Fluorescence of $\Delta^{5,7,9(11),22}$-Ergostatetraen-3β-ol in Micelles, Sterol Carrier Protein Complexes, and Plasma Membranes†

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Received June 26, 1984

ABSTRACT: The fluorescent sterol analogue $\Delta^{5,7,9(11),22}$-ergostatetraen-3β-ol (dehydroergosterol) was synthesized and purified by reverse-phase high-performance liquid chromatography. Dehydroergosterol in aqueous solution had a critical micelle concentration of 25 nM and a maximum solubility of 1.3 μM as ascertained from fluorescence polarization and light scattering properties, respectively. Several lines of evidence indicated a close molecular interaction of dehydroergosterol with purified rat liver squalene and sterol carrier protein (SCP). SCP increased the maximal solubility of dehydroergosterol in aqueous buffer. The fluorescence emission spectrum of dehydroergosterol was blue shifted upon addition of SCP. The fluorescence lifetime of dehydroergosterol in aqueous buffer was 2.3 ns; addition of SCP resulted in the appearance of a second lifetime component near 12.4 ns. The SCP increased the fluorescence polarization of monomeric dehydroergosterol in aqueous buffer from 0.033 to 0.086. Scatchard analysis of the binding data indicated that dehydroergosterol interacted with purified rat liver SCP with an apparent K_D = 0.88 μM and B_{max} = 4.8 μM. At maximal binding, 1.0 mol of dehydroergosterol was specifically bound per mole of SCP. The close molecular interaction of dehydroergosterol with SCP was also demonstrated by energy-transfer experiments. The intermolecular distance between SCP and bound dehydroergosterol was evaluated by fluorescence energy transfer from tyrosine residues of SCP to the conjugated triene series of double bonds in dehydroergosterol. The transfer efficiency was 36%, and R, the apparent distance between the tyrosine energy donor and the dehydroergosterol energy acceptor, was 19 Å. The significance of these data obtained in vitro for dehydroergosterol interaction with SCP was also tested in vivo. A dehydroergosterol–protein complex was isolated from the cytosol of LM fibroblasts cultured in the presence of dehydroergosterol. Dehydroergosterol was also a membrane component of these cells. The limiting anisotropy, order parameter, and rotational relaxation time of dehydroergosterol in LM plasma membranes were 0.097, 0.48, and 1.17 ns, respectively. These results demonstrate the utility of dehydroergosterol not only as a membrane sterol probe molecule but also as a probe for sterol–protein interactions.

In the past, characterization of biological and model membrane structure and the dynamic properties of lipids therein have often been explored through the use of synthetic fluorescent lipid analogues. Generally, there has been little consideration of the potential perturbing effects of such molecules, and even more important, comparisons of the physical and biological properties of these fluorescent lipid probe molecules have been few. Current interest in the location of sterols in membranes and in sterol–protein interactions has centered around the use of fluorescent sterol molecules whose structure more closely resembles that of native membrane sterols [for a recent review, see Schroeder (1984)]. In several of these studies, $\Delta^{5,7,9(11),22}$-ergostatetraen-3β-ol (dehydroergosterol), a fluorescent sterol whose structure closely resembles ergosterol and desmosterol, has been utilized to study lipoprotein thermotropic behavior (Bergeron & Scott, 1982) and the asymmetric transbilayer distribution of sterols in plasma membranes (Schroeder, 1981; Hale & Schroeder, 1982). The chemical interaction of dehydroergosterol with digitonin and with polyene antibiotics such as filipin and amphotericin B (Hale & Schroeder, 1982; Archer, 1975), the phase behavior of dehydroergosterol in lipid vesicles as determined by differential scanning calorimetry (Hale & Schroeder, 1982) and by circular dichroism (Yeagle et al., 1982a,b), and the lack of effect of dehydroergosterol on microbial (Archer, 1975) and mammalian (Schroeder, 1981; Hale & Schroeder, 1982) cell growth and membrane bound enzymes (Hale & Schroeder, 1982) all indicate the suitability of using dehydroergosterol as a probe molecule for native sterols. In fact, dehydroergosterol has been found to exist as a naturally occurring, biologically synthesized molecule in eukaryotic organisms such as the yeast *Candida tropicalis* (Sica et al., 1982) and in a single-celled animal, the Red Sea sponge *Biemna fortis* (Delseth et al., 1979), at levels up to 2% of the cellular sterols. When LM fibroblasts were cultured in the presence of dehydroergosterol, synthesis of the native sterol desmosterol was inhibited and dehydroergosterol was incorporated into cellular membranes up to 85 mol % of the total sterol without adverse effect on cellular growth, biochemistry, or membrane structure (Schroeder, 1981; Hale & Schroeder, 1982).

Our interest in dehydroergosterol as a fluorescent sterol analogue is not just to determine how sterols affect membrane structure and function. Instead, the present investigation seeks to extend our knowledge of the properties of sterols themselves to sterol–protein interactions and to the behavior of sterols in membranes. In this report we address the characterization of the fluorescence properties of highly purified dehydroergosterol existing as (1) free, soluble molecules, (2) aqueous micelles, (3) components of LM fibroblast plasma membranes, and (4) bound complexes with purified rat liver sterol carrier

† This work was supported, in part, by National Institutes of Health Grants GM 31651 to F.S. and HL-25512 and HL-28176 to M.E.D. and National Cancer Institute Grant CA 24339 to F.S.

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0006-2960/85/0424-3322$01.50/0

protein.[1] Furthermore, dehydroergosterol was introduced into LM fibroblast cell cultures to demonstrate in vivo binding of dehydroergosterol to soluble cytosolic sterol carrier proteins which may be responsible for intracellular transport of sterols to microsomal and mitochondrial membranes, a function proposed for sterol carrier protein (Dempsey et al., 1981; Grinstead et al., 1983; Dempsey, 1984).

Experimental Procedures

Materials

Rat liver sterol carrier protein (SCP)[2] was isolated as described previously (Dempsey et al., 1981). Ergosterol was purchased from Sigma Chemical Co. (St. Louis, MO). Acetic anhydride and mercuric acetate were obtained from Fisher Scientific Co. (Pittsburgh, PA). A dehydroergosterol standard was obtained from Frann Scientific, Columbia, MO.

Methods

Synthesis and Purification of $\Delta^{5,7,9(11),22}$-Ergostatetraen-3β-ol. The synthesis of dehydroergosterol was a modification of the procedures of Ruyle et al. (1953) and Antonucci et al. (1951). Purification by reversed-phase high-performance liquid chromatography (HPLC)[2] was performed also as described earlier (Fischer et al., 1985).[1] All steps were performed with light-protected glassware to prevent formation of photooxidation products. Likewise, each reaction was carried out under N_2 to prevent sterol oxidation.

Dehydroergosterol Purity and Structure. The purity of dehydroergosterol was determined by high-performance liquid chromatography, by gas chromatography, and by measurement of the absorbance ratio at 282/324 nm as described previously (Fischer et al., 1985).[1] Mass spectroscopy, proton nuclear magnetic resonance spectroscopy, and carbon-13 nuclear magnetic resonance spectroscopy were used to determine the structure and location of double bonds in dehydroergosterol also as described earlier (Fischer et al., 1985).[1] Retention times, absorbance ratios, mass spectra, and NMR spectra were compared to those of an authentic dehydroergosterol standard obtained from Frann Scientific, Columbia, MO.

Fluorescence Measurements. Fluorescence characterization of dehydroergosterol was performed by simultaneously measuring absorbance-corrected fluorescence, corrected fluorescence emission, relative fluorescence efficiency, and absorbance using a computer-centered spectrofluorometer (Holland et al., 1973) as previously described (Schroeder, 1981; Hale & Schroeder, 1982). The computer corrected for instrumental variables and for the inner filter effect (primary absorbance artifact) during absorbance-corrected fluorescence and corrected fluorescence emission determinations. Light scattering was minimized by using nonfluorescent cutoff filters (Schott Optical Glass, Inc., Duryea, PA) and narrow bandpasses. Fluorescence lifetime, τ, and steady-state fluorescence polarization, P, were determined by using a T-format spectrofluorometer (Weber & Bablouzian, 1966) obtained from SLM Instrument (SLM 4800 subnanosecond spectrofluorometer, SLM Instrument, Inc., Champaign—Urbana, IL) as described earlier (Hale & Schroeder, 1982). Light scattering in fluorescence lifetime determinations was minimized also as described above. During fluorescence lifetime analysis the excitation polarizer was set to 0° and the emission polarizer set to 55° (to eliminate Brownian motion as a determinant of apparent lifetime). Values for τ were obtained by simultaneous measurement of a reference solution of 1,4-bis(4-methyl-5-phenyloxazol-2-yl)benzene (Me_2POPOP) in absolute ethanol as previously described (Lakowicz & Cherek, 1981a,b). Multiple lifetimes (τ_1, τ_2) and the corresponding fluorescence fractions of each signal (F_1, F_2) were resolved by Weber's closed form heterogeneity analysis (Weber, 1981). All measurements were made at 37 °C, unless otherwise stated.

Dynamic properties of dehydroergosterol in membranes were determined by differential polarized phase fluorometry as first described by Weber (1978) and extended by others (Lakowicz et al., 1979). This procedure for obtaining $(6R)^{-1}$, the rotational relaxation time in nanoseconds, and r_∞, the limiting anisotropy, is summarized in detail elsewhere (Schroeder et al., 1984a,b). The order parameter, S, was obtained from $S = (r_\infty/r_0)^{1/2}$ as described previously (Jahnig, 1979; Heyn, 1979). The latter calculation assumes that dehydroergosterol behaves as a cylindrical molecule in similar fashion as 1,6-diphenyl-1,3,5-hexatriene.

Fluorescence Energy Transfer. The efficiency of fluorescence energy transfer was determined from quantum yield, lifetime, polarization, and excitation spectra largely as described elsewhere (Stryer, 1978). The method utilizing excitation spectra is especially useful if binding of a fluorescent molecule to a protein may concomitantly alter the conformation of the binding site. The procedure is briefly described as follows:

$$E = [G(\lambda_2)/G(\lambda_1) - \epsilon_A(\lambda_2)/\epsilon_A(\lambda_1)][\epsilon_A(\lambda_1)/\epsilon_D(\lambda_2)] \quad (1)$$

where $G(\lambda)$ is the magnitude of the corrected excitation spectrum of the energy acceptor excited at wavelength λ. The extinction coefficients of the energy donor and acceptor at that wavelength are $\epsilon_D(\lambda)$ and $\epsilon_A(\lambda)$, respectively. G is measured at two wavelengths: λ_1, where the donor does not absorb, and λ_2, where the extinction coefficient of the donor is much larger than that of the acceptor. Energy-transfer efficiency is related to R, the distance separating the donor and acceptor, and to R_0, the distance at which transfer efficiency is 50%, according to Forster's theory where the following relationships are given (Stryer, 1978; Radda, 1975):

$$R = (E^{-1} - 1)^{1/6}R_0 \quad (2)$$

$$R_0 = (JK^2Q_0n^{-4})^{1/6}(9.7 \times 10^3\ \text{Å}) \quad (3)$$

where K^2 is the orientation factor for dipole-dipole interaction and is set equal to 0.476 for proteins (Steinberg, 1971), n is the index of refraction set at 1.5 for aromatic acids in proteins (Steinberg, 1971), Q_0 is the donor quantum yield set at 0.061 for tyrosine in SCP, and J is the overlap integral calculated as follows (Stryer, 1978):

$$J = \left[\int F(\lambda)\epsilon(\lambda)\lambda^4\, d\lambda\right] \Big/ \left[\int F(\lambda)\, d\lambda\right] \quad (4)$$

where $F(\lambda)$ is the fluorescence intensity of the energy donor at wavelength λ (in cm) and $\epsilon(\lambda)$ is the extinction coefficient (in $cm^{-1}\ M^{-1}$) of the energy acceptor.

Energy transfer from SCP tyrosine residues to dehydroergosterol was measured by exciting tyrosine residues at 280 nm and measuring absorbance-corrected fluorescence emission of tyrosine and dehydroergosterol at 315 and 374 nm, respectively. This measurement was performed on dehydroergosterol (0.1 μg/mL), on SCP (50 μg/mL), and then on the combined complex of dehydroergosterol (0.1 μg/mL) plus SCP (50 μg/mL) in PBS.

[1] A portion of this work was previously presented as an abstract at the FASEB Meeting in St. Louis, MO, April 1984 (Fischer et al., 1984).

[2] Abbreviations: PBS, phosphate-buffered saline, pH 7.4; SCP, sterol and squalene carrier protein; HPLC, high-performance liquid chromatography; P, polarization; τ, lifetime; F, fractional fluorescence; r_∞, limiting anisotropy; $(6R)^{-1}$, rotational relaxation time in nanoseconds; S, order parameter; TEA, triethanolamine.

Energy transfer between dehydroergosterol molecules in micelles was determined according to Cathou & Bunting (1976) to determine R from lifetime analysis.

$$R = (R_0)/[T(1/\tau' - 1/\tau)]^{1/6} \quad (5)$$

where τ and τ' represent the dehydroergosterol lifetime in the absence of quenching and presence of quenching, respectively.

Light-Scattering Measurements. Light scattering of dehydroergosterol vesicles was measured at an angle of 90° with the computer-centered spectrofluorometer (Schroeder, 1981; Fischer et al., 1985).[1] Dehydroergosterol prepared in 2 mL of PBS (0.05–10.0 μg/mL) was equilibrated at 37 °C for 15 min followed by determination of relative light scattering with both the excitation and emission monochromator set at 324 nm. This procedure was also repeated at the above dehydroergosterol concentrations in the presence of SCP (50 μg/mL) equilibrated for 15 min at 37 °C.

Polarization and Lifetime Measurements of Dehydroergosterol in Aqueous Solution. The sensitivity of the SLM 4800 fluorometer was at least 10 times greater than that of the computer-centered spectrofluorometer, and therefore, smaller concentrations of dehydroergosterol could be measured. A series of dehydroergosterol concentrations (0.01–1.00 μg/mL of PBS) were equilibrated for 15 min at 37 °C. Fluorescence polarization and lifetime were measured and then repeated at each concentration following the addition of SCP (50 μg/mL) and a second equilibration for 15 min at 37 °C.

Isolation of a Dehydroergosterol–Protein Complex from an LM Fibroblast Cytosolic Fraction. LM fibroblasts, a highly tumorigenic cell line (Kier & Schroeder, 1982), were obtained from the American Type Culture Collection (CCl1.1) and cultured in a suspension with a chemically defined medium as described elsewhere (Schroeder et al., 1976). LM fibroblasts contain 2–3% of their total cellular protein as sterol carrier proteins (Schroeder et al., 1984c). SCP was isolated from LM cells as follows: A 1-L LM fibroblast culture (0.7×10^6 cells/mL) was divided into two parts: a 250-mL control group and a 750-mL group supplemented with dehydroergosterol (20 μg/mL of medium). Both aliquots of cells were incubated for 3 h at 37 °C and then sedimented at 1100g for 10 min. The cells were washed 2 times in their original volume of cold PBS and again sedimented at 1100g at 4 °C. The weighed pellet was suspended and homogenized in a 15 × (pellet wet weight) volume of 0.15 M sucrose and 1 mM TEA, pH 7.4, as described earlier (Schroeder et al., 1976). The cell debris was removed by centrifugation at 1700g for 10 min on an SS-34 rotor (Sorvall Instrument, Du Pont Inc., Wilmington, DE). The supernatant was subsequently centrifuged for 1 h at 33000g with a 50.2 Ti rotor and L5-65 ultracentrifuge (Beckman Instruments, Fullerton, CA) to remove membrane vesicles and subcellular organelles. The supernatant from the 33000g spin was then centrifuged for 15 h at 105000g to sediment any residual microvesicles or membrane fragments. The supernatant from the 105000g spin was then fractionated by a modified version of the procedure of Bloj & Zilversmit (1977). The soluble cytosolic proteins were fractioned first by shifting the pH to 5.1 and then taking a 0–40% ammonium sulfate fraction after addition of saturated ammonium sulfate reagent, pH 7.4. A second 40–90% ammonium sulfate pellet contained the cytosolic protein of interest in this study. The 40–90% ammonium sulfate pellets were suspended in 4.0 mL of PBS, and a protein assay was performed (Lowry et al., 1951). Lipid was extracted from a 2-mL aliquot of the partially purified sterol carrying cytosolic proteins (Bligh & Dyer, 1959) followed by a separation of neutral from polar lipids by silicic acid chromatography (Schroeder et al., 1976). Ergosterol was added as an internal standard prior to the extraction step. The neutral lipids were resolved by analytical HPLC using a methanol–hexane (95:5) mobile phase at a 1.0 mL/min flow rate. The elution retention times for desmosterol, dehydroergosterol, and ergosterol were determined by comparison with the appropriate standards. Desmosterol and ergosterol standards were obtained from Steraloids (Wilton, NH), while a dehydroergosterol standard was provided by Frann Scientific (Columbia, MO).

Incorporation of Dehydroergosterol into LM Cell Plasma Membranes, Microsomes, and Mitochondria. LM fibroblasts cultured in the presence of dehydroergosterol (20 μg/mL) for 72 h were washed and homogenized, and subcellular membranes were separated according to the procedure of Schroeder et al. (1976). The neutral lipids isolated as described above were analyzed by HPLC as described earlier (Fischer et al., 1985).[1]

Results

Purity and Structure of $\Delta^{5,7,9(11),22}$-Ergostatetraen-3β-ol. The purity of the dehydroergosterol utilized herein was 99+% as determined by high-performance liquid chromatography, gas chromatography, and absorbance peak ratios at 282/324 nm. The latter value was 0.29 ± 0.01, similar to that for pure dehydroergosterol of 0.30 ± 0.01. Mass spectroscopy and proton and carbon-13 nuclear magnetic resonance spectroscopy confirmed the structure as $\Delta^{5,7,9(11),22}$-ergostatetraen-3β-ol (Fischer et al., 1985).[1]

Aqueous Solubility of $\Delta^{5,7,9(11),22}$-Ergostatetraen-3β-ol. At the outset of our investigation with dehydroergosterol it was apparent that there was a significant solvent blue shift in the emission spectrum of dehydroergosterol fluorescence emission in PBS vs. hydrophobic organic solvents. In PBS the fluorescence emission spectra showed maxima near 402 and 426 nm (Figure 1A). In micelles, ethanol, and membranes, emission maxima were shifted to 356 and 374 nm (Figure 1B–D). In addition, spectral peak alterations were also noted with decreasing solvent dielectric constant in both absorbance and absorbance-corrected fluorescence excitation spectra. In aqueous solution, dehydroergosterol had ratios of (absorbance at 338 nm)/(absorbance at 324 nm) and of (absorbance-corrected fluorescence at 338 nm)/(absorbance corrected fluorescence at 324 nm) near 0.92 and 0.75, respectively. With decreasing solvent dielectric constant these ratios decreased until a dielectric constant of 25 was reached, at which point the above ratios were 0.65 and 0.60, respectively. The absorbance and absorbance-corrected fluorescence ratios remained constant with further decreases in dielectric constant. These shifts in peak ratios could occur either because of direct interaction of the excited state of the fluorescent molecules with respective solvents or because the dehydroergosterol formed micelles in the solvents with higher dielectric constant. Dehydroergosterol (0.4 μg/mL) formed micelles in PBS but not in ethanol. Therefore, various ratios of ethanol/PBS were prepared to determine the onset of micelle formation in PBS. Fluorescence polarization and corrected fluorescence emission of dehydroergosterol (0.4 μg/mL) in a range of solvent polarities from 100% ethanol to 100% PBS indicated a distinct transition point near 70% PBS (dielectric constant near 72; Figure 2A). Corrected emission spectra obtained at 9%, 50%, 60%, and 65% (v/v) PBS in ethanol gave virtually identical profiles with emission maxima in the 360- and 380-nm region (Figure 2B). These data indicated that micellization occurred at a much higher solvent dielectric constant than 25 or 30 and that the changes in absorbance and absorbance-corrected fluorescence peak ratios in dioxane/H_2O mixtures were due

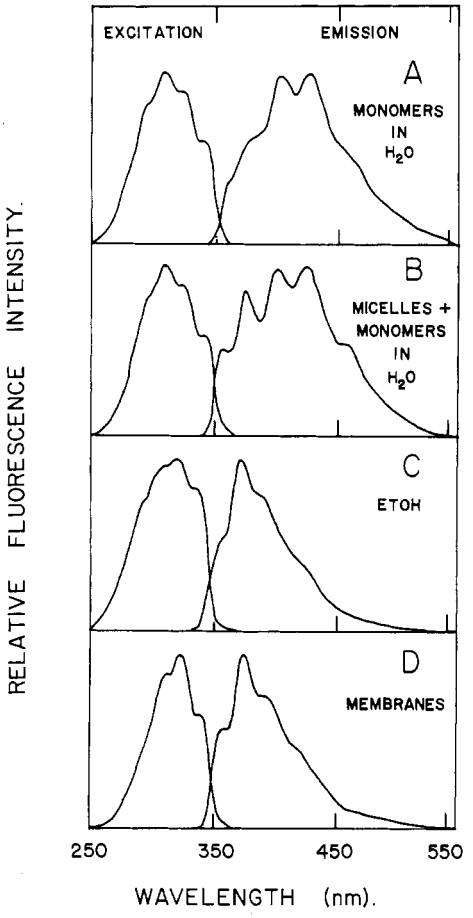

FIGURE 1: Fluorescence characteristics of dehydroergosterol as monomers or micelles in PBS, in EtOH, and in membranes. Fluorescence excitation and emission spectra of dehydroergosterol were obtained from the monomeric form (A) (0.08 ng/mL of PBS), from the micellar form near the critical micellar concentration (B) (0.08 μg/mL of PBS), in 20 μg/mL of EtOH (C), and in plasma membranes (D).

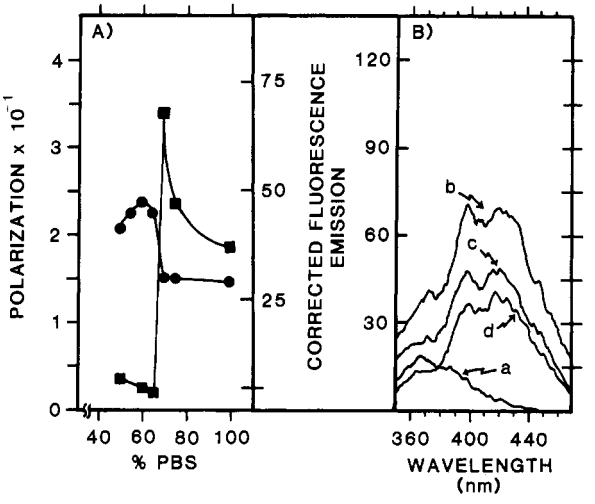

FIGURE 2: Fluorescence properties of dehydroergosterol in EtOH/PBS mixtures. (A) Fluorescence polarization (●) and corrected fluorescence emission (■) of 0.4 μg dehydroergosterol/mL in PBS diluted with various amounts of ethanol at 37 °C. Excitation was at 324 nm during polarization and emission determinations. (B) Corrected fluorescence emission spectra of 0.5 μg of dehydroergosterol/mL in (a) 65% PBS/35% ethanol, (b) 70% PBS/30% ethanol, (c) 75% PBS/25% ethanol, and (d) 100% PBS, as determined by excitation at 324 nm at 37 °C.

to dielectric constant changes and not due to micellization. Since micellization did not account for the peak ratio changes in the dioxane/H_2O mixtures under the conditions used herein,

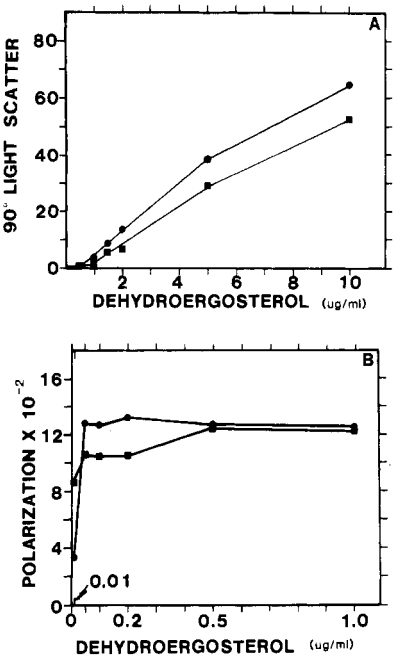

FIGURE 3: Effect of SCP on fluorescence of dehydroergosterol in PBS. (A) Absorbance-corrected fluorescence due to light scattering of dehydroergosterol in PBS (●) and dehydroergosterol plus 50 μg/mL SCP in PBS (■) as a function of dehydroergosterol concentration at 37 °C. Excitation and emission monochromators were set at 324 nm. Samples were incubated for 15 min at 37 °C. (B) Fluorescence polarization of dehydroergosterol in PBS (●) and dehydroergosterol plus 50 μg/mL SCP in PBS (■) as a function of dehydroergosterol concentration at 37 °C.

this solvent system may be used to describe the effect of solvent polarity of dielectric constant on dehydroergosterol fluorescence. The polarization of dehydroergosterol increased from 0.079 in 100% ethanol to 0.350 in 65% PBS. This increase in polarization was probably the result of the formation of micelles. At higher mole percent PBS, the dehydroergosterol fluorescence polarization decreased from a maximum of 0.350 to 0.240. The latter data were consistent with a self-quenching mechanism between like molecules, leading to decreased polarization. The theory for fluorescence depolarization due to self-quenching by energy transfer was previously described (Knox, 1968; Weber, 1954) and confirmed with chlorophyll *b* (Lee, 1975; Kelly & Patterson, 1971). Scattered light is also highly polarized, and as demonstrated below, light scattering of dehydroergosterol in PBS was also concentration dependent. This could account, in part, for the increased fluorescence polarization up to dehydroergosterol concentrations near 0.5 μg/mL. Above 70% PBS the fluorescence polarization further decreased from 0.240 to 0.180 while the fluorescence emission intensity decreased from 70 to 37, indicating a quenching efficiency of 49% and 47%, respectively. Kelly & Patterson (1971) examined chlorophyll concentration dependent self-quenching of polarization, quantum yield, and lifetime. In this system the reduction in fluorescence lifetime and quantum yield showed a different concentration dependence than the reduction in polarization. This difference was interpreted as implying the existence of another rapid energy dissipating process at the site of excitation. However, since polarization and fluorescence intensity of dehydroergosterol both decreased nearly equally with concentration, the results presented here appear to be more consistent with self-quenching via energy transfer between identical molecules.

At low concentrations of dehydroergosterol in PBS (10 ng/mL) the polarization was less than 0.033, indicating very little association between sterol molecules. However, at

dehydroergosterol concentrations between 0.05 and 1.0 μg/mL, polarization increased to a value near 0.130 (Figure 3B), indicating an increased dehydroergosterol rotational anisotropy as a result of micelle formation. The concentration dependence of dehydroergosterol fluorescence polarization was consistent with a critical micelle concentration of dehydroergosterol in PBS near 25 nM (Figure 3B). Light scattering was relatively insignificant below 0.5 μg/mL dehydroergosterol (Figure 3A). At concentrations near the limit of aqueous solubility (above 0.5 μg/mL), there was a significant increase in the light scattering corresponding to the presence of larger aggregates of dehydroergosterol. The maximal solubility of dehydroergosterol in PBS at 37 °C was 1.3 μM.

Interaction of Sterol Carrier Protein with $\Delta^{5,7,9(11),22}$-*Ergostatetraen-3β-ol.* Because of the low solubility of sterols in water, the presence of micelles complicated the use of this fluorescent sterol to monitor sterol/SCP interactions. However, if the SCP actually bound dehydroergosterol, then one may predict that the maximal solubility of dehydroergosterol in PBS should be shifted to higher concentration. Indeed, the results obtained in Figure 3A were consistent with this expectation: the maximal solubility of dehydroergosterol was shifted from 1.3 to 1.9 μM by SCP (50 μg/mL of PBS). Since the molecular weight of rat liver SCP was 14 184 (Gordon et al., 1983), the concentration of SCP in the assay was 3.53 μM. A double-reciprocal plot of [(light scattering of dehydroergosterol in PBS) − (light scattering of dehydroergosterol + SCP in PBS)]$^{-1}$ vs. [free dehydroergosterol]$^{-1}$ indicated that the apparent dissociation constant of dehydroergosterol for SCP was near 0.88 μM (data not shown). The point at which the fitted straight line crossed the ordinate represented the limiting difference in light scattering when the dehydroergosterol binding sites of SCP were fully occupied (B_{max} = 4.8 μM). The maximal binding of dehydroergosterol to SCP calculated therefrom was 1.3 mol/mol of SCP. When corrected for nonspecific binding, the maximal specific binding of dehydroergosterol to SCP was 1.0 mol/mol of SCP. The above apparent K_D and B_{max} were calculated under conditions in which all binding sites for dehydroergosterol were saturated. Therefore, the apparent K_D represents an average value for approximately one specific binding site per mole of SCP. These experiments were also repeated at concentrations far from saturation, and an apparent K_D and B_{max} were calculated from dehydroergosterol fluorescence lifetime data as described under Methods. The apparent K_D and B_{max} obtained thereby were 0.40 μM and 4.0 μM, respectively (data not shown). The apparent K_D's obtained by light scattering and lifetime analysis therefore agreed within a factor of 2. The lifetime data indicated that 1.1 mol of dehydroergosterol was specifically bound per mole of SCP. It should be noted that the light-scattering data were obtained under saturating conditions for SCP while the lifetime data were obtained at concentrations far below saturation of all SCP binding sites.

The molecular interaction of SCP with dehydroergosterol was also supported by spectral analysis. A comparison of the fluorescence emission maxima of dehydroergosterol in PBS and in PBS plus SCP indicated the appearance of a modified corrected fluorescence emission spectrum having not only an emission maximum at 415 nm but also an additional emission maximum at 374 nm (Figure 4). This effect was most apparent at concentrations of dehydroergosterol less than 0.5 μg/mL; at higher concentrations the emission of dehydroergosterol aggregates at 415 nm dominated the spectrum (Figures 4 and 5). The appearance of the emission maximum near 374 nm represented a significant blue shift and may

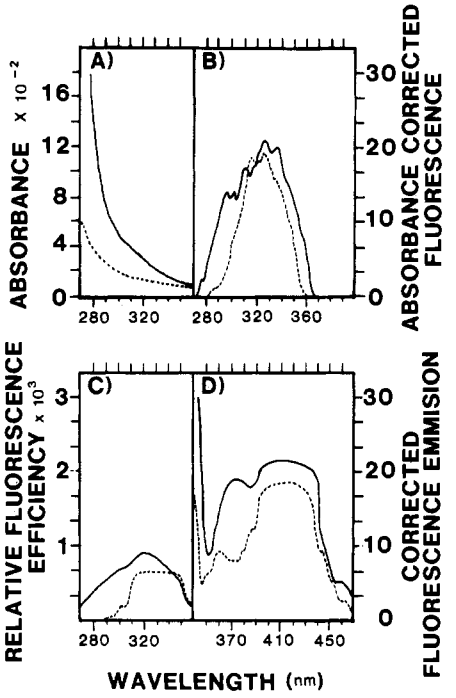

FIGURE 4: Effect of SCP on fluorescence properties of dehydroergosterol. Fluorescence properties of 0.1 μg of dehydroergosterol/mL of PBS (---) and 0.1 μg of dehydroergosterol/mL of PBS plus 50 μg of SCP/mL (—) as a function of wavelength at 37 °C. (A) Absorbance. (B) Absorbance-corrected fluorescence. (C) Relative fluorescence efficiency. (D) Corrected fluorescence emission. Excitation scans (A–C) were measured with the emission at 415 nm. Emission scans (D) were measured with excitation at 324 nm. Samples were incubated 15 min at 37 °C prior to fluorescence determinations.

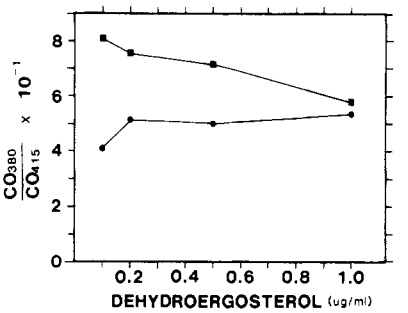

FIGURE 5: Effect of SCP on fluorescence spectral emission of dehydroergosterol in PBS. Ratio of absorbance-corrected fluorescence measured with emission monochromator set at 374 nm to absorbance-corrected fluorescence measured with emission at 415 nm (CO_{280}/CO_{415}) of dehydroergosterol in PBS (●—●) and dehydroergosterol plus 50 μg/mL SCP in PBS (■—■) as a function of dehydroergosterol concentration at 37 °C. Excitation wavelength was 324 nm, and samples were incubated 37 °C for 15 min prior to fluorescence determinations.

denote transfer of the dehydroergosterol from PBS to a more hydrophobic environment other than micelles, e.g., the SCP–dehydroergosterol binding site. The emission maximum of dehydroergosterol in ethanol and in solvents of low polarity was near 374 nm. The fluorescence polarization of dehydroergosterol solubilized by SCP (Figure 3B) also agreed with this finding. At a concentration of 10 ng of dehydroergosterol/mL of PBS (no light scattering due to micelles), in the presence of SCP, polarization of dehydroergosterol increased from 0.033 to 0.086 as a result of sterol interaction with SCP. At higher dehydroergosterol concentration (0.10 μg/mL), dehydroergosterol formed micelles, and the polarization of the fluorescent sterol was 0.142. SCP did not induce micelle formation by dehydroergosterol. Light scattering (Figure 3A)

Table I: Fluorescence Lifetime of Dehydroergosterol upon Binding to SCP[a]

[dehydroergosterol] (μg/mL)	SCP (50 μg/mL)	τ_1 (ns)	F_1 (%)	τ_2 (ns)	F_2 (%)
0.01	-	2.34	100		
0.01	+	2.07	53	12.70	47
0.02	-	2.27	100		
0.02	+	1.76	48	12.40	52

[a] Measurements were made after a 15-min equilibration at 37 °C with excitation energy at 324 nm and emission cutoff below 382 nm. Emission lifetimes were derived by heterogeneity analysis as described under Methods. τ_1 = lifetime 1; F_1 = percent of fluorescence due to component at lifetime 1; τ_2 = lifetime 2; F_2 = percent of fluorescence due to component at lifetime 2.

Table II: Displacement of Dehydroergosterol from SCP by Cholesterol[a]

dehydroergosterol (0.1 μg/mL)	SCP (50 μg/mL)	[cholesterol] (μg/mL)	polarization
+	-		0.142
+	+		0.109
+	+	0.01	0.102
+	+	0.10	0.104
+	+	1.00	0.129

[a] SCP (50 μg/mL) and dehydroergosterol (0.10 μg/mL) were incubated for 15 min at 37 °C prior to additions of cholesterol. Incubations with displacing cholesterol were for 25 min at 37 °C before polarization measurements were made (excitation at 325 nm and emission cutoff below 384 nm).

demonstrated that SCP increased the solubility of dehydroergosterol by impeding the formation of aggregates.

Additional data confirming the close molecular interaction of dehydroergosterol and SCP were obtained from fluorescence lifetime analysis. At low concentration (.02 μg/mL of PBS), dehydroergosterol displayed a single fluorescence lifetime near 2.3 ns (Table I). In the presence of SCP another lifetime component appeared near 12.4 ns. The short lifetime component near 2 ns may be assigned to monomeric dehydroergosterol not bound to SCP whereas the longer lifetime component near 12 ns can be attributed to dehydroergosterol bound to a hydrophobic site in SCP.

Sterol carrier protein contains three tyrosine residues and no tryptophan (Dempsey et al., 1981). Tyrosine emission in SCP was in a broad band (275–390 nm) with a maximum near 315 nm. Dehydroergosterol absorbance was over the range 265–360 nm, with absorption peak maxima near 338, 324, and 308, thus providing excellent overlap for nonradiative energy transfer from SCP tyrosine residues to bound dehydroergosterol. The resulting sensitized emission by dehydroergosterol was maximal at 374 nm. Long-range energy transfer by dipole–dipole interactions can take place if there is overlap of donor fluorescence emission and acceptor absorbance, if the donor and acceptor are separated by a short distance (energy transfer is proportional to r^{-6}), and if the lifetime of the donor is relatively long (Stryer, 1978; Brand & Witholt, 1967). The transfer of energy described below satisfies these criteria but not those of trivial transfer; e.g., the SCP donor emission spectrum was not distorted, and the fluorescence lifetime did not remain constant. In the following experiment, SCP (50 μg/mL of PBS) was incubated with dehydroergosterol (0.1 μg/mL of PBS). The tyrosine residues were excited at 280 nm, and sensitized fluorescence emission due to bound dehydroergosterol was measured at 374 nm. The absorbance-corrected emission intensity at 374 nm was 15% greater for the dehydroergosterol–SCP complex than for SCP alone. However, it is not clear that this difference was due to energy transfer and not simply due to the enhanced quantum yield of dehydroergosterol upon binding SCP. The extinction coefficient of dehydroergosterol is not zero at 280 nm; the ratio of absorbance at 280/324 nm was 0.30 for pure dehydroergosterol. Simply subtracting the fluorescence intensity of dehydroergosterol in water alone is not useful since absorption, emission, and lifetime of dehydroergosterol are sensitive to the environment, as shown above. Therefore, the energy-transfer efficiency, E, was calculated from the excitation spectrum of the energy acceptor as described under Experimental Procedures. This procedure utilized both extinction coefficients of the donor and acceptor. Insertion of the appropriate experimental values yielded $E = 36 \pm 2\%$ for the transfer efficiency for SCP tyrosine to bound dehydroergosterol. This transfer efficiency was confirmed by measurement of percent decrease in SCP protein corrected fluorescence emission intensity. Fluorescence excitation spectra had maxima near 280 nm, while emission was maximal near 315 nm (data not shown). When SCP was excited at 280 nm, added dehydroergosterol decreased the fluorescence emission of SCP at 315 nm by 34 ± 3%. The energy-transfer efficiency can also be related to R, the distance separating the donor and acceptor, and R_0, the distance at which transfer efficiency is 50% according to Forster's theory (described under Experimental Procedures). Since tyrosine emission and dehydroergosterol absorption allowed almost complete overlap, the overlap integral, J, calculated according to eq 4, was 5.56×10^{-15} M^{-1} cm^3. The quantum yield of tyrosine in SCP, Q_0, was 0.061 [experimentally determined for tyrosine in SCP from absorption spectra and fluorescence emission spectra as compared to those of a standard, diphenylhexatriene in benzene (quantum yield 0.80)]. All tyrosines were assumed to have the same quantum yield. The refractive index was set at $n = 1.5$ [for aromatic acids in proteins (Steinberg, 1971)], and K^2 was set at 0.476 [for proteins in which the donor–acceptor pairs are immobile and randomly distributed (Steinberg, 1971)]. These values allowed derivation of $R_0 = 17.3$ Å according to eq 3. Therefore, R, the distance between the centers of the donor and acceptor chromophores, was calculated according to eq 2 and found to equal 19 Å. Alternately, if both the donor and acceptor are assumed to undergo free rotation with a rotational relaxation time much less than the excited state lifetime of the donor, then K^2 equals $^2/_3$ (Stryer, 1978). Substituting this value for K^2 in eq 3 yields $R_0 = 18.3$ Å and $R = 20.1$ Å.

Further evidence for the close molecular interaction between dehydroergosterol and SCP was provided by a competitive binding experiment with a nonfluorescent sterol. The polarization of dehydroergosterol in PBS (0.10 μg/mL) was 0.142 (Table II). Upon addition of SCP (50 μg of protein/mL of PBS; equivalent to a SCP/dehydroergosterol mole ratio of 17/1), dehydroergosterol associated with SCP to give a polarization of 0.109. Addition of cholesterol (0.01–0.10 μg/mL) did not perturb the established dehydroergosterol–SCP equilibrium significantly since there was a 16-fold excess of available uncomplexed SCP to bind cholesterol. When 1.0 μg/mL of cholesterol was added (a 10-fold molar sterol excess over dehydroergosterol), displacement readily occurred as evidenced by a fluorescence polarization of 0.129, a value close to that of free micellar dehydroergosterol in PBS (0.142; see Table II). Concomitantly, lifetime analysis indicated the disappearance of the dehydroergosterol lifetime component near 12 ns.

Identification of Bound Dehydroergosterol in LM Fibroblast Cytosolic Lipid Transfer Proteins. Although the above results demonstrated the binding of dehydroergosterol to SCP in vitro, the possible significance of such an interaction in vivo has not been established. Therefore, we attempted isolation

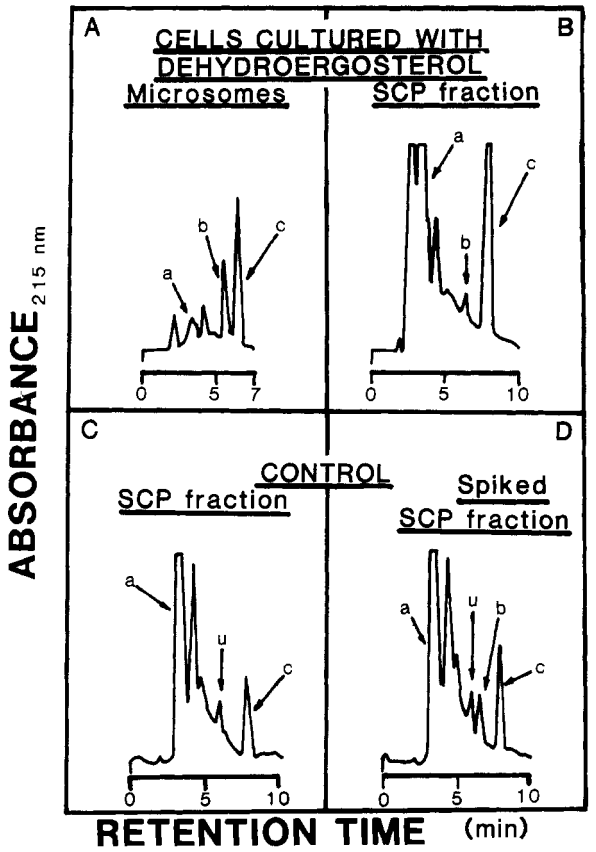

FIGURE 6: HPLC of neutral lipids from (A) LM fibroblast microsomal membranes and (B–D) soluble cytosolic protein fractions enriched in lipid-transfer proteins. (A) The chromatograph of microsomal membrane lipids from cells cultured with dehydroergosterol 72 h prior to fractionation had two distinguishable neutral lipids other than (a) desmosterol and (b) dehydroergosterol; (c) was the ergosterol internal standard. HPLC of the membrane was run with a 90:10 mixture of methanol–hexane at 1.0 mL/min on a reverse-phase column. (B) Neutral lipids from the lipid transfer protein enriched cytosol of cells given dehydroergosterol 3 h prior to fractionation showed a peak coinciding with R_T for a dehydroergosterol standard. (C) Neutral lipid extract of the lipid transfer protein enriched cytosol of control cells having an unidentified compound (U), eluting near the dehydroergosterol retention time. (D) Control sample as in (C) with 0.5 μg of pure dehydroergosterol added to the sample showing complete resolution from the unknown. The lipid transfer protein enriched cytosols in (B–D) were run at a 95/5 ratio of methanol to hexane.

of an SCP–dehydroergosterol complex from a mammalian system. Cultured LM fibroblasts readily incorporate dehydroergosterol without adverse effect (Schroeder, 1981; Hale & Schroeder, 1982), and immunoprecipitation with antibody to rat liver SCP indicated the presence of significant amounts of SCP in LM cells (Schroeder et al., 1984c). LM cells were cultured in suspension, and a culture in log phase was exposed to dehydroergosterol. The cytosol was isolated and fractionated to obtain a protein fraction enriched in sterol carrier proteins. This fraction was lipid extracted and subjected to HPLC analysis. After a 3-h incubation of LM cells with dehydroergosterol, the cytosolic sterol carrier protein extract demonstrated an absorbance peak, measured at 215 nm, with a retention time of 6.40 min by HPLC. Control cells had a component with a retention time of 6.0 min (Figure 6B,C). Pure dehydroergosterol was resolved at a retention time near 6.3 min, and spiking the control cell extract with 0.5 μg of dehydroergosterol demonstrated complete resolution of dehydroergosterol at 6.3 min from the neighboring unknown peak at 6.0 min (Figure 6D). Incubation of dehydroergosterol (0.1 μg/mL of PBS) with the control LM fibroblast SCP fraction (50 μg of protein/mL) resulted in a 1.7-fold enhancement of dehydroergosterol fluorescence.

LM Membrane Properties of Dehydroergosterol. Incorporation of dehydroergosterol into LM cell membranes was followed to study the membrane distribution of the probe after a 3-day incubation with dehydroergosterol. The dehydroergosterol to desmosterol ratio in plasma membranes, microsomal membranes (Figure 6A), and mitochondrial membranes was 2.86, 1.92, and 0.30, respectively. Correspondingly, the fluorescence polarization of dehydroergosterol in plasma membranes, microsomal membranes, and mitochondrial membranes was 0.305, 0.295, and 0.286, respectively. The fluorescence lifetime, limiting anisotropy, rotational relaxation time, and order parameter of dehydroergosterol in LM fibroblast plasma membranes were 1.6 ns, 0.097, 1.17 ns, and 0.48, respectively.

Discussion

In the work presented here we have utilized highly purified dehydroergosterol of known structure to address several fundamental questions regarding the chemical and physical properties of dehydroergosterol that are pertinent to its use as a probe for investigation of lipid–sterol and protein–sterol interactions. With few exceptions (Bergeron & Scott, 1982), most previous investigations with synthetic dehydroergosterol (Schroeder, 1981; Hale & Schroeder, 1982; Rogers et al., 1979; Archer, 1975; Schroeder et al., 1979; Yeagle et al., 1982a,b), provided few assessments of probe molecule purity. The presence of even small amounts of impurities or oxidized sterols must be strictly avoided. Fluorescence anisotropy, differential scanning calorimetry, and ^{31}P NMR demonstrated that oxidized derivatives of cholesterol did not suppress the melting phase transition of phospholipid liposomes as did pure cholesterol (Vincent & Gallay, 1983; Gallay et al., 1984). Oxidized sterols did not bind to the same site on oxysterol carrier protein as cholesterol (Kandutsch & Shown, 1981). Even trace amounts of oxidized sterols can be toxic to cultured cells (Kandutsch & Chen, 1975). Therefore, the use of impure dehydroergosterol at 1–50 mol % in membrane lipids may allow serious potential artifacts. The preparations of dehydroergosterol used in the present investigation were 99+% pure. Perhaps most important for its use as a biological probe for sterols, the structure of chemically synthesized dehydroergosterol was identical with that synthesized biologically by *C. tropicalis* and *B. fortis* (Sica et al., 1982; Delseth et al., 1979).

The fluorescent dehydroergosterol readily formed micelles in aqueous solution. The critical micellar concentration (25 nM) was determined by fluorescence polarization while the maximum aqueous solubility (1.3 μM) was ascertained by light-scattering methods. The apparent critical micellar concentration for cholesterol in aqueous solution, determined from rates of dialysis, was 25–40 nM (Haberland & Reynolds, 1973). However, the size range (M_r N 10 000–478 000) of cholesterol micelles was highly heterogeneous. More important, the maximum solubility of cholesterol in aqueous solution was 4.7 μM. Thus, dehydroergosterol had a similar maximal aqueous solubility (1.3 vs. 4.7 μM) and critical micellar concentration (25 vs. 25–40 nM) as cholesterol. It should be noted that initially the fluorescence polarization of dehydroergosterol (near 25 nM) increased while at higher concentrations there was a gradual decrease in polarization to a nonzero level. Theory predicts that energy transfer (by concentration self-quenching) would decrease the fluorescence polarization (Knox, 1968; Weber, 1954; Lee, 1975; Kelly & Patterson, 1971). Thus, the initial increase in fluorescence polarization near the critical micelle concentration indicated a different type of dehydroergosterol–dehydroergosterol interaction or

Table III: Summary of Dehydroergosterol Spectral Parameters in Aqueous Systems[a]

conditions	emission maxima (nm)	CO_{374}/CO_{426}	P	τ_1 (ns)	F_1 (%)	τ_2 (ns)	F_2 (%)
free dehydroergosterol	402, 426, 456		0.033	2.34	100		
dehydroergosterol bound to SCP	357, 374		0.086	2.07	53	12.70	47
dehydroergosterol in micelles	357, 374, 394, 415	0.513	0.133	0.57	30	5.26	70
dehydroergosterol micelles with SCP	374, 415	0.753	0.105	2.30	75	10.40	25
dehydroergosterol in LM plasma membrane	357, 374, 394, 417	1.97	0.261	2.00	100		

[a] Free dehydroergosterol (0.01 μg/mL of PBS); dehydroergosterol bound to SCP (0.01 μg/mL per 50 μg of protein/mL of PBS); micellar dehydroergosterol (0.2 μg/mL of PBS); micellar dehydroergosterol with SCP (0.2 μg/mL per 50 μg/mL protein); dehydroergosterol in LM plasma membranes (25 μg/mL protein in PBS). Samples were corrected for small contributions of protein fluorescence (SCP or LM membranes) to the fluorescence emission. Excitation was 324 nm.

packing that did not allow energy transfer. At higher concentration, the dehydroergosterol may pack into larger micelles or planar arrays wherein the orientation allowed more efficient energy transfer. As shown in Table III, the fluorescence lifetime of dehydroergosterol at low concentration displayed only one component near 2 ns, while at high concentration a second component near 5 ns was evident. The latter phenomenon was surprising since simple self-quenching would be expected to result in a shortened fluorescence lifetime. Indeed, the short lifetime component normally seen at 2.34 ns in dilute aqueous solution decreased to 0.57 ns (Table III). However, the appearance of the longer lifetime component at 5.26 ns was indicative of another type of interaction or packing, as yet undescribed. The appearance of a second lifetime component also occurred in energy-transfer quenching of 12-anthroylstearate by heme in cytochrome *c* (Radda & Vanderkooi, 1972).

SCP binding of dehydroergosterol altered the fluorescent properties of the sterol as well as those of the protein. The interaction of the fluorescent sterol with a hydrophobic pocket of SCP resulted in restricted mobility, a blue shift of the emission spectrum, increased lifetime, increased polarization, and increased rotational relaxation time as compared to the aqueous monomer. Increased viscosity or rigidity of the sterol in the SCP binding site is expected to restrict the mobility of dehydroergosterol. Indeed, when the fluorescence lifetime of dehydroergosterol was determined in 1-palmitoyl-2-oleoylphosphatidylcholine vesicles as a function of temperature, the limiting anisotropy and order parameter increased from 0.097 and 0.492, respectively, at 65 °C to 0.145 and 0.602 at 24 °C, respectively (F. Schroeder and T. E. Thompson, unpublished observation). Below the critical micellar concentration the fluorescence polarization of free dehydroergosterol increased from 0.033 to 0.086 upon binding to SCP. The binding of a cytotoxic alkaloid, camptothecin, by albumin resulted in an increased fluorescence polarization of the drug from 0.013 to 0.240 (Chignell, 1976). Many of the fluorescence properties of protein-associated dehydroergosterol were distinguishable from those of micelles or from those of membrane-bound probes. Protein binding to other fluorescent molecules has also been observed to blue shift the maximal emission (Mollay & Kreil, 1973; Reeves et al., 1973) and to give rise to a second lifetime component (Radda & Vanderkooi, 1972).

The binding of dehydroergosterol by SCP shifted the maximal aqueous solubility of dehydroergosterol from 1.3 to 1.9 μM. Shifts of the critical micelle concentration of other micellar systems, e.g., CTBR by glucagon, have been noted (Bornet & Edelhoch, 1971). Herein, the use of light-scattering data to construct a binding curve allowed determination of the apparent K_D = 0.88 μM and a 1:1 molar interaction for dehydroergosterol with SCP. Unfortunately, we were unable to confirm the K_D obtained from light scattering directly by measurement of fluorescence intensity before and after separation of bound from free sterol. Separation of bound from free probe was complicated by the presence of micelles. However, an apparent dissociation constant and maximal number of binding sites were also calculated from fluorescence lifetime data (0.40 μM and 4.0 μM, respectively), leading to 1.1 mole of dehydroergosterol bound per mole of SCP. These results were consistent with those of others using a functional assay for cholesterol transfer to conclude that SCP_2, a completely different sterol binding protein, also had one cholesterol binding site per molecule of protein (Chanderbhan et al., 1982).

Using the excitation spectrum of the energy acceptor, dehydroergosterol, to measure energy transfer from tyrosine of SCP, we obtained an apparent donor (SCP tyrosine) to acceptor (dehydroergosterol) distance of 19 Å. We recognize, however, that the transfer distance calculated as described under Experimental Procedures is only an approximation since several assumptions are made in order to use the equations for SCP–dehydroergosterol energy transfer. First, the orientation factor, K^2, depends upon both the donor and acceptor. Although this factor can vary from 0 to 4, in proteins or viscous environments in a variety of proteins it is near 0.476 (Steinberg, 1971), the value assumed and used herein. It has been shown that this approximation introduces an uncertainty of less than 10% if the fluorescence polarization is less than 0.3 (Haas et al., 1978; Stryer, 1978). Second, SCP contains three tyrosine residues (Dempsey et al., 1981) that may differ in quantum yield. Tyrosine quantum yields vary from 0.02 to 0.07 in proteins (Steinberg, 1971). Our experimentally determined value of 0.061 is within this range. Since R_0 is proportional to the sixth root of the quantum yield, the maximal inaccuracy due to variation in quantum yield would be about 17% (0.061 vs. 0.02). It is also possible that tryosine–tyrosine energy transfer may also take place, thereby providing somewhat inflated transfer efficiencies. The amino acid composition of the rat liver SCP preparation used, herein, was determined by chemical analysis (Dempsey et al., 1981) and cloned cDNA analysis (Gordon et al., 1983). Both procedures showed the presence of three tyrosine and no tryptophan residues. Upon excitation at 280 nm the fluorescence emission of this SCP was maximal near 315 nm. This finding is consistent with that of other proteins containing tyrosine but no tryptophan and maximally emitting near 305–312 nm (e.g., insulin, RNase, zein, and ovomucoid) (Weber, 1960; Teale, 1960). In contrast, proteins containing both tyrosine and tryptophan or tryptophan alone fluoresce maximally near 335 nm (Teale, 1960). In fact, human serum albumin contains only one tryptophan and 17 tyrosine residues and emits maximally near 335 nm (Teale, 1960). The latter phenomenon appears due to the efficient intramolecular transfer of energy from tyrosine to tryptophan. Amino acid analysis and cloned cDNA analysis of rat liver SCP showed the presence of two tyrosine and no tryptophan residues (Dempsey et al., 1981;

Gordon et al., 1983). Similar observations were reported for human liver and yeast SCP (Dempsey et al., 1981; Dempsey, 1977) and for related preparations (Bloj et al., 1978; Ketterer et al., 1976). However, another report states that SCP_2, a protein completely different from SCP, contains one tryptophan and no tyrosine. Yet another class of lipid binding proteins, fatty acid binding protein, contains one tryptophan and one to five tyrosine residues (Dempsey et al., 1981; Poorthuis et al., 1981; Dempsey, 1984; Noland et al., 1980). Comparisons between these proteins are difficult because differences in properties appear to be due to the fact that they may be completely different proteins. In any case, the important point is that SCP interacts closely with dehydroergosterol such that fluorescent amino acids approach within 19 Å of dehydroergosterol upon binding. Third, small variations in refractive index will have only a slight effect on measurement of the intermolecular distance since eq 3 contains an n^{-4} term. Values for the refractive index in proteins vary between 1.33 and 1.60 (Steinberg, 1971), thereby introducing only a 4% maximal variation in determination of R_0. Thus, although the actual distance separating dehydroergosterol from tyrosine in SCP is probably slightly different from that estimated here, the data do indicate that dehydroergosterol is in close proximity to SCP. Dehydroergosterol has also been shown to interact with glycophorin, a red cell membrane protein (Yeagle et al., 1982a,b). The R_0 calculated here for the dehydroergosterol–SCP complex (17.3 Å), was similar to that obtained for cholestatrienol–LDL or –HDL tryptophan (25.4 Å) (Smith & Green, 1974) and other fluorophore–protein interactions in the range 10–50 Å (Kouyama et al., 1983; Chignell, 1970; Herman & Fernandez, 1982; Katsumata et al., 1978). Last, the energy-transfer data allow speculation of the size or shape of SCP. If SCP is a spherical, globular protein, then its diameter calculated from its molecular weight should be about 33 Å (Chignell, 1970). Since R, the distance between SCP tyrosine and the cholestatrienol, was 19 Å, the SCP molecule could easily be a globular protein.

The demonstration of in vivo dehydroergosterol binding to LM fibroblast soluble cytosolic lipid binding proteins confirmed the biological importance of this interaction. These proteins account for as much as 8% of cytosolic protein (Dempsey et al., 1981). The data in Figure 6 were consistent with the possibility that dehydroergosterol inhibited LM cell microsomal sterol specific synthetic enzymes and caused accumulation of desmosterol precursers in the SCP. This observation is consistent with that of others who demonstrated that cholestatrienol, a fluorescent sterol very similar to dehydroergosterol, inhibited microsomal HMG CoA reductase of cultured fetal lung cells (Shakespeare & Wilton, 1980).

Previous investigations using radiolabeled cholesterol did not demonstrate the presence of cholesterol in purified SCP (Dempsey et al., 1981) because, in in vitro assays for binding, cholesterol caused aggregation of SCP to higher molecular weight oligomers (Grabowski et al., 1976; Daum & Dempsey, 1979). This phenomenon in combination with sterol micellization and nonspecific binding (Kandutsch & Shown, 1981; Ishibashi & Bloch, 1981) precluded the determination of cholesterol binding by gel filtration assays. The value of the fluorescent sterol, dehydroergosterol, lies in the sensitivity of its spectral properties to binding by SCP. Properties such as lifetime and polarization, especially, are concentration-independent parameters that are sensitive to molecular interactions. Brominated cholesterol has also been used to monitor sterol protein interactions (Simmonds et al., 1982). However, this method provided information only on alterations in the protein fluorescence. Dehydroergosterol, a fluorescent sterol, provided data on the behavior of the sterol as well and may even be used to detect sterol–protein interactions in which fluorescent amino acid residues may be absent from the protein or may not participate in the sterol binding site. Since sterols such as brominated cholesterol and dehydroergosterol are not identical in structure to cholesterol, it is possible that the binding data obtained with sterols such as dehydroergosterol may not reflect those of cholesterol. However, dehydroergosterol displayed many similar properties to those of cholesterol (Hale & Schroeder, 1982; Archer, 1975; Yeagle et al., 1982a,b; Rogers et al., 1979). Cholesterol and dehydroergosterol also bound to the same site of SCP with approximately equal affinity. Therefore, the SCP binding results obtained with dehydroergosterol are likely to also reflect those of cholesterol, at least qualitatively.

In summary, the data described here indicate the utility of the fluorescent sterol, dehydroergosterol, for probing the behavior of sterols in micellar solutions, in membranes, and especially in sterol–protein interactions.

ACKNOWLEDGMENTS

We gratefully acknowledge the following people for their support in contributing to this research effort. The secretarial work was expendiently and accurately accomplished by Genie Eckenfels. The LM fibroblast cells were cultured by Lynn Whitmer and Ellen Moezpoor. Rat liver SCP was isolated by Carol D. Olson. We also acknowledge the competent technical support of Gene Hubert and Larissa Schuster in the HPLC and fluorescence analyses.

Registry No. Dehydroergosterol, 516-85-8; squalene, 111-02-4; cholesterol, 57-88-5.

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