rovertin, 11002-90-7; efrapeptin, 56645-91-1; sodium azide, 26628-22-8.

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Interactions of Cholesterol Hemisuccinate with Phospholipids and $(Ca^{2+}$-$Mg^{2+})$-ATPase†

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ABSTRACT: Cholesterol hemisuccinate has been shown to equilibrate readily with liposomes and with the $(Ca^{2+}$-$Mg^{2+})$-ATPase from sarcoplasmic reticulum and has been used to modify the sterol content of these membranes. Cholesterol hemisuccinate incorporates into dioleoylphosphatidylcholine (DOPC) up to a molar ratio of 3:1 sterol to DOPC. Effects on lipid order as detected by electron spin resonance and fluorescence polarization are comparable to those of cholesterol. Binding constants have been determined, and the uncharged form of the sterol binds more strongly than the anionic form. Binding to DOPC and to the lipid component of the ATPase system is comparable. From use of the fluorescence quenching properties of 1,2-bis(9,10-dibromooleoyl)phosphatidylcholine and dibromocholesterol hemisuccinate, two classes of binding sites on the ATPase have been deduced. At the lipid/protein interface, the binding constant for cholesterol hemisuccinate is considerably less than that for DOPC. At the second set of sites (nonannular sites), binding occurs with K_d = 0.55 in molar ratio units. The effect of cholesterol hemisuccinate on the activity of the ATPase depends on the phospholipid present in the system: ATPase reconstituted with DOPC is inhibited whereas ATPase reconstituted with dimyristoleoylphosphatidylcholine is activated. We conclude that changes in membrane fluidity are not important in determining ATPase activity in these systems.

Membrane fluidity has been thought to be an important determining factor in a variety of membrane processes (Shinitzky & Henkart, 1979; Kates & Kuksis, 1980). It is known that many organisms alter the phospholipid compositions of their membranes in response to a variety of environmental changes, and it has been suggested that these changes occur in order to maintain an optimal fluidity for the membrane (Sinensky, 1974, 1980). A classic example is provided by the increase in saturation of the phospholipid acyl chains in bacteria with increasing growth temperature (McElhaney, 1982; Melchior, 1982). Cholesterol is also postulated to be

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an important factor in determining membrane fluidity since addition of cholesterol to simple phospholipid bilayers in the liquid-crystalline state is known to cause a marked decrease in fluidity (Demel & de Kruijff, 1976). Thus, for example, Shinitzky & Inbar (1974) reported a lower level of cholesterol in malignant lymphoma cells than in normal lymphocytes, which they correlated with the higher membrane fluidity in the lymphoma cells as measured by the fluorescence polarization of the probe diphenylhexatriene. The changes in fluidity were postulated to be important in the development of leukemia. These ideas have been summarized in the concept of "homoviscous adaptation" (Sinensky, 1974). In response to an environmental challenge, an organism can increase the fluidity of its membrane by decreasing the average chain length or increasing the average unsaturation of its phospholipid acyl chains or by decreasing the cholesterol content of its membranes. Correspondingly, membrane fluidity can be decreased by the opposite changes. According to the concept of homoviscous adaptation, the response of a cell to an environmental challenge will be to attempt to restore the fluidity of its membrane to an optimal value through the appropriate changes in the phospholipid and cholesterol content of its membrane.

It has been suggested that one reason why lipid fluidity is important is because the rate of operation of membrane proteins is determined to a large extent by the viscosity of the membrane (Yuli et al., 1981). Recent studies on reconstituted membrane protein systems, however, provide little support for this suggestion. Studies with the $(Ca^{2+}$–$Mg^{2+})$-ATPase suggest that enzyme activity is determined predominantly by the chemical structure of the surrounding phospholipids rather than by their fluidity, as long as the phospholipids remain in the liquid-crystalline phase (Warren et al., 1974; Johannsson et al., 1981; London & Feigenson, 1981; East & Lee, 1982). Thus, the activity of the $(Ca^{2+}$–$Mg^{2+})$-ATPase reconstituted with dimyristoleoylphosphatidylcholine is only ca. 20% of that reconstituted with dioleoylphosphatidylcholine (Johannsson et al., 1981; Simmonds et al., 1982) although the shorter chain lipid is expected to be the more "fluid". Further, the activities of the ATPase reconstituted with dimyristoleoylphosphatidylcholine and dimyristoylphosphatidylcholine are very similar at 37 °C (East & Lee, 1982; Simmonds et al., 1982) so that introduction of a double bond into the phospholipid fatty acyl chain has little effect on activity as long as the phospholipids are in the liquid-crystalline phase [the activity of the ATPase is markedly less if the surrounding phospholipid is in the gel phase (Warren et al., 1974)].

Studies with these reconstituted systems also suggest that effects of cholesterol on enzyme activity cannot be attributed solely to effects on fluidity. Thus, cholesterol has little effect on the activity of the ATPase reconstituted with dioleoylphosphatidylcholine and causes a large *stimulation* of the ATPase reconstituted with dimyristoleoylphosphatidylcholine (Simmonds et al., 1982).

Since cholesterol has been so widely used to alter membrane fluidity, we wished to study these effects in more detail. Unfortunately, because of its low aqueous solubility, it is not easy to modify the cholesterol content of a membrane. A variety of methods have been used including exchange from cholesterol-loaded liposomes or lipoproteins and detergent reconstitution, but all are difficult to quantitate. An alternative is to use water-soluble derivatives of cholesterol that will partition directly into the membrane from aqueous solution.

The esters cholesterol hemisuccinate and cholesterol betainate have been used in this way (Heron et al., 1980; Efrati et al., 1980; Yuli et al., 1981). Here we characterize the interaction between cholesterol hemisuccinate and lipid bilayers and use the fluorescence quenching properties of brominated derivatives of phospholipids and cholesterol hemisuccinate to study binding to the $(Ca^{2+}$–$Mg^{2+})$-ATPase purified from sarcoplasmic reticulum of rabbit skeletal muscle.

Competition between sterols and phospholipids for binding sites at the phospholipid/protein interface (annular sites) can be studied by using the fluorescence quenching properties of 1,2-bis(9,10-dibromooleoyl)phosphatidylcholine (BRPC)[1] (East & Lee, 1982; Simmonds et al., 1982). The $(Ca^{2+}$–$Mg^{2+})$-ATPase contains a large number of tryptophan residues in hydrophobic regions of the protein structure whose fluorescence can be quenched by hydrophobic compounds, and the fluorescence intensity of the tryptophan residues in the ATPase reconstituted with BRPC by the lipid titration procedure is 40% of that for the ATPase reconstituted with dioleoylphosphatidylcholine (DOPC) (East & Lee, 1982). Displacement of BRPC from annular sites on the ATPase by non-brominated sterols reduces fluorescence quenching, which can be quantitated in terms of binding constants.

Studies with fluorescence probes have also detected a small number of binding sites for hydrophobic molecules on the ATPase distinct from the annular sites (Lee et al., 1982). The presence of such nonannular sites has also been deduced from fluorescence quenching studies with brominated derivatives of fatty acids and cholesterol (Simmonds et al., 1982).

Materials and Methods

Cholesterol (BDH) was recrystallized from diethyl ether/acetone. Egg yolk phosphatidylcholine and dioleoylphosphatidylcholine (DOPC) were from Lipid Products, cholesterol hemisuccinate was from Sigma, and nitroxide-labeled fatty acids were from Syva. Cholesterol was brominated to give 5,6-dibromocholestan-3β-ol as described in Simmonds et al. (1982). Cholesterol hemisuccinate was brominated in diethyl ether/acetic acid (2:1 v/v) in the presence of anhydrous sodium acetate. After rotary evaporation of the diethyl ether and addition of water, the product 5,6-dibromocholestan-3β-ol hemisuccinate was extracted into chloroform/methanol and recrystallized from acetone by addition of water. The product was characterized by bromine analysis and thin-layer chromatography [petroleum ether (60–80 °C)/acetone, 9:2 v/v]. The specific optical rotation, α_D, of cholesterol hemisuccinate in chloroform was −31°, and for 5,6-dibromocholestan-3β-ol hemisuccinate, $\alpha_D = -41°$. These values can be compared to the corresponding values of −37 and −43.4° for cholesterol and 5,6-dibromocholestan-3β-ol, respectively (Barton & Miller, 1950; Simmonds et al., 1982), and confirm that the product is in the planar trans-diaxial (5α,6β) form: the kinked trans-diequatorial (5β,6α) form of 5,6-dibromocholestan-3β-ol shows an α_D of +47° (Barton & Miller, 1950). Dioleoylphosphatidylcholine was brominated to give 1,2-bis(9,10-dibromooleoyl)phosphatidylcholine (BRPC) as described in East & Lee (1982). Dimyristoleoylphosphatidylcholine (DMPC) was synthesized by the method of Patel et al. (1979) with the fatty acid anhydride synthesized by the method of Selinger & Lapidot (1966). Radiolabeled cholesterol hemisuccinate was prepared by refluxing cholesterol (100 mg) doped with

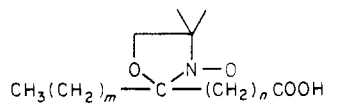
[1] Abbreviations: DOPC, dioleoylphosphatidylcholine; BRPC, 1,2-bis(9,10-dibromooleoyl)phosphatidylcholine; DMPC, dimyristoleoylphosphatidylcholine; DPH, diphenylhexatriene; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*',*N*'-tetraacetic acid; ESR, electron spin resonance; Tris, tris(hydroxymethyl)aminomethane.

[26(27)-^{14}C]cholesterol (Amersham) with succinic anhydride (300 mg) in dry pyridine (20 mL) for 20 h. After addition of water and acidification, the product was extracted into diethyl ether. The ether solution was washed with saturated $NaHCO_3$, and the aqueous phase was separated, acidified to pH 5, and reextracted with diethyl ether. The product was recrystallized twice from acetone/water and ran as a single spot on thin-layer chromatography (chloroform/methanol/water/ammonia, 65:35:3:1 by volume). Radiolabeled phosphatidylcholine was prepared from egg yolk phosphatidylethanolamine (Lipid Products) and [^{3}H]methyl iodide (Amersham) by the method of Smith et al. (1977) and purified by preparative thin-layer chromatography with a solvent system of chloroform/methanol/water/acetic acid (65:35:7:3 by volume). *N*-Palmitoyl-L-tryptophan *n*-hexyl ester was prepared by esterification of tryptophan with thionyl chloride in hexanol followed by coupling to palmitic acid with *N*,*N*'-dicyclohexylcarbodiimide.

(Ca^{2+}–Mg^{2+})-ATPase was purified from hind leg muscle of rabbit as described in East & Lee (1982). The final preparation contained 30 lipid molecules per ATPase, under the assumption of a protein molecular weight of 115000. Lipid substitutions, measurement of ATPase activity, and fluorescence measurements were made as in East & Lee (1982) and Simmonds et al. (1982).

Fluorescence polarization measurements were made with an Aminco-Bowman spectrofluorometer, equipped with quartz Polacoat filters. Lipid (100 μM), diphenylhexatriene (DPH, 1 μM), and the appropriate concentration of cholesterol hemisuccinate dissolved in chloroform/methanol were mixed and evaporated to dryness under a stream of nitrogen. Buffer (40 mM HEPES, 0.1 M NaCl, and 0.1 mM EGTA, pH 7.2 at 37 °C) was added and the mixture shaken for 30 min at 37 °C to allow equilibration before measurement. ATPase was labeled with DPH by incubation for 60 min at room temperature in buffer (40 mM HEPES, 0.1 M NaCl, and 1 mM EGTA, pH 7.2 at 37 °C). Aliquots of cholesterol hemisuccinate were added from a 10 mM stock solution in methanol and allowed to equilibrate for 20 min before measurements.

ESR spectra were run on a Bruker ER 200D spectrometer, interfaced to a Cromemco microcomputer system for data analysis. The spin-labeled (*m*,*n*)stearic acids were added and



incubations with cholesterol hemisuccinate carried out essentially as described for DPH. Order parameters were calculated with the corrected method given by Gaffney (1976).

Binding of radiolabeled cholesterol hemisuccinate to liposomes and to ATPase was determined by a centrifugation assay. A number of water-soluble cholesterol esters have been shown to form liposomes in the absence of phospholipid (Brockerhoff & Ramsammy, 1982). In agreement, we find that prolonged centrifugation of dispersions of cholesterol hemisuccinate in water produces pelleting. In the centrifugation assay of binding to phospholipids, it was therefore necessary to use low concentrations of cholesterol hemisuccinate and short centrifugation times. Under these conditions, a firm pellet of phospholipid was not obtained. The procedure adopted was therefore as follows. The appropriate concentration of [^{14}C]cholesterol hemisuccinate was mixed with [^{3}H]phosphatidylcholine (1.3 μM) in buffer (8 mL; 10 mM potassium phosphate, 10 mM NaCl, and 0.1 mM EGTA, pH 7.2) and incubated for 45 min at 20 °C. The sample was then centrifuged at $100000g_{av}$ for 20 min, and three samples were taken for counting: the upper 7 mL of supernatant, the bottom 1 mL of supernatant (including pelleted material), and finally a methanol rinse of the tube. Controls to determine the extent of cholesterol hemisuccinate pelleting and binding to the tubes were carried out as above in the absence of phosphatidylcholine, and were found to be between 3 and 14% and 4 and 10%, respectively, over the concentration range employed. The proportion of phosphatidylcholine pelleted decreased slightly with increasing concentration of cholesterol hemisuccinate from 91 to 85%. Binding of cholesterol hemisuccinate to the ATPase was studied in an essentially similar manner, except that smaller volumes (150 μL) were used and centrifugation was performed in 5 × 20 mm cellulose–propionate tubes in a Beckman airfuge.

Measurements of electrophoretic mobility were made on a Rank Brothers Mark 1 microelectrophoresis apparatus. Care was taken to focus at the stationary layer.

Analysis of Binding to Lipid. We have shown elsewhere (Rooney et al., 1983) that, for a negatively charged compound that mixes randomly with lipid, binding can be described by a pair of Langmuir adsorption isotherms:

$$\sigma^A = (\sigma^{max} - \sigma^A - \sigma^{HA})[A^-]_{x=0}/K^A \quad (1)$$

$$\sigma^{HA} = (\sigma^{max} - \sigma^A - \sigma^{HA})[HA]_{x=0}/K^{HA} \quad (2)$$

where σ^A and σ^{HA} are, respectively, the number of molecules of the charged form of the compound, A^-, and of the uncharged form, HA, adsorbed to the membrane per unit area; σ^{max} is the maximum number of molecules adsorbed per unit area; K^A and K^{HA} are dissociation constants for binding of A^- and HA, respectively, and $[A^-]_{x=0}$ and $[HA]_{x=0}$ are the aqueous concentrations of A^- and HA, respectively, at the membrane solution interface, $x = 0$. The relative proportions of A^- and HA in the aqueous phase are given by the Henderson–Hasselbalch equation:

$$pH = pK + \log([A^-]/[HA]) \quad (3)$$

The two dissociation constants for binding are related by (Lee, 1978)

$$K^A/K^{HA} = \exp(2.303\Delta pK) \quad (4)$$

The concentration of HA close to the surface will be equal to the bulk concentration, but the concentration of A^- at the surface will be less than the bulk concentration of A^- because of the negative charge on the membrane that results from the binding of A^-. The charge effect can be described by the Boltzmann relationship:

$$[A^-]_{x=0} = [A^-]_{bulk} \exp[F\psi_0/(RT)] \quad (5)$$

where ψ_0 is the electrostatic potential in the aqueous phase adjacent to the membrane. Elsewhere (Rooney et al., 1983), we detail how ψ_0 can be related to σ^A and how electrophoretic mobilities can be used to calculate ψ_0 and thus the binding constants K^A and K^{HA}. Binding of Na^+ to the bound anions will reduce the negative surface charge density, σ^-, according to

$$\sigma^A = \sigma^-(1 + K_a[Na^+]_{x=0}) \quad (6)$$

where

$$[Na^+]_{x=0} = [Na^+]_{bulk} \exp[-F\psi_0/(RT)] \quad (7)$$

Analysis of the electrophoresis data for the interaction of cholesterol hemisuccinate with phospholipid bilayers will be more complex if the mixtures formed are nonideal. In par-

ticular, for cholesterol there is evidence for clusters of cholesterol–phospholipid complex separating from regions of pure phospholipid [see Rogers et al. (1979) and Presti et al. (1982)]. Such clustering for cholesterol hemisuccinate will, by producing regions of high surface potential within the membrane, significantly alter the pattern of binding. The simplest approach to this problem is to assume equilibrium between sterol molecules in clusters and dispersed randomly in the bulk phospholipid phase, described by a simple partition coefficient, K_p:

$$[HA]_c = K_p[HA]_b \quad (8)$$

$$[A]_c = K_p[A]_b \exp[(\psi_c - \psi_b)F/(RT)] \quad (9)$$

where subscripts c and b represent concentrations of HA and A^- in clusters and bulk phospholipid phases, respectively. The partition of the anionic species, A^-, will be modified by the difference in surface potential between the cluster, ψ_c, and the bulk phospholipid, ψ_b. The surface potential in the clusters is easily calculated if the clusters are of a sufficient size for "edge effects" to be ignored, since the surface charge density in the cluster depends only on the relative molecular areas of sterol and phospholipid molecules and their stoichiometry and on the pH, which determines the degree of ionization (Rooney et al., 1983).

The concentration of bound sterol participating in cluster formation can be calculated as follows. The apparent pK's of membrane-bound molecules depend on the surface potential according to

$$pK_b = pK + \Delta pK - \psi_b F/(2.303RT) \quad (10)$$

$$pK_c = pK + \Delta pK - \psi_c F/(2.303RT) \quad (11)$$

where pK_b and pK_c are the apparent pK's in the bulk phospholipid and in the cluster, respectively. If we let

$$C_c = 10^{pH-pK_c}/(1 + 10^{pH-pK_c}) \quad (12)$$

and

$$C_b = 10^{pH-pK_b}/(1 + 10^{pH-pK_b}) \quad (13)$$

we can write

$$[A]_b = ([D]_0 - [A]_c/C_c)C_b \quad (14)$$

where $[D]_0$ is the total concentration of bound sterol. From (9), we have

$$[A]_c = K_p[D]_0 C_b \alpha/(1 + K_p \alpha C_b/C_c) \quad (15)$$

where

$$\alpha = \exp[(\psi_c - \psi_b)F/(RT)] \quad (16)$$

The total sterol concentration $[D]_c$ in the cluster is therefore

$$[D]_c = [A] + [HA] = [A]_c/C_c \quad (17)$$

and the total sterol concentration $[D]_b$ in the bulk phospholipid phase is

$$[D_b] = [D]_0 - [D]_c \quad (18)$$

The completely general case of partition of sterol between aqueous phases, clusters, and bulk phospholipid phases is mathematically intractable. However, for cholesterol hemisuccinate we show below that at least 99% of the sterol will be bound under the conditions of the electrophoresis experiment, so that $[D]_0$ can be set equal to the total sterol concentration. In the electrophoresis experiment, an average ζ potential is measured, which is related (Rooney et al., 1983) to the average surface charge density, σ^-_{av}, given by

$$\sigma^-_{av} = [(\sigma^-_b \times \text{area}_b) + (\sigma^-_c \times \text{area}_c)]/(\text{area}_b + \text{area}_c) \quad (19)$$

where area_c and area_b are, respectively, the areas (Å^2) of the cluster and bulk phospholipid regions of the membrane surface and σ^-_c and σ^-_b are the surface charge densities in the cluster and bulk phospholipid, respectively. For any given value of K_p, σ^-_{av} can be calculated by a simple iterative method by assuming a particular sterol to phospholipid stoichiometry in the clusters if molecular areas are known. This determines σ^{max} in the cluster, and ψ_c may be calculated (Rooney et al., 1983). A value for ψ_b, the surface potential in the bulk lipid, is estimated, and eq 15–17 are solved to give the total concentration of sterol participating in cluster formation. If all the sterol present is membrane bound, then eq 18 applies, and $[A]_b$ may be calculated from eq 14. The surface charge density in the bulk lipid, σ^-_b, is calculated, and ψ_b is recalculated with the Grahame equation (Rooney et al., 1983). The initial estimate of ψ_b is adjusted until there is no significant difference between successive estimations. The average surface potential, ψ_{av}, is calculated with the Grahame equation, and ζ potentials are calculated from ψ_{av} by assuming that the hydrodynamic plane of shear at which the ζ potential is measured is 2 Å from the plane of the membrane surface charges (Rooney et al., 1983).

These equations are too complex to allow nonlinear least-squares fitting to the experimental data. Rather, calculated curves for ζ potentials vs. concentration were compared visually to the experimental data: variations in the fitted values by more than 10% from the given values result in an obviously worse fit.

In a medium of high ionic strength, where charge effects can be ignored, binding to phospholipid bilayers can be described by an effective dissociation constant, K_d^{eff}:

$$K_d^{eff} = [\text{lipid}]^{free}[D]^{free}/[D]^{bnd} \quad (20)$$

where $[D]^{free}$ and $[D]^{bnd}$ are, respectively, the total free and membrane-bound concentrations of sterol and $[\text{lipid}]^{free}$ is the number of unoccupied binding sites in the phospholipid bilayer. For unlimited binding (simple partition), $[\text{lipid}]^{free}$ in eq 20 is put equal to the total phospholipid concentration.

Binding to the ATPase. In previous studies (Lee et al., 1982, 1983; Simmonds et al., 1982), we have analyzed drug binding to the ATPase system in terms of binding to the lipid component and to two classes of site on the ATPase, referred to as annular and nonannular sites. Under conditions of high ionic strength, binding to lipid can be described by eq 20. Since, as will be seen, under the conditions of our experiment most of the added sterol is present in the lipid phase of the membrane, the obvious concentration unit in which to describe binding to protein sites is the molar ratio of sterol in the lipid component of the membrane, x^D (choice of concentration unit is equivalent to choice of thermodynamic standard state). For the annular class of protein sites, we then have

$$K_d^{ann} = [S]_{ann}^{free} x^D/[S]_{ann}^{bnd} \quad (21)$$

where $[S]_{ann}^{free}$ and $[S]_{ann}^{bnd}$ refer to the concentrations of unoccupied and occupied annular sites, respectively (expressed as moles per liter). A corresponding equation describes binding to nonannular sites. The above set of transcendental equations describing binding can be solved numerically by the Bolzano method (McCormick & Salvadori, 1964).

Results

Interaction with Phospholipid Bilayers. Figure 1A shows the results of a centrifugation assay of the binding of chole-

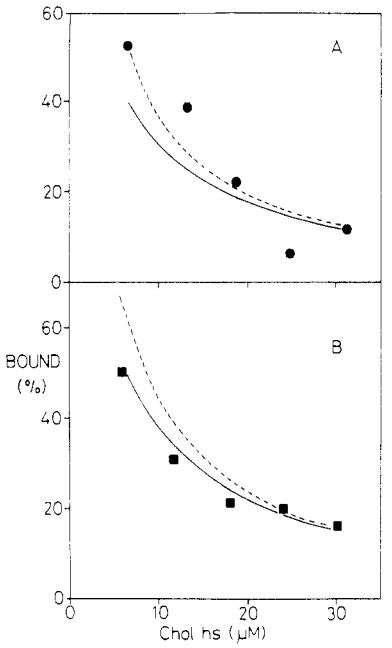


FIGURE 1: Binding of [^{14}C]cholesterol hemisuccinate to (A) phosphatidylcholine (1.33 μM) and (B) (Ca^{2+}–Mg^{2+})-ATPase (0.044 μM protein, equivalent to 1.33 μM phospholipid). In (A), the solid and dashed lines are calculated binding curves for K_d^{eff} = 2 and 0.5 μM, respectively, with a sterol to phospholipid stoichiometry of 3:1. The dotted line represents unlimited binding (simple partition) with K_d^{eff} = 2 μM. In (B), for the ATPase, the corresponding calculated binding curves all included binding to the ATPase, with 30 annular and 3 nonannular sites, with K_d values (in molar ratio units) of 2.0 and 0.55, respectively (see text). The estimated error in the measurement of percent bound cholesterol hemisuccinate is ±7%.

sterol hemisuccinate to phosphatidylcholine and a fit to the simple binding equation (eq 20) with K_d^{eff} = 0.5 μM and a stoichiometry for binding of cholesterol hemisuccinate to phospholipid of 3:1. Less good fits to the data are obtained with binding stoichiometries of 2:1 and 4:1 but, because of the inaccuracies of the method, probably cannot be ruled out. The data, however, cannot be made to fit either unlimited binding (simple partition) or a binding stoichiometry of 1:1.

The binding constants derived from this experiment are such that cholesterol hemisuccinate should be largely bound under the conditions used in the fluorescence experiments to be described below. This has been confirmed by comparing the fluorescence quenching properties of dibromocholesterol and dibromocholesterol hemisuccinate. As shown in Figure 2, dibromocholesterol quenches the fluorescence of the hydrophobic tryptophan analogue *N*-palmitoyl-L-tryptophan *n*-hexyl ester incorporated into liposomes of dioleoylphosphatidylcholine. The quenching caused by dibromocholesterol hemisuccinate is very similar to that caused by dibromocholesterol at phospholipid concentrations of both 10 and 30 μM (Figure 2), showing that under these conditions the dibromocholesterol hemisuccinate must be largely bound to the liposomes. Assuming that bound dibromocholesterol hemisuccinate and dibromocholesterol cause equal quenching, the expected quenching profiles for dibromocholesterol hemisuccinate can be calculated as a function of K_d^{eff}. The upper limit to provide a good fit to the data (broken line in Figure 2) is 10 μM.

In a previous paper (Rooney et al., 1983), we have shown how measurements of electrophoretic mobility can be used to determine binding parameters for fatty acids to phospholipid bilayers. Figure 3A shows ζ potentials of liposomes of egg yolk phosphatidylcholine as a function of the concentration of cholesterol hemisuccinate and dibromocholesterol hemisuccinate at pH 8.65, and Figure 3B shows data at a fixed

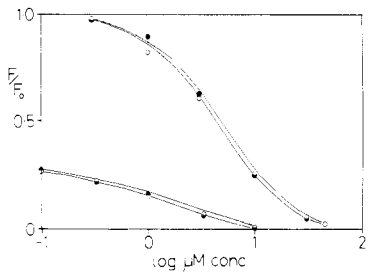


FIGURE 2: Quenching (F/F_0) of the fluorescence of *N*-palmitoyl-L-tryptophan *n*-hexyl ester in liposomes of dioleoylphosphatidylcholine at concentrations of 30 (upper curves) and 10 μM (lower curves) as a function of the concentration (micromolar) of added dibromocholesterol (●) and dibromocholesterol hemisuccinate (○) at 37 °C. The molar ratio of tryptophan to phospholipid was 1:30. The broken lines represent the calculated fluorescence quenching caused by dibromocholesterol hemisuccinate for a K_d^{eff} for binding of 10 μM, on the basis of the fluorescence quenching curves for dibromocholesterol given by the solid lines. The buffer was HEPES (40 mM), NaCl (100 mM), and EDTA (1 mM), pH 7.2.

sterol concentration as a function of pH. It is clear that the behavior of cholesterol hemisuccinate and dibromocholesterol hemisuccinate is identical. The data can be analyzed by the procedure developed for fatty acids (Rooney et al., 1983). The pK of cholesterol hemisuccinate was set equal to 4.77, the value determined for succinyl monoesters in 10% acetone solutions (Bardinet, 1947). The membrane surface area occupied by cholesterol hemisuccinate was taken to be equal to the value for cholesterol, 40 Å^2 (Demel et al., 1972). The data were then fitted to eq 1–7 as described in Rooney et al. (1983) to give K^{HA}, the dissociation constant for binding of the uncharged form, σ^{max}, the maximum number of steroid molecules bound per angstrom squared, and ΔpK, the shift in pK value on binding. The association constant, K_a, between Na^+ and the bound anionic form of the steroids was taken as 0.6 M^{-1}, the value determined by Eisenberg et al. (1979) for the binding of Na^+ to phosphatidylserine. As found previously, it is possible to fit the data over a range of σ^{max} and K^{HA} as long as the ratio K^{HA}/σ^{max} is maintained constant. Figure 3 shows a fit of the data to $K^{HA} = 3 \times 10^{-9}$, ΔpK = 2.2, and σ^{max} = $^1/_{60}$, corresponding to a sterol to phospholipid stoichiometry of 3:1. Over the concentration range employed, this implies that over 99% of the sterol is bound, so that the K^{HA} value will only represent an upper limit on the dissociation constant. The effective dissociation constant, K_d^{eff}, calculated from these binding constants for the conditions of the radiolabeling experiment is 2 μM. Comparison of the lower pK value for succinic acid in 10% acetone solution (Bardinet, 1947) with values in 0.1 M salt solutions (Sillen & Martell, 1971) suggests that the pK value for succinyl monoesters in buffer might be ca. 0.25 lower than that in 10% acetone. With a pK value of 4.5, the best fit to the electrophoresis data is obtained with ΔpK = 2.5.

The above analysis assumes that the distribution of bound sterol within the membrane is random. Because the fit to the data shown in Figure 3 is relatively poor (especially in the pH plot, Figure 3B, where the slope is rather too steep), and because it is likely that the distribution of cholesterol is nonrandom (Rogers et al., 1979; Presti et al., 1982), we have also analyzed the data in terms of cluster formation, eq 8–19. Assuming that all the sterol is bound, and with a sterol to phospholipid stoichiometry of 3:1 in the cluster, a good fit to the data is obtained with the partition coefficient between clusters and bulk phospholipid; K_p = 10 and ΔpK = 1.5 (solid line, Figure 3). The essential difference between the cluster and random-distribution models is the high surface potential

Table I: Effects of Sterols on the Fluorescence Anisotropy r of Diphenylhexatriene at 37 °C[a]

molar ratio of sterol to phospholipid	anisotropy r: cholesterol and DOPC	cholesterol hemisuccinate and DOPC	dibromocholesterol hemisuccinate and DOPC	cholesterol hemisuccinate and $(Ca^{2+}$-$Mg^{2+})$-ATPase
0	0.058	0.058	0.058	0.158
0.5	0.099	0.093	0.102	0.184
1.0	0.146	0.126	0.136	0.194

[a] The molar ratio of phospholipid to diphenylhexatriene was 100:1 for experiments with DOPC and 52:1 for experiments with ATPase. Buffer was HEPES (40 mM), NaCl (100 mM), pH 7.2, and EGTA at 0.1 and 1 mM for the phospholipid and ATPase experiments, respectively.

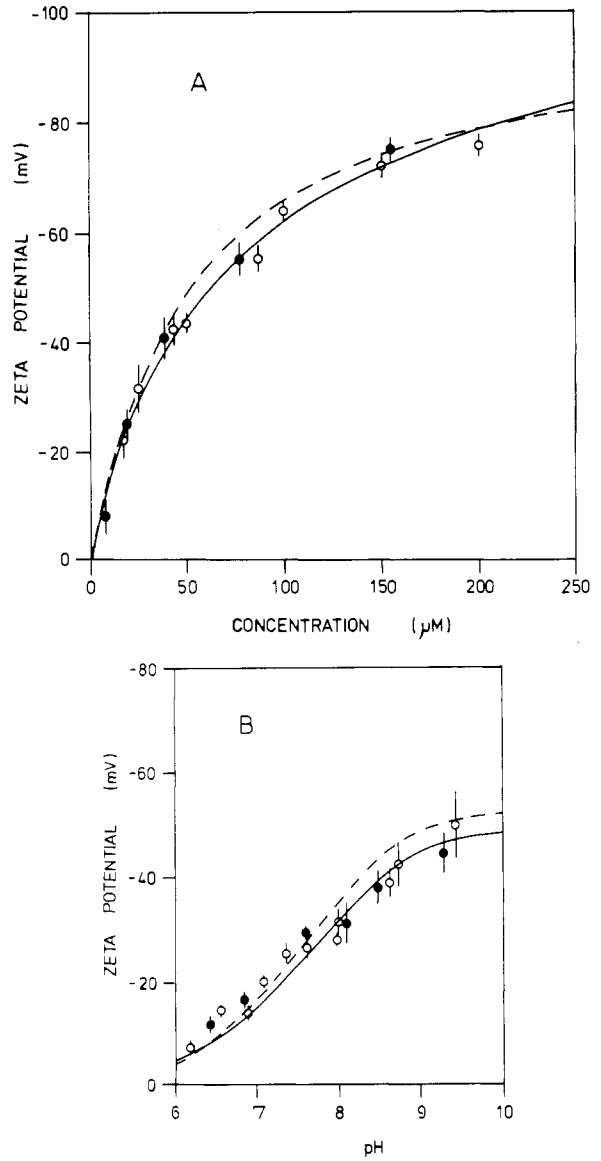


FIGURE 3: (A) ζ potentials of liposomes of egg phosphatidylcholine (0.67 mM) as a function of cholesterol hemisuccinate (○) or dibromocholesterol hemisuccinate (●) concentration at pH 8.65 in 10 mM Tris-HCl, 10 mM NaCl, and 0.1 mM EDTA at 25 °C. Symbols represent potentials calculated from the means ±SD of at least 10 mobility measurements. The dashed line represents the predicted variation of ζ potential with sterol concentration, calculated by assuming a random distribution of sterol in the membrane, with pK = 4.77, ΔpK = 2.2, $K^{HA} = 3 \times 10^{-9}$, and $\sigma^{max} = {}^{1}/_{60}$ Å^2. The solid line was calculated by assuming that all the sterol was bound and distributed between clusters with a 3:1 sterol to lipid stoichiometry and the bulk lipid with K_p = 10, pK = 4.77, and ΔpK = 1.5. (B) As in (A), but as a function of pH in the presence of 43.5 μM sterol. In both experiments, Na^+ binding to bound anion was described by an association constant of 0.6 M^{-1}.

attained in the cluster (ψ_c = −134 mV at pH 8.65 in the presence of 10 mM Na^+). This makes the formation of clusters unfavorable until the surface potential, ψ_b, in the bulk phospholipid phase rises to values comparable to ψ_c. Thus, in Figure 3A, the apparent value of K_p (=$[D]_c/[D]_b$) is 0.29 at 25 μM sterol, increasing to 1.92 at 250 μM. As a result, the potential vs. concentration plot is flatter than that for a random distribution of sterol, providing a better fit to the data. The flatter potential vs. pH plot is also in better agreement with experiment (Figure 3B).

Table II: Effects of Sterols on the Order Parameter S of Nitroxide-Labeled (12,3)Stearic Acid in Dioleoylphosphatidylcholine at 20 °C[a]

molar ratio of sterol to phospholipid	order parameter S: cholesterol	cholesterol hemisuccinate	dibromocholesterol hemisuccinate
0	0.64	0.64	0.64
0.33	0.68	0.66	0.68
0.5	0.69	0.67	0.70
0.66	0.70	0.68	0.71
1.0	0.71	0.71	0.73

[a] Molar ratio of fatty acid to phospholipid was 1:100. Phospholipid concentration was 32 mM. Buffer was HEPES (40 mM), NaCl (100 mM), and EGTA (0.1 mM), pH 7.2. Order parameters were calculated by the method of Gaffney (1976).

Table I shows the effects of cholesterol hemisuccinate and dibromocholesterol hemisuccinate on the fluorescence anisotropy of diphenylhexatriene incorporated into liposomes of dioleoylphosphatidylcholine. The effects on anisotropy are comparable to the effects caused by cholesterol. From use of the binding constants above for random distribution of cholesterol hemisuccinate within the bilayer, it is calculated that >98% of the sterol will be bound under these conditions. Diphenylhexatriene fluorescence is 70% quenched by dibromocholesterol hemisuccinate, so that anisotropy values for this system will not be directly comparable with the others. However, if quenching is largely static [as it is for other comparable systems—see Simmonds et al. (1982)] with relatively little effect on the fluorescence lifetime of the diphenylhexatriene, then any correction term will be small.

Table II lists the effects of cholesterol, cholesterol hemisuccinate, and dibromocholesterol hemisuccinate on the ESR order parameter of nitroxide-labeled (12,3)stearic acid incorporated into liposomes of DOPC, and Figure 4 shows the effect of increasing concentrations of cholesterol hemisuccinate on the spectra of nitroxide-labeled (5,10)stearic acid in DOPC. Again, it is clear that the effects of the sterols are all similar and that they all produce an increase of order within the bilayer.

Interaction with the $(Ca^{2+}$-$Mg^{2+})$*-ATPase.* As we prepared it, the purified ATPase system had a molar ratio of phospholipid to ATPase of 30:1. The results of a centrifugation assay of the binding of [^{14}C]cholesterol hemisuccinate to the ATPase system is shown in Figure 1B. It is clear that the extent of binding is similar to that for an equivalent concen-

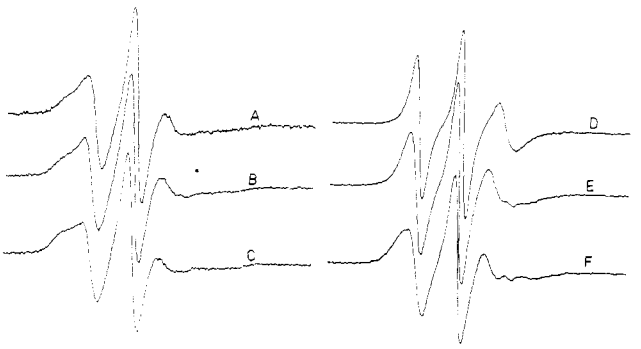


FIGURE 4: ESR spectra at 37 °C of nitroxide-labeled (5,10)stearic acid incorporated at a molar ratio of phospholipid to probe of 52:1 in (A–C) (Ca^{2+}–Mg^{2+})-ATPase and 120:1 in (D–F) dioleoylphosphatidylcholine. Cholesterol hemisuccinate to phospholipid molar ratios were (A and D) 0, (B and E) 0.5, and (C and F) 1.0.

tration of phosphatidylcholine (Figure 1A). In fitting the data to a theoretical binding plot, account must be taken of the binding of cholesterol hemisuccinate to sites on the ATPase itself, but, with the parameters derived below, such binding makes only a relatively small contribution to the total. As shown in Figure 1B, the data fits reasonably well to the parameters that describe binding to simple lipid bilayers.

Table I shows the effect of cholesterol hemisuccinate on the fluorescence anisotropy of diphenylhexatriene incorporated into the ATPase system. The anisotropy value (0.158) in ATPase alone is in agreement with the value reported by Gomez-Fernandez et al. (1979) for a dimyristoylphosphatidylcholine-substituted ATPase but is lower than the value (0.21) reported by Moore et al. (1978), which is also higher than the values of Seelig et al. (1981). As for simple phospholipid bilayers, addition of cholesterol hemisuccinate results in an increase in fluorescence anisotropy.

Figure 4 shows the effect of cholesterol hemisuccinate on the ESR spectra of nitroxide-labeled (5,10)stearic acid incorporated into the ATPase system. As for other protein systems, the spectra are clearly composed of at least two components, one of which (the one with the smaller splitting) has the characteristics of fatty acid incorporated into the phospholipid component of the membrane [see Jost & Griffith (1980)]. Without attempting a detailed analysis, Figure 4 shows an increase in order on addition of cholesterol hemisuccinate.

In previous papers (East & Lee, 1982; Simmonds et al., 1982), we have shown that quenching of the fluorescence of tryptophan residues in the ATPase by brominated derivatives of phospholipids and cholesterol can be used to measure binding constants to the ATPase. We have shown that fluorescence quenching of the ATPase by BRPC can be fitted to

$$F' = F/F_0 = 0.4 + 0.6[(N_{ann}P - L^*)/(N_{ann}P)]^{1.6} \quad (22)$$

where F_0 is the fluorescence intensity in the absence of quenching lipid, F is the fluorescence intensity when the concentration of BRPC bound to annular sites on the ATPase is L^*, N_{ann} is the number of annular sites, and P is the concentration of ATPase. As shown in Figure 5, reconstitution of the ATPase with BRPC reduces the fluorescence intensity by 60%. Addition of cholesterol hemisuccinate up to 60 μM, corresponding to a 1:1 molar ratio of sterol to phospholipid, produces only a slight increase in fluorescence intensity, showing that cholesterol hemisuccinate can bind only weakly to the annular sites. Binding of cholesterol hemisuccinate to annular sites with concomitant displacement of BRPC can be described by eq 21. Assuming binding to the phospholipid

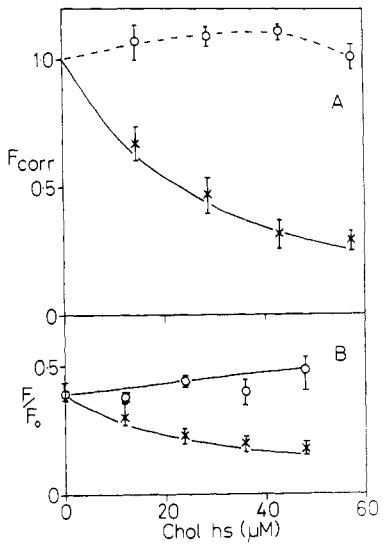


FIGURE 5: Effects of cholesterol hemisuccinate (○) and dibromocholesterol hemisuccinate (×) on the fluorescence intensities of DOPC–ATPase (A) and BRPC–ATPase (B). ATPase concentration in (A) was 0.06 μM and in (B) 0.05 μM. The lipid to protein molar ratio was 970:1. In (A), the fluorescence intensity change observed with dibromocholesterol hemisuccinate was normalized with respect to that seen with an equal concentration of cholesterol hemisuccinate. Points are experimental (mean ± SD of three measurements) values. Solid lines are theoretical calculations (see text).

component of the ATPase with K_d^{eff} = 2 μM and a sterol to phospholipid stoichiometry of 3:1, the observed increase in fluorescence can be fitted by eq 22 with K_d^{ann} = 2.0 (molar ratio units) (Figure 5). As shown in Figure 5, addition of dibromocholesterol hemisuccinate to BRPC–ATPase causes further fluorescence quenching, as previously observed for other brominated molecules (Simmonds et al., 1982). We have shown that the total fluorescence quenching in such systems can be described by

$$F_{total} = 0.4F_A + 0.6F_AF' \quad (23)$$

where F_A represents fluorescence quenching caused by binding of dibromocholesterol hemisuccinate to sites on the ATPase distinct from the annular sites (referred to as nonannular sites). We have shown that this extra quenching can be fitted by assuming that quenching that results from binding at the postulated nonannular sites is directly proportional to the degree of occupation of the sites, so that

$$F_A = 1 - x^D/(K_d^{nonann} + x^D) \quad (24)$$

where K_d^{nonann} is the dissociation constant for binding at the nonannular sites. Figure 5 illustrates that a good fit can be obtained to the fluorescence quenching data with dibromocholesterol hemisuccinate for both DOPC–ATPase and BRPC–ATPase with K_d^{ann} = 2.0 and K_d^{nonann} = 0.55. In these experiments, the concentration of sites on the ATPase is much less than the concentration of added sterol, so that the numbers of such sites cannot be determined.

Figure 6 shows that addition of dibromocholesterol hemisuccinate to native ATPase also results in fluorescence quenching. The observed data can be fitted by using the binding constants used to fit the data of Figure 5. In the native ATPase system, the molar ratio of phospholipid to protein of 30:1 compares to 970:1 in the reconstituted system, and the concentration of dibromocholesterol hemisuccinate to produce quenching is correspondingly less. The concentration of sites on the ATPase is now more comparable to the concentration of added sterol, and the data can be used to set approximate limits on the number of these sites: between approximately

Table III: Reversibility of Effects of Sterols on the Activity of the (Ca^{2+}-Mg^{2+})-ATPase[a]

system	sterol to phospholipid molar ratio	activity (%) before DOPC addition	DOPC added (μM)	activity (%) after DOPC addition
native ATPase	0	100	103	119
+ cholesterol hemisuccinate (1 μM)	1	84	52	107
+ dibromocholesterol hemisuccinate (1 μM)	1	66	52	100
+ cholesterol hemisuccinate (11 μM)	10	20	520	85
+ dibromocholesterol hemisuccinate (11 μM)	10	6	520	41
DOPC-ATPase	0	100	412	102
+ cholesterol hemisuccinate (20 μM)	1	50	412	79
+ dibromocholesterol hemisuccinate (20 μM)	1	54	412	73

[a] Duplicate samples were incubated with sterol and either assayed for activity or incubated with DOPC for 20 min at 37 °C and then assayed for activity to test for reversibility.

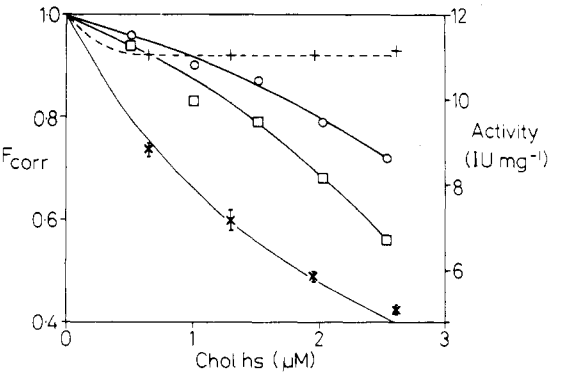


FIGURE 6: Effects of cholesterol hemisuccinate (+) and dibromocholesterol hemisuccinate (×) on the fluorescence intensity of the native ATPase (0.09 μM) and effects of cholesterol hemisuccinate (○) and dibromocholesterol hemisuccinate (□) on the activity of the ATPase (0.07 μM) at 37 °C. The fluorescence intensity change observed with dibromocholesterol hemisuccinate was normalized with respect to that seen with an equal concentration of cholesterol hemisuccinate, and the solid line through the points is the theoretical curve (see text). Points are experimental values (mean ± SD of three measurements).

1 and 7 for the nonannular sites and 20 and 40 for the annular sites.

The binding parameters listed above were those that gave the best agreement with the binding of [^{14}C]cholesterol hemisuccinate (Figure 1) and with the fluorescence quenching experiments (Figures 5 and 6). Variation of the parameters by more than 20% gave obviously worse fits to the data. As shown in Figure 6, both cholesterol hemisuccinate and dibromocholesterol hemisuccinate decrease the activity of the ATPase by about 30% at a 1:1 molar ratio of sterol to phospholipid, with the effect of the brominated derivative being slightly greater. Figure 7 shows inhibition of reconstituted DOPC–ATPase and BRPC–ATPase, inhibition of the latter system being slightly greater. In contrast, as shown in Figure 8, addition of both sterols to DMPC–ATPase produces a marked stimulation at about a 1:1 molar ratio of sterol to phospholipid, followed by inhibition. Finally, Figure 9 illustrates the effects of very high concentrations of sterols on native ATPase. The reversibility of these effects was shown by addition of a large excess of DOPC to bind the sterol (Table III).

Discussion

It has been clearly demonstrated that changing the cholesterol content of biological membranes results in large changes in membrane fluidity or order (Demel & de Kruijff, 1976). Since it has also been demonstrated that changing the cholesterol content of membranes results in alteration in many of the enzymatic activities associated with the membrane, it has often been concluded that the effects of cholesterol are mediated through effects on membrane fluidity (Demel & de

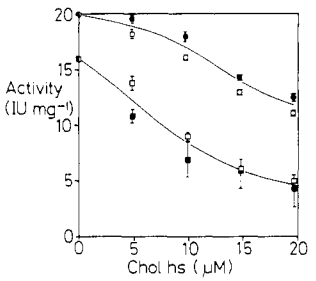


FIGURE 7: Effects of cholesterol hemisuccinate (○, □) and dibromocholesterol hemisuccinate (●, ■) on the activity of DOPC–ATPase (○, ●) and BRPC–ATPase (□, ■) at 37 °C. The ATPase concentration was 0.02 μM with a molar ratio of phospholipid to protein of 970:1. Points are experimental values (mean ± SD of three measurements).

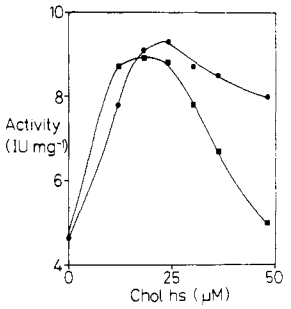


FIGURE 8: Effects of cholesterol hemisuccinate (●) and dibromocholesterol hemisuccinate (■) on the activity of DMPC–ATPase at 37 °C. The ATPase concentration was 0.02 μM with a molar ratio of phospholipid to protein of 1110:1.

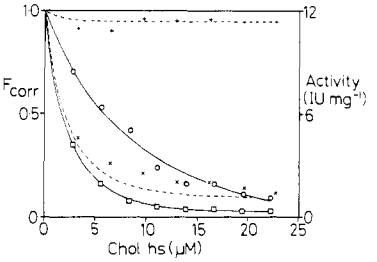


FIGURE 9: Effects of cholesterol hemisuccinate (+, ○) and dibromocholesterol hemisuccinate (×, □) on the relative fluorescence intensity (+, ×) and activity at 37 °C (○, □) of the native ATPase. ATPase concentrations for fluorescence and activity measurements were 0.08 and 0.04 μM, respectively. The fluorescence intensities with dibromocholesterol hemisuccinate (×) are normalized with respect to those seen with an equal concentration of cholesterol hemisuccinate. The broken line through the points (×) is the theoretical profile (see text).

Kruijff, 1976; Sinensky, 1980). However, it is also possible that the effects of cholesterol follow directly from the interaction of cholesterol with membrane proteins. These two

possibilities are not readily distinguished experimentally because of the difficulty of manipulating the cholesterol content of membranes in a quantifiable manner. In earlier studies, we reconstituted the (Ca^{2+}–Mg^{2+})-ATPase into bilayers of defined phospholipid/cholesterol composition by a detergent-dilution procedure and showed that cholesterol could either increase or decrease the activity of the ATPase, depending on the chemical structure of the phospholipid present in the system (Simmonds et al., 1982). This technique is, however, only readily applicable to the study of membrane proteins that can be obtained in a relatively pure state. Here we show that the more water-soluble cholesterol hemisuccinate can be used to modify directly the sterol content of membranes without the use of detergents and that results with the hemisuccinate are very similar to those obtained with cholesterol. Equilibration of cholesterol hemisuccinate with membranes is complete after 10–20 min.

It has been suggested (Demel & de Kruijff, 1976) that the structural features of sterols essential for their interaction with membranes include a planar (trans-fused) tetracyclic ring system, an aliphatic side chain, and an unblocked equatorial –OH at C-3. Although the –OH in cholesterol hemisuccinate is esterified, cholesterol hemisuccinate still binds strongly to lipid bilayers, with a maximum binding stoichiometry of sterol to phospholipid of 3:1. For cholesterol, it has been suggested that beyond a 1:1 molar ratio of cholesterol to phospholipid, crystalline cholesterol separates out (Ladbrooke et al., 1968), but in sonicated liposomes stoichiometries up to 3:1 have been reported (Horwitz et al., 1971; Cooper et al., 1978). It has been suggested that the stoichiometry will in part be determined by interaction with the phospholipid head group (Loomis et al., 1979). Such interactions would be expected to be different for cholesterol and cholesterol hemisuccinate as suggested, for example, by the observations that whereas cholesterol forms crystals in excess water (Loomis et al., 1979), a variety of polar cholesterol esters form bilayer structures (Brockerhoff & Ramsammy, 1982).

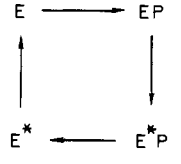
Effects of cholesterol and cholesterol hemisuccinate on order or fluidity of phospholipid fatty acyl chains are comparable, as shown by fluorescence polarization of DPH (Table I) and by ESR with spin-labeled fatty acids (Table II, Figure 4). In the ESR spectra for the ATPase system (Figure 4), separate mobile and immobile components can be seen and, although a detailed analysis has not been attempted, effects of cholesterol hemisuccinate on the mobile component appear comparable to the effects in simple phospholipid bilayers (Figure 4).

Analysis of both the radiolabel binding and electrophoresis data show that under the conditions used in most of the experiments reported here, cholesterol hemisuccinate is predominantly membrane bound. The electrophoresis experiments show that binding of cholesterol hemisuccinate to phospholipid bilayers introduces significant negative charge into the bilayers. The experiments are consistent with the formation of separate phospholipid–sterol clusters in the membrane, analogous to the separation of phospholipid–sterol complexes in bilayers of cholesterol and excess phospholipid (Rogers et al., 1979; Presti et al., 1982). Within probable experimental error, the data are, however, also consistent with a random distribution of cholesterol hemisuccinate within the membrane. The pH dependence of electrophoretic mobility (Figure 3B) suggests that the uncharged form of the sterol binds more strongly than the charged form, with a shift in pK value on binding of between 1.5 and 2.5, depending on the model used to analyze the data. The radiolabeling experiment gives an effective binding constant, which is a weighted average for that of the uncharged and charged form and which includes an effect of charge on the binding of the charged form. The value obtained (0.5–2 μM) is consistent with the electrophoresis data, and with fluorescence quenching data (Figure 2).

Binding of the sterols to the ATPase can be studied by fluorescence quenching. From the displacement of BRPC from annular sites on the ATPase (Figure 5), a dissociation constant for sterol binding at annular sites of 2.0 can be estimated where the concentration unit is molar ratio within the phospholipid component of the membrane. This corresponds to a relative binding constant for sterol relative to DOPC of 0.5. To explain the fluorescence quenching caused by dibromocholesterol, it is necessary to propose a second set of sites (nonannular sites) on the ATPase, with a dissociation constant for binding of 0.55 (molar ratio units). Very similar parameters were determined for cholesterol binding to the ATPase, with no significant binding to annular sites (up to a 1:1 molar ratio of cholesterol to phospholipid) and K_d = 0.7 (molar ratio units) for binding at the nonannular sites (Simmonds et al., 1982). Griffith et al. (1982) have also reported that binding of a spin-labeled analogue of cholesterol to the annular sites on the ATPase could be relatively weak.

Binding sites on the ATPase for hydrophobic molecules, distinct from the annular binding sites, have also been deduced from fluorescence titrations with dansylundecanoic acid (Lee et al., 1982), and the number of these sites (three) is consistent with the experimental data presented here. If the ATPase is present in the membrane in dimeric form (Napolitano et al., 1983), then these nonannular binding sites could be at protein/protein interfaces in the dimer (Simmonds et al., 1982).

Effects of cholesterol hemisuccinate on the activity of the native ATPase and of the ATPase reconstituted with DOPC and DMPC are shown in Figures 6–8. The concentration of sterol required to affect activity depends markedly on the phospholipid concentration of the system, consistent with strong binding of the sterol to the phospholipid component. Whereas the sterols inhibit native ATPase or ATPase reconstituted with DOPC, they cause a marked increase in the activity of the ATPase reconstituted with DMPC. The effects of the sterols up to a molar ratio of 2:1 sterol to phospholipid are largely reversible. At much higher molar ratios of sterol (Figure 9), inhibition of ATPase activity becomes almost complete and only partially reversible, and fluorescence quenching by dibromocholesterol hemisuccinate is greater than that calculated from the parameters deduced from fluorescence quenching at lower molar ratios of sterols. It seems likely that at these high sterol molar ratios there is extensive disruption of the membrane. Detailed analysis of the effects of the sterols on the activity of the ATPase is not possible on the basis of our steady-state kinetic measurements at saturating Ca^{2+} and ATP concentrations. The overall reaction pathway of the ATPase can be represented as



and includes two steps, EP → E*P and E* → E, that could be sensitive to alterations in the membrane milieu [see de Meis (1981)]. Preferential binding, for example, to the E conformation of the ATPase could tend to increase the overall rate of reaction by favoring the E* → E step but would tend to reduce the rate by favoring the conversion E*P → EP. Thus,

if binding of short-chain phospholipids favored the E* form and long-chain phospholipids favored the E form and sterols bound preferentially to the E form, then sterols could activate the ATPase reconstituted with a short-chain phospholipid such as DMPC but decrease the activity for the ATPase reconstituted with a long-chain phospholipid such as BRPC. Consistent with such an interpretation, activation of DMPC–ATPase closely follows the calculated binding to nonannular sites, and inhibition follows binding to annular sites. For BRPC–ATPase, inactivation matches calculated binding to annular and nonannular sites.

It is clear that effects of sterols on the activity of the ATPase cannot be attributed to effects on phospholipid fluidity but that specific interactions with the ATPase have to be considered. It has been shown previously that phospholipid binding to the ATPase is relatively nonspecific although the activity of the ATPase depends markedly on the chemical structure of the surrounding phospholipid (London & Feigenson, 1981; East & Lee, 1982). The relatively weak binding of cholesterol to the phospholipid/protein interface probably reflects poor interaction between the rigid sterol ring system and the molecularly rough hydrophobic surface of the ATPase.

Registry No. DOPC, 10015-85-7; BrPC, 61596-55-2; DMPC, 77285-90-6; ATPase, 9000-83-3; cholesterol, 57-88-5; cholesterol hemisuccinate, 1510-21-0; 5,6-dibromocholestan-3β-ol hemisuccinate, 88730-76-1; *N*-palmitoyl-L-tryptophan *n*-hexyl ester, 81591-68-6; tryptophan, 73-22-3; thionyl chloride, 7719-09-7; palmitic acid, 57-10-3.

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