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Incorporation of Dansylated Phospholipids and Dehydroergosterol into Membranes Using a Phospholipid Exchange Protein†

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ABSTRACT: A nonspecific phospholipid exchange protein (PLEP) preparation was used to transfer dansyl[^{3}H]phosphatidylethanolamine (DNS-PE), dansyl[^{3}H]phosphatidylserine (DNS-PS), and dehydroergosterol (DHE) from sonicated lipid vesicles to electroplax plasma membrane fragments enriched in Na^+,K^+-ATPase with retention of 80–90% of Na^+,K^+-ATPase activity. The transfer of individual fluorescent lipid molecules was distinguished from a nonspecific association of lipid vesicles and membranes by including [^{14}C]triolein, a lipid that is not transferred by PLEPs, in the vesicles. Dansyl ^{3}H-labeled phospholipids (DNS-[^{3}H]PLs) or DHE was considered "incorporated" into the Na^+,K^+-ATPase membranes when fluorophores pelleted with the Na^+,K^+-ATPase preparation without the nonexchangeable [^{14}C]triolein. The locations of incorporated DHE and DNS-PLs were also described by iodide quenching experiments. DHE was not accessible to iodide for quenching, while 75% of the DNS-PLs incorporated into Na^+,K^+-ATPase membrane fragments were accessible to iodide. After a technique was developed for using PLEP to incorporate fluorescent lipids into membranes with the Na^+,K^+-ATPase preparation, DNS-PE, DNS-PS, and DHE were then analogously incorporated into electroplax plasma membranes enriched in acetylcholinesterase (AChE) and into erythrocyte ghosts in order to evaluate the fluorophores as membrane probes. In the subsequent evaluation, the fluorescent properties of membrane-incorporated DNS-PE, DNS-PS, and DHE were systematically compared to the fluorescent properties of the molecules in lipid vesicles. The fluorescence polarizations of both DNS-PLs were increased by the presence of protein in a bilayer. The fluorescence polarization of DNS-PS was greater than the polarization of DNS-PE in both membranes and vesicles. In contrast, the polarization (and the lifetime) of DHE was the same whether the fluorescent sterol was in a membrane preparation or in vesicles. Fluorescence polarization and intensity of all three fluorophores were measured in the bilayer preparations as a function of temperature. The intensities of all three probes and the polarization of DNS-PE in both membranes and vesicles decreased biphasically with a change in slope occurring at 26.0–27.5 °C. DNS-PS in lipid vesicles was depolarized biphasically with increasing temperature, but when incorporated into membranes, DNS-PS was depolarized linearly without a change in slope. The polarization of DHE in either membranous or vesicle bilayers did not change with temperature. Finally, when the Na^+,K^+-ATPase membrane preparation was used as a model system, the question was asked whether or not the fluorescent parameters of the lipid probes (i.e., intensity, polarization, and lifetime) could detect conformational changes in an intrinsic membrane enzyme. Results were negative despite localization of the fluorophores near the protein.

In the past decade, the structure and dynamics of biological membranes have been investigated with fluorescent probes. The application of such probes, however, requires scrutiny of at least two fundamental issues. First, the majority of these probes [e.g., 8-anilino-1-naphthalenesulfonate, diphenylhexatriene, (anthroyloxy)stearic acid, 2-(octadecylamino)-naphthalene-6-sulfonic acid, pyrene, and perylene] do not resemble the lipid molecules present within a membrane (Badley et al., 1973). The molecules may therefore perturb the surrounding environment to an extent where their emission reflects the environment they created rather than that of the membrane (Krishnan & Balaram, 1975; Cadenhead et al., 1975; Curtain et al., 1978). In addition, fluorescent lipid probes lacking an amphipathic structure will not be under the same restraints as membrane lipids. Their fluorescent properties will reflect events at the surface of the bilayer or within the hydrophobic core without the influence of covalent linkage to the other region. Second, the location of fluorescent probes

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within a bilayer is often undefined. Fluorophores are exogenously added to an aqueous membrane suspension, partitioning into the hydrophobic regions of the bilayer due to partition coefficients that favor nonaqueous environments. Association with a membrane is detected by an increase in the fluorescence intensity of the probes. This does not define fluorophore location, especially when the bilayer is a complex membrane. It has been suggested that these limitations could be circumvented by using fluorescent "analogues" of lipids (Azzi, 1975) placed in a membrane by the action of phospholipid exchange proteins (PLEPs)[1] (Monti et al., 1978; Waggoner & Stryer, 1970).

PLEPs are soluble cytosolic proteins that act to extract and insert phospholipid (PL) molecules into membranes (Wirtz, 1974). Although most of these proteins exchange only one class of PL, other PLEPs have been shown to nonspecifically transfer several classes of lipids, including cholesterol (Dyatlovitskaya et al., 1978; Bloj & Zilversmit, 1977; Crain & Zilversmit, 1980). We presumed that the nonspecific PLEPs offered the greatest potential for incorporating fluorescent lipid analogues within a membrane, provided that the risk of altering membrane lipid composition could be minimized (Muczynski et al., 1981). Also the use of PLEP in combination with nonexchangeable lipid markers should provide a means of localizing lipid fluorophores within a membrane.

The purpose of this report is 2-fold. In the first part, a protocol is described for incorporating two derivatives of PLs [dansyl[^{3}H]phosphatidylethanolamine (DNS-[^{3}H]PE) and dansyl[^{3}H]phosphatidylserine (DNS-[^{3}H]PS)] and an analogue of cholesterol [dehydroergosterol (DHE)] into membrane fragments enriched in Na^+,K^+-ATPase by using a nonspecific PLEP preparation (Crain & Zilversmit, 1980). The second part of this report focuses on how these fluorophores can be used as membrane probes, or more specifically, it aims to determine which spectral parameters of the probes correlate with membrane organization of lipids and proteins.

Our approach to the evaluation of the DNS-PLs and DHE as membrane probes was as follows. The spectral parameters of the fluorophores incorporated into membrane preparations were compared with those of the fluorophores in donor lipid vesicles (used with PLEP for the incorporation procedure) and in vesicles formed from lipids extracted from the membranes. In this manner the protein dependence or independence of a particular fluorescent parameter was assessed. Where differences in the vesicle and membrane preparations were found, sonicated vesicle models containing fluorescent lipid were examined to determine if variations in lipid composition or fluorophore concentration could account for the discrepancies. Finally for those fluorescent parameters that appeared to be dependent on the presence of integral membrane protein, the value of the parameter in three membrane systems was compared to determine if it was specific for a given membrane preparation or if it reflected some property of biological membranes in general.

The membranes selected for this study included fragments isolated from the plasma membrane of the electric organ of *Electrophorus electricus* enriched in either Na^+,K^+-ATPase or in acetylcholinesterase to approximately 85–90% homogeneity (Cantley et al., 1978) and human erythrocyte ghosts (Dodge et al., 1963). Selection of these membrane preparations offered several advantages to this investigation. First, the procedures for their isolation did not require salt or detergent, thereby minimizing alteration of native membrane structure. Second, the similarities and differences in the three membrane preparations offered a means whereby the specificity of the spectral properties of the DNS-PLs and DHE in a given membrane system could be evaluated. The Na^+,K^+-ATPase and AChE membrane fragments were similar in that each was enriched in a single multisubunit protein isolated from the same *E. electricus* electroplax plasma membranes. In contrast, the erythrocyte ghosts represented a multiprotein preparation from an unrelated tissue and organism. Third, the Na^+,K^+-ATPase membranes were used to determine whether the spectral properties of the incorporated fluorescent lipids were sensitive to functionally significant conformational changes in intrinsic membrane proteins. Extensive study of the Na^+,K^+-ATPase has shown that the activity of the enzyme requires lipid (Kimbelberg & Papahadjopoulous, 1972; Goldman & Albers, 1973; Depont et al., 1978; Noguchi & Freed, 1971) and is associated with conformational changes of the protein (Harris & Stahl, 1977; Cantley et al., 1978; Karlish, 1979; Taniguchi et al., 1980). Finally, the DNS-PLs and DHE could be incorporated into all three membrane preparations with PLEP.

Materials and Methods

Fluorescent Lipid Probes. DHE was synthesized from ergosterol (Aldrich) by oxidation with mercuric acetate (Bergmann & Stevens, 1948). It was selected for a sterol probe over cholesta-5,7,9-trien-3,8-diol because of its reported stability (Rogers et al., 1979). DHE melted at 140–144 °C. It eluted from a silica gel high-performance liquid chromatography column (Waters microporocil) with hexane–acetonitrile–diethyl ether (97:5:2.5:1 v/v) as a single peak (detected at 210 nm) just behind ergosterol. DHE has an absorption maximum in 25–100% dioxane in water solutions at 326 nm with shoulders at 311 and 340 nm. The extinction coefficient for DHE in dioxane at 326 nm was determined to be 10 600 ± 300 M^{-1} cm^{-1}. DHE has a $\lambda_{ex_{max}}$ at 327 and 340 nm and a $\lambda_{em_{max}}$ at 374 and 394 nm; DNS-PLs have a $\lambda_{ex_{max}}$ at 356 nm and a $\lambda_{em_{max}}$ that varies from 460 to 530 nm with increasing solvent polarity.

DNS-[^{3}H]PE and DNS-[^{3}H]PS were synthesized from egg yolk phosphatidylcholine (PC) (Biran & Bartley, 1961) by the following sequence of reactions: PC was converted to phosphatidic acid (PA) with phospholipase D (Ito et al., 1975). Phospholipase D was isolated from savoy cabbage (Yang, 1969). PA was reacted with cytidine 5′-monophosphoromorpholidate to form a cytidine diphosphate diglyceride (CDP-diglyceride) (Agranoff & Suomi, 1963). To synthesize [^{3}H]PS, CDP-diglyceride was incubated with L-[^{3}H(G)]serine in the presence of an *E. coli* ribosomal preparation of PS synthetase (Raetz & Kennedy, 1972, 1974). Ribosomes were precipitated twice with 30 mM $MgCl_2$ to remove the soluble

[1] Abbreviations: PLEP, phospholipid exchange protein; Na^+,K^+-ATPase, (Na^+ and K^+)-stimulated adenosinetriphosphatase; *E. coli*, *Escherichia coli*; CDP, cytidine diphosphate; PL, phospholipid; DHE, dehydroergosterol; DNS-PE, dansylphosphatidylethanolamine; DNS-PS, dansylphosphatidylserine; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositoi; PS, phosphatidylserine; Sph, sphingomyelin, PG, phosphatidylglycerol; PA, phosphatidic acid; $\lambda_{em_{max}}$, emission wavelength maxima; λ_{ex}, excitation wavelength; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; BSA, bovine serum albumin; EDTA, ethylenediaminetetraacetic acid; NaCl, sodium chloride; Tris, Trizma base; HCl, hydrochloric acid; ATP, adenosine triphosphate; $MgCl_2$, magnesium chloride; KCl, potassium chloride; TLC, thin-layer chromatography; NaI, sodium iodide; AChE, acetylcholinesterase; SD, standard deviation; τ, actual excited-state lifetime of a fluorophore; DPPC, dipalmitoylphosphatidylcholine; DMPC, dimyristoylphosphatidylcholine; nm, nanometer; $I_{\parallel}$, intensity parallel; $I_{\perp}$, intensity perpendicular; P, polarization; P_i, inorganic phosphate; MRC, maximum recoverable cpm; cpm, counts per minute; ME, 2-mercaptoethanol; ND, not determined.

PS decarboxylase that contaminated the PS synthetase preparation. Half of the [^{3}H]PS was decarboxylated to [^{3}H]PE with PS decarboxylase according to the conditions of Dowhan et al. (1974). PS decarboxylase was prepared from *E. coli* (Kanfer & Kennedy, 1964). Finally, [^{3}H]PS and [^{3}H]PE were reacted with dansyl chloride [DNS-Cl, 5-(dimethylamino)-naphthalene-1-sulfonyl chloride] as described by Waggoner & Stryer (1970). DNS-[^{3}H]PLs were isolated by sequential extraction with acid, base, and then water. DNS-[^{3}H]PE was purified by chromatography on silicar CC-7 (Mallinckrodt); DNS-[^{3}H]PS was purified by chromatography on DEAE-cellulose and then silicar CC-7 (Martin & Lagunoff, 1979). The final DNS-[^{3}H]PLs migrated as a single radioactive, fluorescent spot on silica gel 60 F-254 thin-layer plates developed in either chloroform–methanol–water (65:26:4 v/v) or 1-propanol–methyl acetate–chloroform–methanol–0.25% KCl (25:25:25:10:9 v/v). [The complete detailed synthesis of the DNS-[^{3}H]PLs is available as supplementary material (see paragraph at end of paper regarding supplementary material).] Both DNS-PLs had absorption maxima at 335 nm in methanol hexane. Specific activities of DNS-[^{3}H]PLs were adjusted to 400 cpm/nmol. (Although the isotope symbol is deleted under Results and Discussion, all DNS-PLs used in this study were labeled with tritium.)

Membrane Preparations. Na^+,K^+-ATPase was isolated from *E. electricus* according to the procedure of Cantley et al. (1978). An eel preparation enriched in acetylcholinesterase (AChE) was obtained by collecting fractions with a refractive index of 1.3475–1.3530 from the first sucrose gradient in the Na^+,K^+-ATPase preparation. These fractions were pooled and processed according to the protocol used for Na^+,K^+-ATPase. Erythrocyte ghosts were prepared from a normal pool of whole human blood, according to the procedure of Dodge et al. (1963).

PLEP Preparation. A preparation containing nonspecific PLEPs was purified from bovine liver according to the procedure of Crain & Zilversmit (1980). The procedure had to be carried through the heat coagulation step in order to eliminate lipolytic activity. Further purification by chromatography on octylagarose resulted in a PLEP preparation which upon SDS–PAGE had one major band just below a lysozyme molecular weight marker. Both preparations of PLEP were used for incorporating lipid fluorophores into the membrane fragments; however, the octylagarose-purified PLEP preparation was used within 2 weeks of its isolation.

Preparation of Lipid Vesicles. Lipid vesicles were prepared in the following manner: PL, sterol, [^{14}C]triolein, and butylated hydroxytoluene (0.1 mol % of the total lipid) in chloroform were dried under nitrogen and brought to a final total lipid concentration of 0.5 mM with 20 mM Tris-HCl and 2 mM EDTA (pH 7.4). Preparations were vortexed and then sonicated to clarity in an ice bath with a Branson probe sonifier. "Donor" lipid vesicles, i.e., those vesicles containing fluorophores that were to be transferred to the membrane preparations by PLEP, were centrifuged at 100000*g* for 1 h just prior to use to remove large vesicles that would pellet when centrifuged with the membrane fragments. Only the donor lipid vesicles contained [^{14}C]triolein.

Because the PLEP preparation has been shown to alter membrane composition (Muczynski et al., 1981), the following precautions were observed in the formation of the donor lipid vesicles that were incubated with the membrane preparations and PLEP: (1) Lipids used in the vesicles were isolated from *E. electricus* electric organ microsomes or erythrocyte ghosts (Biran & Bartley, 1961), so that any PLs removed from the membranes by the PLEP preparation would be replaced with PLs of the same acyl chain composition. (2) The PL:sterol molar ratio of the vesicles was matched to the PL:cholesterol molar ratio of the membranes, 1:0.75. DHE did not cause nonspecific adherence of vesicles to membranes, and therefore, it was substituted for the entire quantity of cholesterol in vesicles containing the fluorescent sterol. (3) The PL composition of the vesicles was matched as closely as possible to that of the membrane preparations. However, since negatively charged lipids caused nonspecific adherence of vesicles to membranes (DiCorleto et al., 1977), the amount of negatively charged PLs that was included in donor vesicles was limited. Vesicles containing high concentrations of DNS-PLs also nonspecifically adhered to the membranes. Therefore, the maximum amount of DNS-PL that could be included in the vesicles without nonspecific adherence of vesicles to membranes had to be determined empirically. This was done by preparing vesicles containing varying concentrations of DNS-PLs, incubating them in an airfuge tube with and without membranes at a concentration that was used for the incorporation incubations, and centrifuging for 20 min at 80000*g* to pellet the membranes. Aliquots of supernatant were counted. The cpm values obtained from the supernatant of the tube containing only vesicles and buffer were used as the maximum recoverable cpm (MRC). Where greater than 95% of the MRC of [^{14}C]triolein were recovered in the supernatant of the tube containing vesicles and membranes, nonspecific vesicle adherence was judged to be insignificant. The maximum amount of DNS-PE and DNS-PS that could be included in the donor lipid vesicles without nonspecific adherence to the membrane preparations was used. Specifically, donor vesicle PL compositions (in terms of percent total PL) were as follows: DNS-PE vesicles = 6.5% DNS-[^{3}H]PE, 20.5% PE, and 73% PC; DNS-PS vesicles = 4.5% DNS-[^{3}H]PS, 27% PE, and 68.5% PC; DHE vesicles = 27% PE and 73% PC. Trace quantities of [^{14}C]triolein were included in all of the vesicles as a nonexchangeable marker.

Unless otherwise stated, lipids used in vesicle studies were obtained from the following sources: PE and PS were purchased from Sulpelco, cholesterol was purchased from Eastman, and PC and isolated from egg yolks.

Incorporation of Lipid Probes into Membranes Using PLEP. Prior to the incubation with PLEP for the incorporation of lipid fluorophores, Na^+,K^+-ATPase membrane fragments (at a concentration of 5–8 mg of protein/mL) were preincubated on ice for 10 min with 100 mM NaCl, 10 mM ATP, 5 mM $MgCl_2$, and 5 mM *p*-nitrophenyl phosphate. Other work in our laboratory involving the fluorescent labeling of Na^+,K^+-ATPase sulfhydryl groups had shown that these ligands prevented loss of Na^+,K^+-ATPase activity. For this reason, as well as the need for a membranous Na^+,K^+-ATPase preparation containing incorporated lipid fluorophores that could subsequently be used in experiments designed to study the interactions of both lipids and sulfhydryls, the ligands were added to the Na^+,K^+-ATPase membrane fragments before the addition of the other incubation reagents. Ligands were not removed after being allowed to react with the Na^+,K^+-ATPase preparation. Instead, the membrane fragments were directly incorporated with lipid fluorophores according to the conditions described below.

Membranes [preincubated Na^+,K^+-ATPase membrane fragments (from above), untreated AChE membranes, or erythrocyte ghosts] were placed in 50 mM Tris-HCl, pH 7.4 (0.35 mg of protein/mL of incubation volume, 0.73 μmol of total lipid/mL of incubation volume), with mixed lipid vesicles

Table I: Incorporation of DNS-[^{3}H]PE into Na$^+$,K$^+$-ATPase Membrane Fragments Distinguished by the Assay for PLEP[a]

condition	required elements: PLEP[b]	required elements: DNS-[^{3}H]PE in donor lipid vesicles[c] (%)	results of PLEP assay: supernatant recovery of radiolabeled lipids[d] (%): [^{14}C]triolein	results of PLEP assay: supernatant recovery of radiolabeled lipids[d] (%): DNS-[^{3}H]PE
(1) incorporation	+	6.5	101	60
(2) aggregation	+	6.5	9	22
(3) vesicle adherence	−	100	4	4

[a] Data in duplicate from a representative set of experiments. [b] (+) or (−) indicates the presence or absence, respectively, of PLEP. [c] Donor lipid vesicles contained tracer amounts of [^{14}C]triolein (a lipid not exchanged by PLEP) and DNS-[^{3}H]PE. The composition of the 6.5% vesicles is described under Materials and Methods; 100% DNS-[^{3}H]PE vesicles contained only fluorescent lipid and triolein. [d] Na$^+$,K$^+$-ATPase membranes were separated from donor lipid vesicles and PLEP, by centrifugation of the incubation mixture. ^{14}C and ^{3}H in the supernatant were detected on a dual channel β scintillation counter with regions at 0–12 MeV for ^{3}H and at 13–156 MeV for ^{14}C. Although regions were adjusted to minimize the energy overlap between β particles emitted from the respective radioisotopes, it was still necessary to correct for ^{14}C decays registering in the ^{3}H channel. This was done by using the channels ratio of [^{14}C]triolein alone. Values represent the corrected percent of the total cpm recovered in the supernatant. A total of 150 μL of supernatant was routinely counted. The maximum recoverable corrected cpm in this aliquot was approximately 1000 cpm each of DNS-[^{3}H]PE and [^{14}C]triolein.

containing fluorescent lipid probe (0.2 μmol of total lipid/mL of incubation volume), PLEP (0.2–0.4 mg of protein/mL of incubation volume), 10 mg of fatty acid poor BSA/mL, 10 mM EDTA, and 10 mM 2-mercaptoethanol (ME) and incubated with agitation at 32 °C for 40 min in a centrifuge tube. The total incubation volume was 5–15 mL. The incorporation reaction mediated by PLEP was terminated by centrifugation (20 min × 80000g) to pellet the membranes. Aggregation and nonspecific adherence of lipid substrate to membranes were calculated from the loss of [^{14}C]triolein in the supernatant as described below. Membrane fragments were washed 3 times by suspending the preparations in 10 mL of each of the following sequence of buffers, centrifuging (20 min × 80000g), and removing the supernatant by aspiration: (1) 100 mM NaCl, 10 mM 2-mercaptoethanol, and 50 mM Tris-HCl (pH 7.4); (2) 50 mM Tris-HCl (pH 7.4); (3) 20 mM Tris-HCl and 2 mM EDTA (pH 7.4). Preparations were stored at a concentration of 1–10 mg of protein/mL in 20 mM Tris-HCl and 2 mM EDTA (pH 7.4) at −80 °C.

The DHE content of Na$^+$,K$^+$-ATPase membranes was varied by incubating different quantities of donor PL:DHE (molar ratio 1:0.75) vesicles with a constant amount of Na$^+$,K$^+$-ATPase and PLEP for a given time period.

Analytical Techniques. Laemmli (1970) continuous SDS–polyacrylamide gradient gels ranging in acrylamide concentration from 6.5 to 17.5% were used to identify the entire protein molecular range of Na$^+$,K$^+$-ATPase and PLEP. We found that α and β subunits of Na$^+$,K$^+$-ATPase have apparent molecular weights of about 100 000 and 45 000, respectively, and PLEP has an apparent molecular weight of 13 000. Protein molecular weight standards were phosphorylase B (92 500), BSA (66 200), ovalbumin (45 000), carbonic anhydrase (31 000), soybean trypsin inhibitor (21 500), and lysozyme (14 400) (Bio-Rad).

Na$^+$,K$^+$-ATPase activity was assayed under standard conditions at 30 °C in 50 mM Tris-HCl containing 3 mM ATP, 20 mM KCl, 3 mM $MgCl_2$ ± 100 mM NaCl (pH 7.4). The method of Lanzetta et al. (1979) was used to determine inorganic phosphate liberated with ATP hydrolysis. Na$^+$,K$^+$-ATPase activity is the increment in inorganic phosphate formed in the presence of sodium. Activity was calculated from assays where less than 5% of the total ATP was hydrolyzed. Protein was determined by the method of Lowry et al. (1951) with BSA as the standard.

The PL:cholesterol ratio of the Na$^+$,K$^+$-ATPase was not altered after the incorporation of lipid fluorophores. PL was determined on a chloroform–methanol (2:1 v/v) lipid extract of Na$^+$,K$^+$-ATPase by a modified Lowry & Lopez (1946) assay after perchloric acid digestion. Cholesterol was assayed by the method of Bhandaru et al. (1977).

PLEP activity, aggregation, and nonspecific adherence of lipid vesicles to membranes were determined in the following manner. Tubes containing the following reagents at the concentrations cited for the incorporation procedure were incubated for 40 min: tube 1, vesicles in buffer containing BSA, EDTA, and ME; tube 2, vesicles plus membranes in buffer containing BSA, EDTA, and ME; tube 3, vesicles plus membranes plus PLEP in buffer containing BSA, EDTA, and ME. Tubes 1 and 2 were incubated at 37 °C and tube 3 at 0 °C. Tubes were centrifuged after incubation (20 min × 80000g), and an aliquot of supernatant was counted on a dual channel β scintillation counter to determine both DNS-[^{3}H]PL cpm and [^{14}C]triolein cpm. The cpm values from tube 1 represented the maximum recoverable cpm (MRC). The cpm values from tube 2 were used to estimate the contribution of nonspecific vesicle adherence. The recovery of [^{14}C]triolein from tube 3 was one estimate of aggregation. The recovery of DNS-[^{3}H]PL from tubes 2 and 3 after taking into account any [^{14}C]triolein loss represented the amount of fluorescent PL transferred to the membranes in the absence of PLEP activity.

The amount of DNS-[^{3}H]PL incorporated into the membrane preparations was determined from the known specific activities of the lipid probes and by counting an aliquot of washed membrane. TLC of extracted membranes containing DNS-[^{3}H]PLs revealed a single fluorescent spot identical in R_f to that of the DNS-[^{3}H]PL stocks. DHE was quantitated by extracting the lipids of the membranes with chloroform–methanol (2:1 v/v) and determining the optical density of an aliquot of the organic phase in dioxane at 326 nm.

Instrumentation. Excitation and emission spectra (uncorrected) were obtained with a Perkin-Elmer MPF-2A fluorescence spectrophotometer interfaced with a Houston Instrument 2000 X-Y recorder (Bausch & Lomb). Cuvette temperature was maintained constant with a circulating water bath. All other fluorescence measurements were determined on an SLM 4800 nanosecond spectrofluorometer equipped with a circulating water bath to regulate sample temperature. The sample chamber of the SLM instrument was maintained in a nitrogen atmosphere and contained a thermistor for cuvette temperature measurements. Schott kV colored glass long-pass filters and a Ditric Optics interference filter were used to determine emission wavelength.

Fluorescence Spectroscopy. Vesicle and protein preparations used for fluorescence were diluted with 20 mM Tris-HCl and 2 mM EDTA (pH 7.4) to 0.3 mM total lipid (PL plus

cholesterol) and 143 μg of protein/mL, respectively. Deviations from this are stated in the figure legends.

Steady-state polarization is defined as $P = (I_\parallel - I_\perp)/(I_\parallel + I_\perp)$ in which $I_\parallel$ and $I_\perp$ are the components of the fluorescent intensity parallel and perpendicular to the excitation polarization directions, respectively. Polarizations of the lipid probes in the membrane preparations were corrected for membrane light scattering by measuring $I_\parallel$ and $I_\perp$ with a blank composed of all constituents except the fluorophore (i.e., membrane and buffer) and subtracting these values from the $I_\parallel$ and $I_\perp$ of samples containing fluorophore. Polarization was then calculated from the corrected intensity values according to the equation $P = (R_c - 1)/(R_c + 1)$, where $R_c = (A_{0,0}/B_{0,9})/(A_{9,0}/B_{9,9})$, with A and B being the intensity determined on photomultiplier tube A and B respectively, the first subscript, the position of the excitation polarizing filter (0 = 0° from vertical position = parallel, 9 = 90° from the vertical position = perpendicular), and the second subscript, the position of the polarizing filter proceeding the respective photomultiplier tube.

Lifetime determinations were made by exciting samples with sinusoidal light modulated at a known frequency and measuring the demodulation $[\tau_M = [1/(2\pi f)][1/D_2 - 1]^{1/2}]$ or the shift in the phase angle of the emitted light $[\tau_\theta = [1/(2\pi f)]$ tan $\theta]$. (Demodulation and phase shift are dependent on the lifetime of the fluorophore as indicated in the above equations.[2]) If all fluorophores in a sample have the same excited-state lifetime, τ_M and τ_θ should agree. The presence of two or more fluorophore lifetimes is indicated by divergence in the values of τ_M and τ_θ, the former being longer than the weighted average of the lifetimes, while the latter is shorter than the weighted average (Spencer & Weber, 1969).

Oyster glycogen was used as a scattering reference for excitation modulation. Steady-state intensities of oyster glycogen scatter (without an emission cutoff filter) and lipid probe fluorescence were matched by adjusting glycogen concentration and imposing neutral density filters or screens between the reference cuvette and the photomultiplier tube. Lifetime measurements were not corrected for "color delay" (Jameson & Weber, 1981). Lifetime determinations of DHE and the DNS-PLs were made at the following wavelengths: λ_{ex} = 330 nm, λ_{em} = 370 nm cutoff and λ_{ex} = 356 nm, λ_{em} = 408 nm cutoff, respectively.

Results

The results of this report were conveniently divided into two major sections. The first describes a protocol for incorporating fluorescent lipids, specifically DNS-PE, DNS-PS, and DHE, into membranes. These studies utilized membrane fragments containing Na^+,K^+-ATPase. The advantage of using this preparation was to have a lipid-dependent membrane function, i.e., hydrolysis of ATP, by which to assess the effect of PLEP-mediated fluorophore incorporation on the membranes. In the second section, DNS-PE, DNS-PS, and DHE were incorporated into three different membrane preparations in an attempt to determine how these fluorophores might be used as membrane probes.

Incorporation of DNS-PE, DNS-PS, and DHE into Na^+,K^+-ATPase Membranes. (1) Membrane Localization of Lipid Fluorophores Defined by the PLEP Assay. By use of DNS-[^{3}H]PE as an example, Table I summarizes the results that can be obtained when PLEP was used to transfer fluorescent lipid from donor mixed lipid vesicles to Na^+,K^+-ATPase membranes. Donor mixed lipid vesicles contained [^{14}C]triolein, as a nonexchangeable marker, and DNS-[^{3}H]PE. DNS-[^{3}H]PE was defined as incorporated when the [^{14}C]triolein remained in the supernatant and ^{3}H-DNS-[^{3}H]PE pelleted with the Na^+,K^+-ATPase (Table I, condition 1). Greater than 95% of the MRC of [^{14}C]triolein were recovered in the supernatant, indicating that aggregation and vesicle adherence were low, if not insignificant. Fluorophores were not transferred in the absence of the PLEP preparation or in the presence of high concentrations of NaCl (greater than 100 mM) which inhibit PLEP activity. "Aggregation" (Table I, condition 2) has been defined as the pelleting of both [^{14}C]-triolein and DNS-[^{3}H]PE with Na^+,K^+-ATPase membranes when membranes, vesicles, and PLEP were incubated together. Aggregation resulted when the PLEP preparation was chromatographed on octylagarose and stored for greater than 2 weeks at 4 °C before use. Aggregation was reduced in the presence of 10 mM EDTA or with 100 mM NaCl and 3 mM EDTA. "Vesicle adherence" (Table I, condition 3), defined as the association of both [^{14}C]triolein and DNS-[^{3}H]PE with Na^+,K^+-ATPase membranes when PLEP was not present, was determined by the lipid composition of donor vesicles and the recipient membrane. Inclusion of greater than 10 mol % negatively charged lipid (e.g., PS) or 7 mol % DNS-PE (or 5 mol % DNS-PS) resulted in vesicle adherence to membrane fragments containing Na^+,K^+-ATPase. The PL composition of vesicles containing fluorescent lipids was adjusted empirically to prevent adherence to Na^+,K^+-ATPase membranes (see Materials and Methods). The presence of BSA also reduced nonspecific adherence of vesicles to the membrane Na^+,K^+-ATPase preparation.

The circumstances under which aggregation (Table I, condition 2) occurred suggests that the octylagarose purified PLEP underwent structural changes with storage. We found evidence of such changes from observations of the intrinsic tryptophan fluorescence of the PLEP preparation. On the day that PLEP was purified on octylagarose, the preparation had an uncorrected emission wavelength maxima ($\lambda_{em_{max}}$) at 336 nm (λ_{ex} = 292 nm). One week later, the uncorrected $\lambda_{em_{max}}$ was shifted to 342 nm. The addition of 1 M guanidine hydrochloride, a protein solubilizer, similarly shifted the $\lambda_{em_{max}}$ of the PLEP preparation from 336 to 348 nm. This suggests that with storage at 4 °C, the tertiary structure of PLEP purified by octylagarose chromatography was altered. In addition to the shifts in $\lambda_{em_{max}}$ of PLEP with storage, changes in tryptophan fluorescence lifetime were observed.

Similar results were obtained with attempts to incorporate DNS-PS or DHE into the Na^+,K^+-ATPase membranes. The maximum amount of DNS-PS that could be included in the donor vesicles without nonspecific adherence was 4.5 mol %. The presence of DHE in donor vesicles was not associated with adherence. Interestingly, the phenomenon of vesicle adherence was also dependent upon the nature of the acceptor membrane used in the incorporation procedure. For example, vesicles containing roughly 10 mol % of DNS-PE adhered to

[2] The Lehrer-modified Stern–Volmer relationship is

$$\frac{F_0}{\Delta F} = \frac{1}{[Q]f_a\tau_0 k} + \frac{1}{f_a}$$

where F_0 is the fluorescent intensity in the absence of quencher, ΔF is the difference in fluorescence intensity in the absence of quencher and in the presence of quencher, [Q] is the concentration of quencher, τ_0, is the lifetime of the excited state, k is the rate constant for the deactivation of a fluorophore by a quencher, and f_a is the fraction of total probe available to quencher. The equation variables for the determination of excited-state lifetimes are the following: τ_M is the excited-state lifetime determined by modulation, f is the modulation frequency, D is the modulation of fluorescence/modulation of excitation, τ_θ is the excited-state lifetime determined by phase shift, and θ is the phase shift in degrees caused by the excited lifetime of a fluorophore.

Table II: Incorporation of Varying Amounts of DHE into Na^+,K^+-ATPase Membrane Fragments[a]

amount of DHE in the incorporation incubation (nmol/mg of Na^+,K^+-ATPase protein)	amount of DHE incorporated in Na^+,K^+-ATPase membranes (nmol/mg of Na^+,K^+-ATPase protein) (mol % of total membrane lipid)[b]
44	24 (1.2)
88	52 (2.5)
177	95 (4.6)
371	171 (8.2)
760	278 (13.3)

[a] A total of 1.21 mg of Na^+,K^+-ATPase protein was incubated with 0.728 mg of PLEP and a varying amount of donor lipid vesicles containing PL and DHE in a 1:0.75 ratio. The PL composition of the vesicles was described under Materials and Methods. Incubation conditions for the incorporation of DHE into Na^+,K^+-ATPase membranes was as described under Materials and Methods. Nonspecific adherence and aggregation were judged to be insignificant based on recovery of greater than 95% of the MRC of the [^{14}C]triolein marker included in the vesicles. [b] The amount of DHE incorporated into Na^+,K^+-ATPase membranes washed after the incorporation incubation (see Materials and Methods). DHE was quantitated as described under Materials and Methods. Na^+,K^+-ATPase protein was determined on the washed membrane preparation by the method of Lowry et al. (1951) with BSA as the standard.

Na^+,K^+-ATPase membranes but not to mitochondria. (Mitochondria are the lipid acceptors used in standardized specific activity determinations of PLEPs.) When any of the fluorophores were considered incorporated into a membrane preparation, vesicle composition had been adjusted to prevent significant vesicle adherence (see Materials and Methods) and appropriate PLEP preparations were used to prevent aggregation.

Membranes were incorporated with DNS-PLs by using vesicles containing only the masimum amount of these fluorophores that could be included in them without nonspecific adherence to the membranes. In addition, the length of the incubation period was selected to maximize recovery of Na^+,K^+-ATPase activity. Less optimal conditions were not indicated as incorporating less DNS-PL in the membranes would have significantly decreased the signal-to-noise ratio of the fluorescence intensity of the DNS-PLs, thereby compromising subsequent fluorescent studies. The situation with DHE was different. Since the fluorescent sterol did not cause vesicle adherence to membranes, the amount of DHE incorporated into the Na^+,K^+-ATPase membranes was easily varied. The Na^+,K^+-ATPase preparation was incubated for 40 min at 32 °C with PLEP and varying concentrations of donor lipid vesicles composed of DHE and PL. Incubation of Na^+,K^+-ATPase membranes and PLEP with increasing concentrations of DHE vesicles resulted in increased incorporation of DHE into the Na^+,K^+-ATPase membranes (Table II).

(*2*) *Na^+,K^+-ATPase Membranes with Incorporated Fluorescent Lipids.* Since the membranes used in this investigation were enriched in Na^+,K^+-ATPase, the sodium-dependent hydrolysis of ATP was assayed as a parameter of membrane function. Untreated, native Na^+,K^+-ATPase membranes hydrolyzed 569 ± 12 μmol of inorganic phosphate h^{-1} (mg of protein)$^{-1}$ (mean ± SD for three determinations made on a single representative preparation) at 30 °C. Na^+,K^+-ATPase membranes pretreated with "protective ligands", incubated at 32 °C for 40 min in the presence of BSA, EDTA, and 2-mercaptoethanol, and incorporated with DNS-PE, DNS-PS, and DHE had specific activities of 511 ± 20, 506 ± 8, and 466 ± 20, respectively. This suggests that DNS-PLs and DHE did not perturb membrane structure, at least not to an extent that affected the activity of the Na^+,K^+-ATPase.

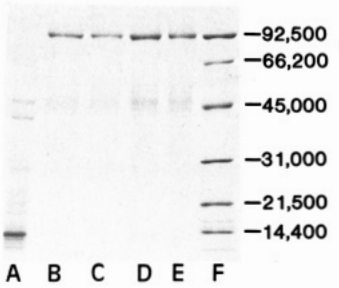

FIGURE 1: SDS–PAGE of *E. electricus* Na^+,K^+-ATPase, untreated (E) and incorporated with DNS-PE (D), DNS-PS (C), or DHE (B) by using a heat-coagulated PLEP preparation (A). Molecular weight standards (F) are described under Materials and Methods. The Na^+,K^+-ATPase preparations exhibited a minor protein band at the molecular weight of PLEP. It is unlikely that the peptide was PLEP because (1) the protein band was present in Na^+,K^+-ATPase preparations that had never been exposed to PLEP (E) and (2) the intensity of the stained peptide did not appear to increase after incubation of Na^+,K^+-ATPase with an equal mass of PLEP (B–D).

SDS–polyacrylamide gel electrophoresis (Figure 1) confirmed that PLEP and BSA, present in the incorporation incubation, were removed from Na^+,K^+-ATPase membrane fragments by the protocol described under Materials and Methods. Therefore, changes in the fluorescent properties of the DNS-PLs or DHE that resulted when the fluorophores were transferred from lipid vesicles to Na^+,K^+-ATPase membranes potentially reflected the presence of Na^+,K^+-ATPase protein rather than association of lipid fluorophores with PLEP or BSA.

(*3*) *Membrane Localization of Fluorescent Lipids Described by Iodide Quenching.* The locations of DHE and the DNS-PLs incorporated into the Na^+,K^+-ATPase were characterized further by iodide quenching experiments. The fluorescence of DNS-PE, DNS-PS, or DHE in Na^+,K^+-ATPase was quenched by iodide and analyzed according to the Stern–Volmer equation as modified by Lehrer (1971)[2], i.e., $F_0/\Delta F = 1/([Q]f_a\tau_0 k) + 1/f_a$, as shown in Figure 2. This relationship relates the degree to which fluorescence is decreased ($F_0/\Delta F$) to the concentration of the quencher ([Q]) and to the accessibility of the fluorescent probe to the quencher (iodide). From such an analysis, the fraction of the total fluorescent lipid available to iodide (f_a) was determined. The low f_a value of 0.083 and the poor correlation between $F_0/\Delta F$ and iodide concentration for DHE incorporated into Na^+,K^+-ATPase are consistent with the fluorescent sterol nucleus being buried within the interior hydrophobic region of the bilayer largely unaccessible to iodide for quenching. In contrast, 75% of both DNS-PE and DNS-PS in Na^+,K^+-ATPase membranes were accessible to iodide (Figure 2) at concentrations ranging from 150 to 500 mM NaI. This suggests that the DNS-PLs are near the surface of the Na^+,K^+-ATPase membranes, consistent with the proposed location in the PL glycerol backbone (Waggoner & Styer, 1970).

DNS-PE, DNS-PS, and DHE as Membrane Probes. The proceeding protocol for using PLEPs to incorporate fluorescent lipids into membranes was used to incorporate DNS-PE, DNS-PS, and DHE into electroplax plasma membranes enriched in either Na^+,K^+-ATPase or AChE and into erythrocyte ghosts. The spectral parameters of the fluorophores were then examined to determine which of them, if any, correlated with

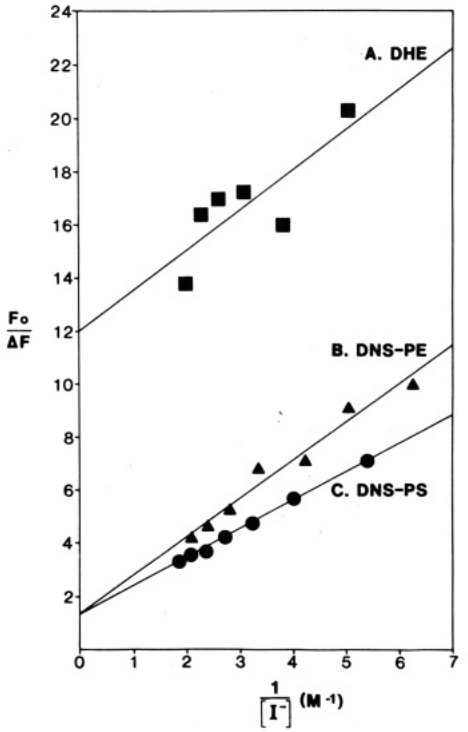

FIGURE 2: Iodide quenching of DNS-PE (▲), DNS-PS (●), and DHE (■) incorporated into Na^+,K^+-ATPase membranes, analyzed by the Lehrer modification of the Stern–Volmer equation (see text). Membranes incorporated with DNS-PE contained 12.3 nmol of the fluorophore/mg of Na^+,K^+-ATPase protein, those with DNS-PS, 8.7 nmol of probe/mg of protein; and those with DHE, 95.5 nmol of probe/mg of protein. To duplicate samples of the membrane preparations were added 5-μL aliquots of 4.829 M NaI or NaCl. F_0 was the fluorescence intensity observed in NaCl. ΔF was the difference between fluorescence intensities measured in NaCl and NaI. Data represent the average of duplicate NaCl additions and duplicate NaI additions. Fluorescence intensities were determined with an SLM 4800 fluorescence spectrophotometer at 22 °C. For the DNS-PL preparations, λ_{ex} = 356 nm, and λ_{em} = 470 nm cutoff (Schott kV filter); for DHE, λ_{ex} = 345 nm, and λ_{em} = 370 nm cutoff (Schott kV filter). Correlation coefficients for curves A, B, and C were 0.81, 0.98, and 0.999, respectively. Values for $1/(f_a\tau_0 k)$ for these curves were 1.52, 1.45, and 1.08, respectively, and f_a values were 0.083, 0.745, and 0.756, respectively.

membrane organization of lipids and proteins.

(*1*) *Fluorescence Polarization.* There was a dramatic increase in the fluorescence polarization of DNS-PE, DNS-PS, and DHE when the probes were transferred from mixed lipid vesicles to membranes (Figure 3). Since increased fluorescence polarization denotes more restricted rotational motion of a fluorescent molecule, it might be assumed that the motion of the DNS-PLs and DHE was more restricted in the membrane preparations than in donor vesicles. The interpretation is not that simple because fluorophore concentration, which was not identical in donor lipid vesicles and in membranes, can have a major effect on depolarizing processes. Therefore, before the increased fluorescence polarization of the DNS-PLs and DHE was attributed to the presence of integral membrane protein, the effect of concentration on polarization for each of the lipid probes in a bilayer was determined at a constant temperature (Figure 4). The fluorescence polarization of DHE, and to a lesser degree the polarization of the DNS-PLs, increased with decreasing probe concentration.

The increased polarization of DHE in the membranes was predicted from Figure 4. For example, when DHE was transferred from mixed lipid vesicles where it represented 42.8% of the total lipid to the Na^+,K^+-ATPase membrane fragments where it represented 6% of the total lipid (Figure

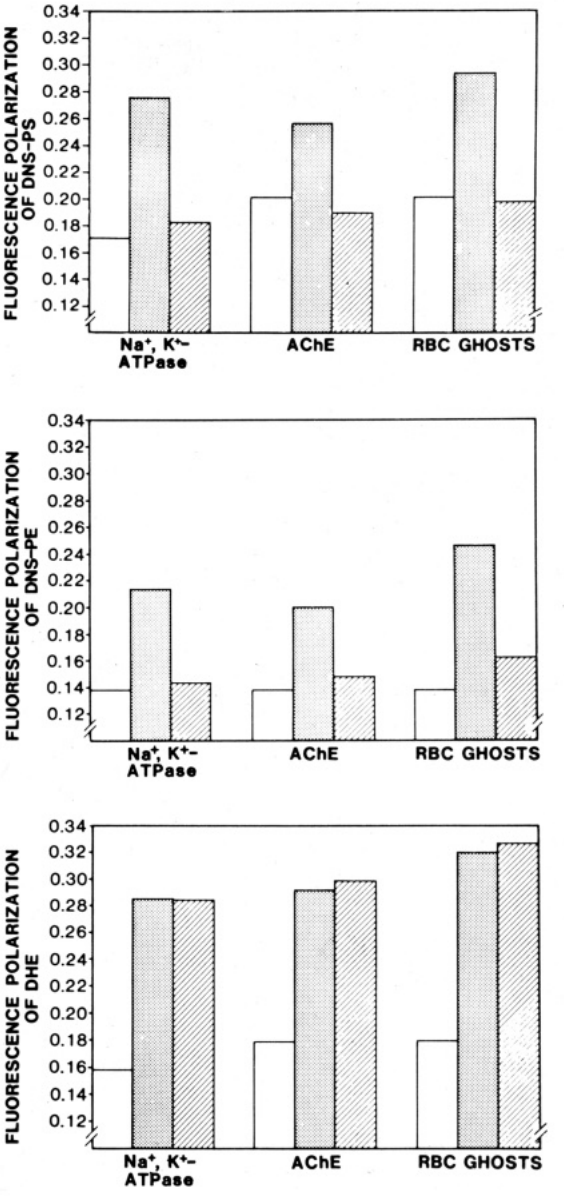

FIGURE 3: Comparison of fluorescence polarization of DNS-PLs and DHE in donor lipid vesicles (open bars), membrane preparations (dotted bars), and vesicles formed of lipids and lipid fluorophores extracted from those membranes (diagonally striped bars). In the chart below and the quantities of fluorescent lipids are given. The

	quantity of incorporated fluorophore (nmol/mg of membrane protein)		
membrane preparation	DNS-PE	DNS-PS	DHE
Na^+,K^+-ATPase	12.3	8.7	124
AChE	14.5	9.8	ND
erythrocyte ghosts	8.5	5.8	ND

amount of DNS-PL incorporated into the membrane preparations represented 1–2 mol % total membrane lipid. Measurements represent the mean of at least five polarization determinations made on a single representative preparation. Standard deviation of each measurement was less than 0.004. Where the polarization of donor vesicles for a given fluorophore differed, membranes were incorporated with fluorescent lipid using different stocks of donor vesicles. Five percent of the fluorescence intensity of the DNS-PLs in the membranes was due to scattered light, and therefore, polarizations were corrected for this contribution as outlined under Materials and Methods. The polarization values of the DNS-PLs in vesicles and of all DHE preparations were not corrected for light scattering because it constituted less than 2% of the fluorescence intensity and thereby did not affect the calculated polarization values. λ_{ex}(DNS-PLs) = 356 nm, and λ_{em}(DNS-PLs) = 470 nm cutoff; λ_{ex}(DHE) = 330 nm, and λ_{em}(DHE) = 370 nm cutoff. Sample T = 19–22 °C.

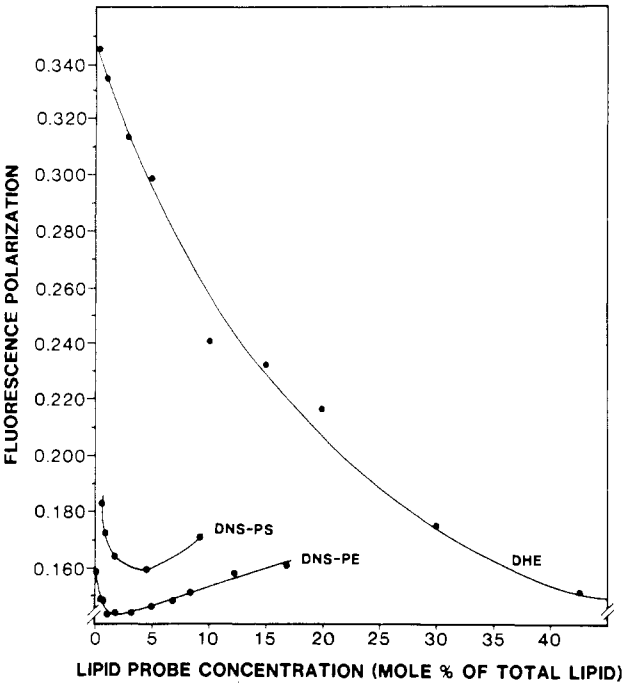

FIGURE 4: Concentration depolarization of DNS-PE, DNS-PS, and DHE in PC:cholesterol vesicles (molar ratio 1:0.75). Polarizations of the DNS-PLs or DHE were determined in sonicated lipid vesicles (see Materials and Methods) containing PC, cholesterol, and a variable amount of probe at a total lipid (PL plus cholesterol) concentration 0.5 mM (note that polarization of a given percentage of DNS-PL or DHE in the total lipid was the same at total cuvette lipid concentrations of 0.3 and 0.5 mM). The PL:sterol ratio was kept constant (at 1:0.75) by substituting DNS-PLs for PC and DHE for cholesterol. λ_{ex}(DNS-PLs) = 356 nm, and λ_{em}(DNS-PLs) = 470 nm cutoff; λ_{ex}(DHE) = 330 nm, and λ_{em}(DHE) = 370 nm cutoff; T = 22 °C. Polarization values were not corrected for light scatter because its contribution to fluorescent intensity was insignificant.

3), it was diluted and should have had an increased polarization. Figure 4 would predict a polarization value of 0.286, assuming the polarization of DHE to be the same whether it is in a mixed lipid vesicle or a membrane fragment. The actual measured value of the polarization of DHE in the Na^+,K^+-ATPase membranes of Figure 3 was 0.287. Furthermore, when the polarizations of DHE in each of the preparations listed in Table III were determined and plotted as a function of the mole percent of total membrane lipid, a curve identical with that of Figure 4 was obtained. Thus, dilution can account for the increased polarization of DHE in the Na^+,K^+-ATPase preparation.

In contrast, the increased fluorescence polarization of the DNS-PLs could not be accounted for by dilution effects. At the concentration of DNS-PL in the vesicles (3–4% of the total lipid) and in the membrane preparations (1% of the total lipid), fluorescence polarization did not significantly vary (Figure 4). This suggests that the polarization of the DNS-PLs was due to the presence of integral membrane proteins.

Further support for the protein dependence of the DNS-PLs polarization and the protein independence of DHE polarization is summarized in Figure 3. DNS-PE, DNS-PS, and DHE were incorporated into the membrane preparations, and then the lipids, including the fluorophores, were extracted from them. Vesicles of extracted lipids were formed and polarization of the fluorescent probes determined at a concentration of probe and total lipid matched to that used for polarization determination of the probes in the membranes. The polarization of the DNS-PLs in the membrane extracts approached the initial value of the probe in donor–mixed lipid vesicles. [Concentration depolarization (Figure 4) accounts for the small difference between the values.] The polarization of DHE in the extracted membrane lipids remained elevated at the level of DHE in the membranes. This was true when DHE constituted 1–13% of the total lipid in a membrane preparation (data not shown).

The data of Figures 3 and 4 indicate that, in general, the polarizations of the DNS-PLs in biological membranes were due to the presence of protein while increases in DHE polarization were a concentration effect. In addition, the polarization values of DNS-PE and DNS-PS in membranes containing Na^+,K^+-ATPase, AChE, and erythrocyte plasma membrane proteins were reproducibly different. In fact, the polarizations of each of the DNS-PLs in Na^+,K^+-ATPase and AChE membrane fragments determined on at least three electric organ membrane preparations isolated independently and incorporated with different stocks of DNS-PL vesicles by using different preparations of PLEP were identical. This suggests that the restriction in the rotational motion of DNS-PE and DNS-PS was characteristic of the protein content of a given membrane preparation.

Another observation from the data of Figures 3 and 4 was that the fluorescence polarization of DNS-PS in a bilayer was greater than that of DNS-PE. This was true whether the bilayer was a sonicated vesicle or a membrane with intrinsic protein and suggests that the carboxyl group of DNS-PS is a restrictive factor to the rotational motion of the DNS-PLs in a bilayer.

A final set of experiments was conducted to assess the effect of phospholipid:sterol ratio and PL composition on DNS-PL fluorescence polarization. The rationale for the work was that small differences in lipid composition might account for the fluorescent data of the membrane preparations. Sonicated lipid vesicles composed of PC and cholesterol at a molar ratio varied from 1:0 to 1:1 (PC:cholesterol) were prepared at a total lipid concentration (PL plus cholesterol) of 0.5 mM (see Materials and Methods). DNS-PE or DNS-PS was included in the vesicles at a concentration of 1% of the total lipid. Fluorescence polarization of the DNS-PLs, determined under the conditions used for polarization determinations of the membrane preparations, was not significantly affected by the ratio of PL to cholesterol (data not shown). In a similar set of experiments, sonicated vesicles were formed with a constant PL:cholesterol ratio of 1:0.75 and a constant concentration of DNS-PL (1% of the total lipid) but with the following PL compositions (given in terms of percent total vesicle phospholipid): (1) 27% PE and 73% PC; (2) 18% PS and 82% PC; (3) 27% PE, 18% PS, and 55% PC. Fluorescence polarization of the DNS-PLs were found to be the same as the polarizations of the probes in vesicles of PC and cholesterol. In other words, the vesicle PL compositions listed above had no significant effect on the fluorescent polarizations of the DNS-PLs. These compositions were selected as they approximate the amount of the major PLs present in Na^+,K^+-ATPase membranes (unpublished data) and in erythrocyte ghosts (Muczynski et al., 1981).

(*2*) *Excited-State Lifetimes.* An attempt was made to use the excited-state lifetimes of the fluorophores to distinguish between fluorescent lipids incorporated into a membrane preparation from probes in sonicated mixed lipid vesicles. The lifetime of DHE incorporated into the three membrane preparations at a concentration of 3–6% of the total lipid was homogeneous and identical with the lifetime of DHE in donor–mixed lipid vesicles, 1.5–2.5 ns as measured by demodulation and phase shift (see Materials and Methods). Furthermore, when the fluorescent parameters of DHE were

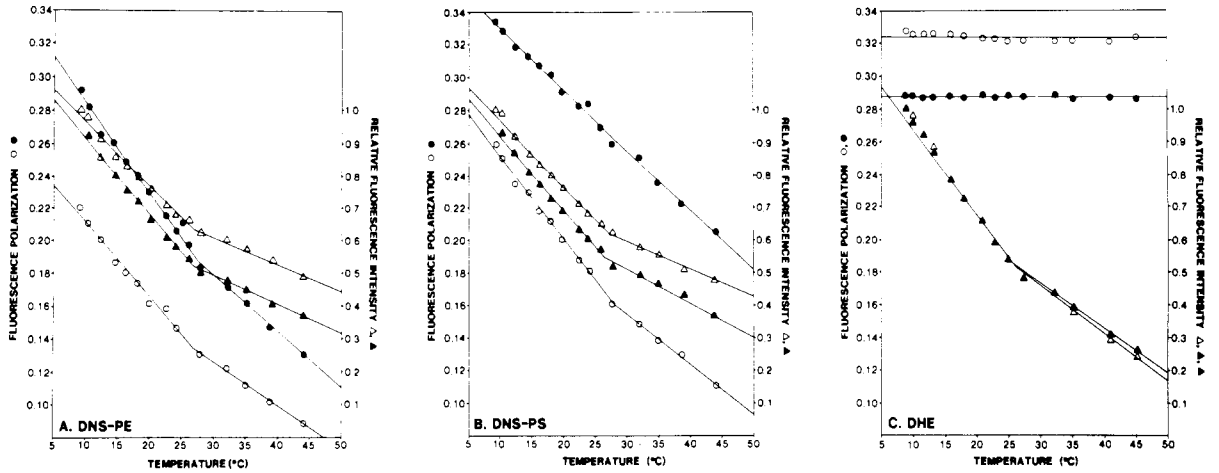

FIGURE 5: Effect of temperature on relative fluorescence intensity (triangles) and polarization (circles) of DNS-PLs and DHE in mixed lipid vesicles (open symbols) and incorporated into Na^+,K^+-ATPase membrane fragments (solid symbols). Symbols represent the mean ± SD of 40 separate values on a single representative preparation. Curves were fitted by linear regression. Correlation coefficients varied from −0.997 to −0.9994 (mean correlation coefficient ± SD = −0.997 ± 0.001). Measurements were corrected for light scatter. Mixed lipid vesicles were made from lipids isolated from *E. electricus* microsomes and consisted of PL:sterol = 1:0.75, where (on a mol % basis) PS = 18% of total PL, PE = 27%, and PC = 53.8–55%. DNS-PL = 1.2% of total vesicle PL, and DHE = 5% total vesicle lipid; 0.6–1.0% of the total Na^+,K^+-ATPase membrane lipid = DNS-PL [Na^+,K^+-ATPase membranes contained 2.09 μmol of total lipid (PL plus cholesterol) per mg of protein]; 6% of the total Na^+,K^+-ATPase membrane lipid = DHE; i.e., 124 nmol of DHE was incorporated into membranes containing 1 mg of Na^+,K^+-ATPase protein. DNS-PL: λ_{ex} = 356, and λ_{em} = 470 nm cutoff. DHE: λ_{ex} = 345 nm, and λ_{em} = 370 nm cutoff.

determined in sonicated lipid vesicles, it was found that the lifetime of DHE was not affected by its concentration in the vesicles (all preparations in Figure 4 had a lifetime of 1.5–2.5 ns) nor by the vesicle phospholipid:sterol ratio. It should be kept in mind, however, that the resolution of lifetime measurements on the SLM 4800 spectrofluorometer is on the order of 1 ns.

The lifetime data gathered on the DNS-PLs were more difficult to interpret due to the contribution of scattered light to the fluorescent intensity of the fluorophores in the membrane preparations. In donor–mixed lipid vesicles in which the DNS-PLs comprised 3–4 mol % of total vesicle lipid and light scatter constituted less than 5% of the fluorescent intensity, DNS-PE and DNS-PS appeared to have a homogeneous lifetime of approximately 12 ns measured by phase and modulation at three modulation frequencies. This homogeneity was likely since τ_θ and τ_M values converged and lifetimes were constant at the three modulation frequencies. In contrast, τ_θ and τ_M for the DNS-PLs incorporated into the membranes at a concentration of 1 mol % of the total lipid, uncorrected for the increased light scatter which constituted 10–15% of the total fluorescence intensity of membrane-incorporated DNS-PL using a 408 nm cut-off filter, diverged, suggesting fluorophore heterogeneity. Membrane preparations were then extracted and the lifetimes of the DNS-PLs determined in vesicles formed from the extracted membrane lipids. τ_θ and τ_M did not approximate the homogeneity seen in donor–mixed lipid vesicles, nor did they approximate the measured lifetime values of the DNS-PLs incorporated into Na^+,K^+-ATPase membrane fragments. Since the effect of light scattering on DNS-PL excited-state lifetime was not quantitatively analyzed, an accurate interpretation of the heterogeneity in the lifetime data could not be made. Vesicle studies examining the effect of DNS-PL concentration of the fluorophore's lifetime revealed that when DNS-PL constituted less than 1–3% of the total vesicle lipid fluorophore excited-state lifetime was also heterogeneous. This would be consistent with the heterogeneity of DNS-PL in the membranes being due to light scattering. More importantly it suggests that the lifetime heterogeneity of DNS-PL in a membrane was not solely an effect of membrane protein.

(*3*) *Temperature Effects.* DNS-PE has been demonstrated to be a reliable indicator of crystal–liquid-crystal transitions in bilayers of PC (Vanderkooi & Change, 1972; Faucon & Lussan, 1973). Specifically the polarization of DNS-PE in sonicated vesicles of dipalmitoylphosphatidylcholine (DPPC) and dimyristoylphosphatidylcholine (DMPC) exhibits breakpoints in polarization vs. temperature plots at the temperatures for the order–disorder transitions of the aliphatic chains of DPPC and DMPC (Ladbrooke & Chapman, 1969). On the basis of these data, the effect of temperature on the fluorescence polarization of the DNS-PLs incorporated into a membrane and in mixed lipid vesicles having the same lipid composition as the membrane was evaluated. Na^+,K^+-ATPase membrane fragments were initially used for these experiments (Figure 5).

The polarization of DNS-PE in Na^+,K^+-ATPase membrane fragments or in vesicles decreased biphasically with increasing temperature (Figure 5A). The polarization of DNS-PS in the two preparations decreased, but a biphasic relationship was observed only when DNS-PS was present in vesicles (Figure 5B). In the Na^+,K^+-ATPase membrane fragments, depolarization of DNS-PS was linear with temperature, without a breakpoint, over a temperature range of 5–45 °C. The DNS-PL preparations that displayed biphasic depolarization with increasing temperature showed a change in slope at 26.0–27.5 °C, just below the 30 °C where Na^+,K^+-ATPase activity was maximized.

Quenching of DNS-PL fluorescence intensity in Na^+,K^+-ATPase membrane fragments and in mixed lipid vesicles was also evaluated as a function of increasing temperature (Figure 5). A change in slope occurred at 26.0–27.5 °C for both DNS-PE and DNS-PS. This breakpoint was independent of the presence of protein since it was exhibited in both Na^+,K^+-ATPase membranes and in lipid vesicles.

Polarization and fluorescence intensity of DNS-PS incorporated into AChE membrane fragments, and erythrocyte ghosts, were also measured as a function of temperature. Both membrane preparations produced data analogous to those of Figure 5B for the Na^+,K^+-ATPase preparation, i.e., fluorescent intensity was quenched biphasically with a change in slope occurring at 26–27 °C (data not shown). DNS-PS in the two

membranes was depolarized linearly with increasing temperature without a change in slope over a temperature range of 5–45 °C.

Rogers et al. (1979) have suggested that the fluorescence intensity of DHE in DPPC bilayers is sensitive to the phase transition in DPPC observed calorimetrically. When fluorescence intensity was plotted as a function of temperature, a marked drop in fluorescence intensity centered at the temperature of the phase transition for DPPC. We determined the effect of temperature on the fluorescence intensity of DHE in Na^+,K^+-ATPase membranes and in mixed lipid vesicles (Figure 5C). As with the DNS-PLs the sterol probe was quenched biphasically with increasing temperature. A change in slope that was independent of the presence of intrinsic membrane protein occurred at 26.0–27.5 °C. The polarization of DHE was not affected by temperature (Figure 5C) whether the sterol was in mixed lipid vesicles or incorporated into Na^+,K^+-ATPase membrane fragments. Although data in Figure 5C are shown only to 50 °C, polarization of DHE was still constant at 65 °C.

(4) Propagation of Intrinsic Protein Conformations to Membrane Lipid. A wealth of literature has emerged in the past decade concerning the regulation of membrane enzymes by lipids [review by Sandermann (1978)]. In this regard, membranous Na^+,K^+-ATPase has been extensively studied as an enzyme whose activity depends upon the presence of lipid. Parallel efforts have been directed toward using fluorescent probes bound to the Na^+,K^+-ATPase to demonstrate conformational changes in the enzyme in response to cation binding and phosphorylation of the enzyme. Attempting to synthesize the direction of the Na^+,K^+-ATPase work into the whole of how membrane enymes might be regulated by lipids, Gupte et al. (1979) used the fluorescent lipid probes DNS-PE and 12-(anthroyl)stearate to detect conformational changes in the Na^+,K^+-ATPase that might be propagated to boundary or bulk lipid. Although their results were negative, it could not be concluded that specific changes in the conformation of the Na^+,K^+-ATPase were not propagated to boundary lipid because (1) the locations of the fluorescent probes were not well characterized, (2) the Na^+,K^+-ATPase used in the study had been purified with detergents (a procedure potentially disruptive to native lipid organization), and (3) only fluorescence intensity and $\lambda_{em_{max}}$ were evaluated.

In the present investigation we looked for changes in the fluorescent parameters of DNS-PE, DNS-PS, and DHE incorporated into Na^+,K^+-ATPase membrane fragments with the addition of ligands reported to induce conformational changes in the Na^+,K^+-ATPase. Specifically, the following ligands were added individually and in combination to Na^+,K^+-ATPase membrane fragments incorporated with a fluorescent lipid: $MgCl_2$, NaCl, and Tris–ATP; $MgCl_2$, ouabain, and P_i; KCl. Ligands were added at concentrations used in the assay of Na^+,K^+-ATPase acty (see Materials and Methods). Fluorescence intensity, polarization, and excited-state lifetimes were determined. Intensity and polarization were determined at various temperatures between 5 and 45 °C. No significant changes in the fluorescent parameters of the probes were induced by the ligands. Insignificant changes in fluorescence intensity and polarization (less than 10% deviation from the initial value) were measured in the DNS-PLs with 5–10 mM $MgCl_2$. However, this was not specific to the Na^+,K^+-ATPase membrane preparation since these same changes were observed in donor–mixed lipid vesicles.

The argument could be made that fluorescent lipids were distantly located from the Na^+,K^+-ATPase and that therefore minor changes in the conformation of the enzyme would not be expected to affect their fluorescent parameters. We believe this to be unlikely because resonance energy transfer from tryptophan to both DNS-PLs[3] and quenching of tryptophan by DHE[4] were demonstrated, suggesting that at least a fraction of each of the fluorescent lipids was a close proximity to the Na^+,K^+-ATPase.

Discussion

Before any fluorescent probe can be useful in reporting on the properties of a system, it is essential to know its location within the system. The DNS-PLs and DHE were considered to be incorporated into membrane fragments on the basis of the known mode of action of PLEPs and through the use of a nonexchangeable lipid marker. By PL monolayer techniques, PLEPs have been demonstrated to extract and insert PL molecules into membranes (Wirtz, 1974; Demel et al., 1973). This activity is assayed by measuring the transfer of radiolabeled PL or cholesterol from sonicated unilamellar vesicles to another membrane system that can be separated from the vesicles by centrifugation. Triglyceride, labeled with another isotope, is included in the sonicated vesicles. Since PLEPs do not transfer this class of lipid (Crain & Zilversmit, 1980), triglyceride serves as a nonexchangeable marker. The transfer of PL was determined from the decreased ratio of labeled PL to labeled triglyceride in the supernatant after incubation with PLEP. We assumed that the DNS-PLs and DHE were incorporated into the membrane preparations when fluorophores pelleted with the Na^+,K^+-ATPase without the nonexchangeable [^{14}C]triolein.

In theory, the "incorporation" of fluorescent lipids into membranes by PLEPs was straightforward. However, the nature of the membrane, the stability of the PLEP preparation, and the charge on the fluorophore created technical difficulties exemplified in Table I with DNS-PE and Na^+,K^+-ATPase membranes.

The amount of DNS-PLs that could be incorporated into the membranes was limited primarily by the nonspecific adherence of vesicles to the membranes that resulted when vesicle PL composition consisted of DNS-PE or DNS-PS in excess of 6.5% of the total PL or 4.5% of the total PL, respectively. Vesicle adherence was not a problem when DHE was incorporated into the membranes and thereby the concentration of the fluorescent sterol within a membrane preparation was easily manipulated. Furthermore, we predict that if one were to use PLEPs to incorporate PLs with fluorescent parinaric acid esterified onto the fatty acyl positions of the glycerol backbone, vesicle adherence would be minimal, provided that neutral PLs were used. If this were true, then the amount of

[3] Resonance energy transfer was demonstrated from the tryptophan residues of the Na^+,K^+-ATPase to both DNS-PE and DNS-PS on the excitation spectra of the DNS-PLs incorporated into Na^+,K^+-ATPase membranes. λ_{em} was held constant at 500 nm while λ_{ex} was varied. A peak at λ_{ex} = 292 nm was observed in both DNS-PL–Na^+,K^+-ATPase preparations but not in DNS-PL lipid vesicles. This peak in the excitation spectra resulted from energy transferred from protein tryptophan to DNS-PLs. The ratios of the fluorescence intensities, λ_{ex} = 292 nm, λ_{em} = 500 nm:λ_{ex} = 356 nm, λ_{em} = 500 nm, for DNS-PE and DNS-PS incorporated into a given Na^+,K^+-ATPase membrane preparation were identical but varied with the preparation ranging from 0.28 to 0.43.

[4] Although resonance energy transfer between tryptophan and DHE was not observed on excitation spectra of DHE incorporated into Na^+,K^+-ATPase membranes, quenching of tryptophan fluorescence by DHE was demonstrated. The quenching of tryptophan was dependent upon DHE concentration and adhered to a plot of the Stern–Volmer equation as modified by Lehrer (1971)[2] with a correlation coefficient = 0.9993. f_a = 0.813, and $1/(k\tau_0)$ = 0.134.

the parinaric acid PL derivatives that could be incorporated into membranes should be easily modulated, as was the case for DHE. In other words, if fluorescent probes that minimize vesicle adherence are selected, the concentration of the fluorophore that can be incorporated into the membrane can be readily varied.

The lipid fluorophores, DNS-PE, DNS-PS, and DHE, offer several advantages for the interpretation of their fluorescence in terms of membrane protein–lipid interactions. First, perturbation of Na^+,K^+-ATPase membrane by the probes is minimal. Two criteria for excluding gross membrane perturbations due to the presence of the probes are the preservation of membrane protein function after incorporation of fluorophores and the use of fluorescent analogues of natural membrane components (Azzi, 1975). With respect to the latter, structurally the DNS-PLs resemble their nonfluorescent lipid analogues PE and PS, being derivatized from these PLs. DNS-PE and DNS-PS maintained the amphipathic structure required to form vesicles in an aqueous environment. DHE is a naturally occurring sterol and as demonstrated by Rogers et al. (1979) is able to replace the native sterols in the membranes of the protozoan, *T. pyriformis*, without any deleterious effect on the growth of the organism. Preservation of enzyme activity as a criteria for the exclusion of membrane perturbation was also demonstrated. At least 80% and usually 90% of Na^+,K^+-ATPase enzymatic activity was retained after incorporation of lipid fluorophores. A second advantage in using the DNS-PLs and DHE for the interpretation of fluorescence data is that the fluorophores can be transferred from a sonicated vesicle to a membrane by using PLEP, thereby providing a basis for defining prove location as incorporated into the membrane. Third, the DNS-PLs represent a pair of fluorophores that differ only by a carboxyl group, providing a means for asking how PL head-group charge could influence protein–lipid interactions. Finally, investigating protein–lipid interactions using DNS-PLs and DHE provides data on two distinct regions of Na^+,K^+-ATPase membrane fragments, the glycerol backbone and the hydrophobic sterol core, respectively. Iodide quenching data supported these fluorophore locations.

After data on the incorporation and localization of DNS-PE, DNS-PS, and DHE in Na^+,K^+-ATPase membranes were presented, the sequela was to determine how these fluorescent lipids can be used as membrane probes. We have shown that the fluorescence polarization of both DNS-PE and DNS-PS in a bilayer is increased by the presence of intrinsic membrane protein and that this increased polarization is reproducibly characteristic of a given membrane preparation. The issue arises whether we are looking at an interaction of the dansyl moiety with protein or whether the polarization data reflect an interaction between lipid and protein. We believe that the latter is so for the following reasons: (1) DNS-PLs were shown to be "incorporated into" membranes with PLEP, as opposed to simply adhering to the membrane surface, without a change in the activity of a membrane enzyme marker. Therefore, even if the dansyl moiety is interacting with the membrane proteins, its interaction is presumably occurring within the boundary or bilayer structure of the membrane and should thereby reflect restraints conferred by the phospholipid portion of the molecule. In addition, if the dansyl group were binding directly to the protein, one might predict modulation of protein activity, which clearly does not occur. (2) We compared the increase in the DNS-PL polarization attributed to the presence of intrinsic membrane protein with the polarization of the probes in the presence of the PLEP, a soluble nonmembranous protein with which the DNS-PLs obviously interact. Both DNS-PE and DNS-PS in sonicated mixed lipid vesicles exhibited increases in polarization in the presence of PLEP, but this increase was small compared to that of the membrane preparations. For example, in one representative experiment where protein (as PLEP) to lipid ratios and total component concentrations approximated those of DNS-PL-containing membranes, the polarization of DNS-PE in vesicles increased 10.6% (from 0.1746 ± 0.0015 to 0.1931 ± 0.0008) with the addition of PLEP, and similarly for DNS-PS, polarizations increased 6.6% (from 0.2124 ± 0.0012 to 0.2264 ± 0.0004). Clearly these increases, although significant, are lower than the 40–65% increases in polarization found when the fluorophores were present in membranes and in vesicles formed from membrane lipid extracts (Figure 3).

If the fluorescence data from the DNS-PLs actually reflect the protein–lipid interactions of their nonfluorescent precursors, PE and PS, then the effect of the PL head-group charge on these interactions can be assessed since DNS-PE and DNS-PS differ in structure only by a carboxyl group which at physiological pH confers a negative charge to the PL. When incorporated into membranes, the DNS-PLs were clearly distinguished by two fluorescent parameters that were related to the presence of protein in the membrane: fluorescence polarization (Figure 3) and temperature depolarization (Figure 4).

The fluorescence polarization of DNS-PS was greater than that of DNS-PE in both vesicles and membranes (Figures 3 and 4). However, if the difference between the polarization of DNS-PE and DNS-PS in vesicles of extracted membrane lipids is compared to the polarization difference between the two probes in the membrane preparation (Figure 3), the latter is greater than the former. This suggests that in addition to the factors determining DNS-PL polarization in vesicles, membrane proteins selectively restrict the motion of DNS-PS to a greater extent than they restrict DNS-PE.

The temperature depolarization curves of DNS-PE incorporated into the membrane preparations were biphasic (Figure 5A); those of DNS-PS were monophasic (Figure 5B). However, in vesicles both DNS-PLs produced biphasic temperature depolarization curves with breakpoints at 26.0–27.5 °C. This suggests that interaction with integral membrane protein prevents the biphasic depolarization of DNS-PS.

The fluorescent properties of DHE in a lipid bilayer were not affected by the presence of intrinsic membrane protein, despite a close association between the sterol and tryptophan residues which resulted in the quenching of protein fluorescence.[4] Specifically, the increased fluorescence polarization of DHE associated with its incorporation into a membrane (Figures 3 and 4) was accounted for by dilution, the lifetime of DHE in a membrane was homogeneous and identical with the lifetime of DHE in sonicated lipid vesicles, and the polarization of DHE in both membranes and vesicles was unaffected by temperature (Figure 5). If DHE is an appropriate structural and functional analogue of cholesterol (Rogers et al., 1979), then one interpretation of the fluorescence data from the sterol is that membrane proteins have little affect on the structural organization of cholesterol and that cholesterol is a notably stable component in a bilayer. A limitation to this interpretation involves the question of whether or not the fluorescence of DHE is actually sensitive to lipid environments. For example, it has been suggested that the reason the polarization of DHE does not change as a function of temperature is that the excited-state lifetime is short relative to the rotational correlation time of DHE in a bilayer. If this is true, it still implies that the rotational motion of the

fluorescent sterol is restricted in a membrane. In addition, it should be noted that Rogers et al. (1979) have demonstrated that the fluorescence intensity of DHE is sensitive to a phase transition in the aliphatic chains of saturated PLs. Furthermore, procedures that disrupt bilayer structure, e.g., organic solvent extraction, depolarized the fluorophore.

In our assessment of the DNS-PLs and DHE as membrane probes, we did not evaluate the emission wavelength maxima, although it is well established that the dansyl group exhibits a large red shift on going from a nonpolar to a polar environment (Waggoner & Stryer, 1970). Instrumentation limitations did not allow us to accurately assess emission spectra of the DNS-PLs in the membrane preparations. The emission spectra of DHE did not exhibit shifts in maxima in solvents that affect DNS-PL emission. Also, we did not quantiatively assess energy transfer from protein tryptophan to the DNS-PLs. Recently, Ghiggino et al. (1981) have demonstrated that the fluorescence spectrum of DNS-PE incorporated into lipid bilayers is time dependent, which thereby complicates the interpretation of any fluorescence quenching or fluorescence energy transfer experiments.

Supplementary Material Available

Detailed synthesis of DNS-[^{3}H]PE and DNS-[^{3}H]PS from egg yolk PC (12 pages). Ordering information is given on any current masthead page.

Registry No. DHE, 516-85-8; iodide, 20461-54-5; phospholipase D, 9001-87-0; PS decarboxylase, 9054-78-8; cytidine 5′-monophosphoromorpholidate, 76742-17-1; L-[^{3}H(G)]serine, 83245-15-2.

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