycin [9]. It is unlikely that the increase in

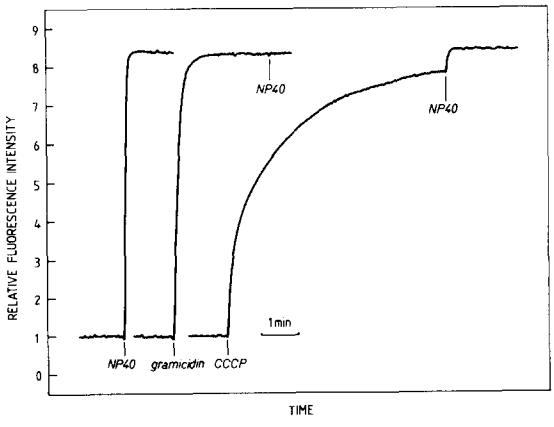

Fig. 3. Triggering of dye releae from sealed vesicles. DMPC vesicles were suspended in 2 ml 50 mM sodium pyrophosphate (pH 7.6) equilibrated at 8°C in a stirred cuvette housed in the sample compartment of a SPEX spectrofluorometer. The vesicles contained a self-quenching concentration (50 mM) of carboxyfluorescein. The fluorescence intensity of each sample was recorded before and after the indicated treatment. The increases in fluorescence intensity correspond to release of dye from the vesicles. Final lipid concentration was $1.3 \cdot 10^{-4}$ M; final concentrations of Nonidet P-40, gramicidin and CCCP were 0.1%, $1.3 \cdot 10^{-6}$ M and 10^{-4} M, respectively.

the rate of ANS translocation resulted from a general disturbance of the lipid matrix caused by the presence of gramicidin, since the antibiotic did not affect the translocation rate of the neutral dye DNS-galactoside across identical DMPC bilayers (data not shown), not did it alter the membrane T_c.

A wide variety of compounds can be trapped within lipid vesicles, and retained against large concentration gradients. The level of retention depends upon the rate at which the entrapped compounds 'leak' out of the vesicles. Rates of leakage are largely a function of each compound's rate of translocation across the lipid bilayer. Thus, in the second series of experiments, the water-soluble dye 5(6)-carboxyfluorescein was used to determine the conditions required to stimulate the release of amphiphilic charged solutes entrapped within lipid vesicles. Small unilamellar lipid vesicles were prepared by sonication of lipid suspensions in aqueous dye solution under an atmosphere of nitrogen.

When the sodium salt of 5(6)-carboxyfluorescein was trapped within sonicated lipid vesicles, and these were stored at 4°C, a temperature well below the lipid's T_c, very little leakage of the dye occurred, even though the concentration gradient of dye across the membrane was high. The results obtained with ANS described above, supported the hypothesis that significant leakage of dye anion or any other charged amphiphile, from lipid vesicles should only occur with the concommitant leakage of counterions such as sodium ions. Therefore, increasing the permeability of the membrane specificially to sodium ions should result in an increase in the rate of leakage of carboxyfluorescein. As shown in Fig. 3, this prediction was confirmed. A 20 μl portion of the vesicle preparation was diluted into 2 ml buffer equilibrated at 8°C in a stirred cuvette housed in the sample compartment of a spectrofluorometer. The fluorescence intensity of each sample was recorded for 1 min before the indicated treatment, and over a period of time after treatment. The rate of increase in fluorescence intensity corresponds to the rate of release of dye from the vesicles. The initial high concentration of dye trapped within the lipid vesicles is self-quenching [11] and displays no fluorescence emission intensity. However, addition of the detergent Nonidet P-40 causes immediate lysis of the vesicles, and release of dye into the external medium. This dilute solution of carboxyfluorescein now displays a brilliant green fluorescence. Addition of the sodium channel-forming antibiotic gramicidin (to a final concentration of 1.3 μM) also promoted release of dye from the vesicles, whereas the addition of the potassium ionophore valinomycin, even in the presence of potassium ions, failed to stimulate dye release (data not shown) presumably because the rate of trans-membrane diffusion for this mobile carrier is extremely slow at the experimental temperature, 14 K below T_c [12].

If retention of trapped carboxyfluorescein within lipid vesicles is indeed the result of electrostatic forces, then it should be possible to induce a stoichiometric release of dye by allowing another anionic species to enter the vesicle freely as leaves.

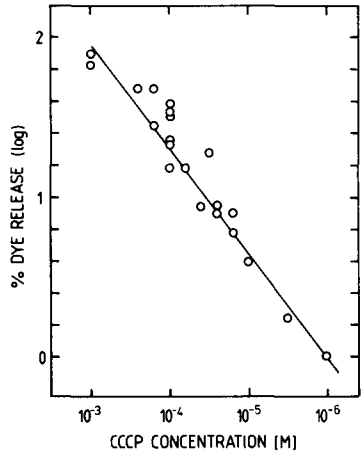

Fig. 4. Stoichiometric release of dye triggered by CCCP. The percentage release of carboxyfluorescein provoked by equilibration with varying concentrations of CCCP in the external medium. Portions (250 μl) of a DMPC vesicle preparation, containing dilute dye (4 mM) and trace quantities of radioactive lactose, were added to polypropylene centrifuge tubes (1.5 ml capacity) previously equilibrated to 4°C and containing 5 μl from stock dilutions of CCCP in ethanol. The mixtures were dialyzed against buffer for 10 h at 4°C with continuous slow agitation, as described under Methods. Following this dialysis, 25-μl samples were removed, diluted to 3 ml with distilled water, and assayed for dye fluorescence. Concentrations of CCCP represent the final concentration of the ligand in the dialysis chambers. Percentage release of dye, and of [^{3}H]lactose were calculated for each sample, values from duplicates were averaged. The results are displayed in the logarithmic plot above, correlation coefficient 0.87 (significance $\gg P = 0.01$). There was no significant release of [^{3}H]lactose under any of the conditions used in this experiment.

Fig. 3 shows that when the hydrophobic anion CCCP was added to the external medium dye release was triggered, as predicted. Equilibrium dialysis measurements using lipid vesicles loaded with a low (4 mM) concentration of carboxyfluorescein and trace quantities of radioactive lactose showed that the addition of a number of different hydrophobic anions including tetraphenylborate and 2,4-dinitrophenoxide, all induced dye release without causing a coincidental release of trapped lactose.

The fact that the lactose remained inside the vesicles indicates that they remained intact throughout the experiment, and that dye was being released by permeation through the membrane rather than by lysis of the vesicles. It is significant to note that ammonium acetate failed to elicit dye release since this is strong evidence against the involvement of a trans-membrane pH gradient in dye retention. Phospholipid bilayers are extremely permeable to ammonium acetate [13] and ammonium chloride [14]. Addition of these salts will efficiently collapse pH gradients in existence across phosphatidylcholine bilayers, even at temperatures well below T_c.

To determine whether CCCP was acting stoichiometrically, or playing a catalytic role, it was tested over the concentration range 10^{-6} to 10^{-3} M. The results, shown in Fig. 4, demonstrate that the amount of dye released is directly proportional to the concentration of CCCP in the external medium. In these experiments the system was allowed to attain equilibrium before measurements of dye release were made. If CCCP had been acting as a catalyst, facilitating proton transport across the bilayer by virtue of the membrane permeability to both the fully protonated and fully dissociated forms of CCCP, then we might expect that varying its concentration would change the rate of equilibration but not the nature of the equilibrium itself.

Similar results were obtained with the other hydrophobic anions such as dinitrophenol and tetraphenylboron, in sub-lytic concentration ranges. The fact that CCCP seems not be acting in a ctalytic role, as a proton translocator, again strongly suggests that dye retention is not maintained by a trans-membrane pH gradient which would develop during net permeation of the protonated form of a dissociable compound such as carboxyfluorescein.

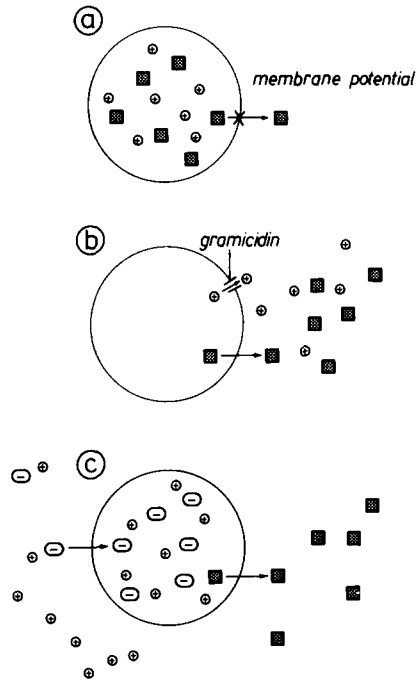

Fig. 5. Mechanism of dye release and retention. (a) The leakage of carboxyfluorescein from lipid vesicles is prevented because of the creation of the trans-membrane diffusion potential ($\Delta\psi$) resulting from the difference between the permeation rates of the dye and its counterion (sodium). (b) Release of dye can be brought about by the addition of gramicidin to the vesicle bilayer (thus increasing the permeability of the membrane towards sodium ions) or (c) by the addition of lipophilic counterions (such as tetraphenylborate) which continuously collapse $\Delta\psi$ and allow the steady release of carboxyfluorescein.

Using model systems, then, experimental results show that the forces allowing certain solutes to be retained within sealed lipid vesicles, against a large concentration gradient, can be primarily electrostatic in nature, with solute permeation being directly coupled to the concommitent permeation of counterions which restore electrical balance. This is shown diagramatically in Fig. 5. One would expect the same type of electrostatic coupling to exist in a steady-state equilibrium maintained for example by the action of an ion- or proton-translocating pump. Such coupling may be involved in several biologically relevant systems such as storage organelles (e.g., the chromaffin granules of the adrenal medulla, or the presynaptic vesicles of cholinergic nerve terminals) where there is clear

evidence that the organelle membranes display intrinsic permeability to the charged solutes (catecholamines and acetylcholine) which are accumulated and stored [15–17]. Similar coupling is probably involved in the specific cellular exclusion or uptake of charged dyes (e.g., the exclusion of Trypan blue from living cells, or the voltage-dependent uptake of carbocyanine dyes) and also in the uptake of drugs such as methochlorpromazine, or anesthetics such as lidocaine [18,19].

What are the practical consequences of these findings? In view of the considerable interest currently being shown in the use of liposomes as drug delivery systems [20], it is critical to appreciate why some agents are capable of entrapment while others are not. Also, vesicles containing trapped dyes as markers are used routinely in studies of membrane lysis [21] and fusion [22]. The results of this study suggest that when the marker solute is an amphiphilic dye like carboxyfluorescein, there may well be a large trans-membrane potential in existence across the membranes of such vesicles which may have an important effect on subsequent events such as membrane fusion or protein insertion into the bilayer. Finally, the experimental procedures described here provide an extremely rapid method for measuring the relative rates at which anionic solutes will permeate across lipid bilayers of defined composition, and for identifying quickly, without the use of radioisotopes, agents which modify the ion permeabilities of membranes; e.g., toxins and certain classes of anesthetics.

Acknowledgments

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