Ruoho, A. E. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 3645–3649.
Vuong, T. M., Chabre, M., & Stryer, L. (1984) *Nature 311*, 659–661.
Wagner, R., Ryba, N. J. P., & Uhl, R. (1987) *FEBS Lett. 221*, 253–259.
Wagner, R., & Ryba, N. J. P., & Uhl, R. (1988) *FEBS Lett. 235*, 103–108.

Rates of Spontaneous Exchange of Synthetic Radiolabeled Sterols between Lipid Vesicles†

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ABSTRACT: ^{14}C-Labeled sterols with structural variation in the polar function [3α-OH, 3-O$(CH_2)_2$O-$(CH_2)_2$O$(CH_2)_2$OH, 3α-NH_2, 3β-NH_2, and 3-OC(O)CHN=N] and at the 7 position (7-oxo, 7α-OH, and 7β-OH) were synthesized and incorporated into unilamellar vesicles for studies of the rates of transfer to an excess of acceptor vesicles. Cholesterol, cholestanol, and epicholesterol underwent full exchange in a single kinetic pool, and 90% of the 3α-triethoxycholesterol was exchangeable in one pool. Biphasic kinetics with full exchangeability were observed for cholesterylamines, which bear a positive charge at the 3 position; the slow phase reflects the high activation energy for inner-to-outer leaflet movement of the charged lipid. Biphasic kinetics were also found for cholesteryl diazoacetate, indicating that this photoaffinity probe and cholesterol have different mechanisms of transfer. Sterols that are more hydrophilic than cholesterol as estimated by reversed-phase high-performance chromatography (elution with acetonitrile–2-propanol, 4:1 v/v, with varying proportions of water) gave faster exchange rates than cholesterol, whereas sterols that are more hydrophobic gave slower exchange rates. However, the rates of [^{14}C]sterol desorption from the lipid–water interface are not correlated with the relative sterol hydrophobicity as estimated by the logarithm of the capacity factors using acetonitrile–2-propanol–water as the mobile phase. These studies suggest that the interaction of sterols with phospholipids provides the principal physical–chemical basis for determining the rates of spontaneous exchange of sterols between bilayers.

There has been considerable interest in recent years in the spontaneous intermembrane movement of unesterified cholesterol. Relatively rapid transfer or exchange of cholesterol has been observed between membrane structures such as vesicles, erythrocytes, lipoproteins, mycoplasmas, and mammalian cells in tissue culture [reviewed by Phillips et al. (1987), Dawidowicz (1987), and Bittman (1988)]. The rate of spontaneous transfer of cholesterol between membranes is a function of the degree of saturation of the phospholipid fatty acyl chains and the sphingomyelin content of the membrane (Phillips et al., 1987; Bittman, 1988) and also of the curvature of the surface from which desorption takes place (McLean & Phillips, 1984; Fugler et al., 1985). Studies of the rates of cholesterol movement between membranes composed of different phospholipids have indicated an inverse relationship between the unidirectional transfer or bidirectional exchange rate and the degree of molecular packing of cholesterol with phospholipids in the bilayer (Fugler et al., 1985; Phillips et al., 1987; Lund-Katz et al., 1988). In addition to the interactions experienced by cholesterol in the lipid matrix of the donor species, the rate of cholesterol release from donor vesicles and transfer to acceptor vesicles is sensitive to factors that modify the aqueous phase solubility of cholesterol, such as the presence of chaotropic salts (Clejan & Bittman, 1984a), bile salts (Vlahcevic et al., 1990), and polar water-miscible organic solvents (Bruckdorfer & Green, 1967; Quarfordt & Hilderman, 1970; Bruckdorfer & Sherry, 1984). A recent comparison of the rates of cholesterol and sitosterol exchange suggested that the rate of sterol exchange between vesicles decreases with increasing sterol hydrophobicity, since the presence of the 24α-ethyl group in sitosterol represents a constraint to the rate of intermembrane movement (Kan & Bittman, 1990, 1991). To evaluate further the effects of structural modifications in cholesterol on the movement of sterols between vesicles, we have synthesized various radiolabeled synthetic analogues of cholesterol. In this paper, we compare the effects of specific structural modifications in the sterol molecule on the rates of efflux from phospholipid vesicles. We have also used sterol transfer kinetics to determine whether the cholesterol photoaffinity probe cholesteryl diazoacetate (Middlemas & Raftery, 1987) behaves analogously to cholesterol with respect to exchange and thus with respect to interactions with donor phospholipids.

Materials and Methods

Chemicals. [4-^{14}C]Cholesterol (specific activity 57.5 mCi/mmol) and [9,10-^{3}H(N)]glycerol trioleate (specific activity 15.4 Ci/mmol) were obtained from Du Pont New England Nuclear (Boston, MA). [4-^{14}C]Epicholesterol (specific activity 57.5 mCi/mmol) was prepared as described previously (Yan & Bittman, 1990). Egg PC,[1] DPPC, DCP,

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[1] Abbreviations: PC, phosphatidylcholine; DPPC, dipalmitoyl-PC; DCP, dicetyl phosphoric acid; k', capacity factor.

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cholesterol, 5α-cholestan-3β-ol (cholestanol), 7-keto- and 7α-hydroxycholesterol, *N*-palmitoyldihydrolactocerebroside, and DEAE-Sepharose CL-6B were purchased from Sigma Chemical Co. The galactosyl-binding lectin *Ricinus communis* agglutin I (RCA-120, referred to as lectin below) was from Vector Laboratories (Burlingame, CA). The solvents used to synthesize the labeled sterols were dried as follows: THF and ether, distilled from sodium/benzophenone before use; pyridine, dichloromethane, benzene, and toluene, distilled from calcium hydride and stored over 4-Å molecular sieves; triethylamine, distilled from potassium hydroxide. Cholesterol was recrystallized twice before use from ethanol–acetone 95:5, and its purity was checked by TLC as described before (Clejan et al., 1981). The purities of the synthetic sterols were checked by HPLC on a C18 Carbosphere column, 4.6 × 250 mm, Phenomenex, Rancho Palos Verdes, CA) using acetonitrile–2-propanol (4:1 v/v) as eluent and a flow rate of 2 mL/min. Gas chromatography was carried out on a 30 m × 0.25 mm i.d., 0.25-μm DB-5 bonded phase fused silica capillary column (J&W Scientific, Folsom, CA).

Preparation of Vesicles. Vesicles were prepared by sonication for about 1 h as described previously (Fugler et al., 1985). Donor vesicles (1 mM total lipid concentration) contained a trace of [^{14}C]sterol as the transferable lipid and acceptor vesicles contained [^{3}H]triolein as a nonexchangeable marker to monitor vesicle recovery. The sizes and homogeneities of the vesicles were estimated by photon correlation spectroscopy by Chris Rutkowski as described elsewhere (Hwang & Cummins, 1982). The sizes of the vesicles prepared from DPPC–sterol–DCP (61:24:15 mol/mol/mol/) were similar: cholesterol, 141-nm diameter; epicholesterol, 129 nm; cholesteryl diazoacetate, 125 nm; triethoxycholesterol, 123 nm; cholestanol, 156 nm. Examination of the donor vesicles by negative-staining electron microscopy revealed that they were entirely unilamellar. Acceptor vesicles (10 mM total lipid concentration) were sonicated using a microtip at 50% duty cycle for 1 h.

Measurement of [^{14}C]Sterol Exchange. The rates of transfer or exchange of [^{14}C]sterols from donor to acceptor vesicles were measured at 37 or 50 °C by two procedures. When the donor vesicles were prepared with dicetyl phosphate (15 mol %), aliquots (200 μL) were withdrawn from the incubation mixture (donor to acceptor vesicle ratio, 1:10) at various time intervals and were applied to DEAE-Sepharose CL-6B columns as described previously (Fugler et al., 1985). When the donor vesicles contained *N*-palmitoyldihydrolactocerebroside (20% w/v), separation of donor and acceptor vesicles was achieved by addition of 7.5 μg of lectin to a 200-μL aliquot of the incubation mixture in an Eppendorf centrifuge tube, followed by centrifugation at room temperature for 2 min. This assay is based on lectin-based assays developed by Curatolo et al. (1978), Backer and Dawidowicz (1979), and Kasper and Helmkamp (1981). The supernatant was transferred to a scintillation vial, 5 mL of Ecoscint (National Diagnostics, Highland Park, NJ) was added, and the mixture was shaken by vortex for about 30 s. The vials were counted for 10 min in a Packard 2000CA liquid scintillation counter. Recovery and fusion were estimated from the ^{3}H dpm in the acceptor fraction; in the DEAE-Sepharose method of separation, recovery was >80%, and in the lectin method of separation, recovery was also >80%. The rates of intervesicular [^{14}C]sterol transfer were analyzed by slight modifications of the procedure we used previously for sterol exchange (Fugler et al., 1985). Briefly, α_t, the fraction of radiolabeled sterol that undergoes exchange at time t, is calculated as follows: $\alpha_t = [^{14}C/^{3}H]_t/[^{14}C/^{3}H]_{mix}$, where $[^{14}C/^{3}H]_t$ and $[^{14}C/^{3}H]_{mix}$ represent the ratio of [^{14}C]sterol to [^{3}H]triolein in the eluate at time t and in the donor–acceptor vesicles without separation, respectively. The initial values of exchange of labeled sterol at times zero and infinity are α_0 and α_∞, respectively. These values were estimated by using the downhill simplex algorithm of Nelder and Mead (1965); for the iterative least-squares programs, see Noggle (1985) and Press et al. (1986). All of the kinetic data (α_t) including experimental points obtained at times approaching the equilibrium value were fit to a function of time, $F(t)$, according to

$$F(t) = \alpha_\infty + [(\alpha_0 - \alpha_\infty) \exp(\text{slope} \times t)] \quad (1)$$

The pseudo-first-order rate constants and half-times of exchange were calculated using the following relationships (for a 10-fold excess of acceptor vesicles):

$$k = -\text{slope}/1.1 \quad (2)$$

$$t_{1/2} = \ln 2/k \quad (3)$$

The size of the exchangeable pool of labeled cholesterol, X_{xch}, was calculated from the relationship

$$X_{xch} = [(\alpha_\infty - \alpha_0)/(1 - \alpha_0)] \times 1.1 \quad (4)$$

Note that the factor of 1.1 is used to correct the rate constant of labeled sterol movement from donor to acceptor particles for back exchange of label (eq 2) and to normalize the equilibrium value of 90.9% expected for random distribution of the label between donor and acceptor particles (acceptor–donor ratio, 10:1) to 100% exchange of [^{14}C]sterol at equilibrium (eq 4). When two kinetic phases (see Figure 3) were observed, eq 5 was used to obtain the rates of the slow (s) and fast (f) phases:

$$F(t) = [\alpha_{\infty s} + (\alpha_{0s} - \alpha_{\infty s}) \exp(\text{slope}_s \times t)] + [\alpha_{\infty f} + (\alpha_{0f} - \alpha_{\infty f}) \exp(\text{slope}_f \times t)] \quad (5)$$

For the lectin assay (Figure 4), eq 5 was simplified to a five-parameter fit by assuming that $\alpha_{\infty s} = \alpha_{\infty f}$.

Synthesis of ^{14}C-Labeled Sterols. [4-^{14}C]Cholesteryl diazoacetate was prepared by the procedure of Middlemas and Raftery (1987), who prepared the ^{3}H-labeled analogue. In the purification, we used two TLC plates to purify the product; first, the crude product was spotted onto a 1-mm thick plate, which was developed in toluene. The product was scraped from the plate, extracted with ether, and then run on a 0.25-mm plate eluted with petroleum ether–ether, 7:1; mp 140–142 °C; R_f 0.40 (petroleum ether–ether, 7:1); ^{1}H NMR (200 MHz, $CDCl_3$) δ 5.45, 4.73.

[4-^{14}C]3α-Triethoxycholesterol [3β-[2-hydroxyethoxy[ethoxy(ethoxy)]]-3α-cholesterol] was prepared by a modification of the procedure of Patel et al. (1984) as follows. To a solution of 39 mg (0.1 mmol) of cholesterol containing 10 μCi of [4-^{14}C]cholesterol in ~1 mL of dichloromethane was added 152 mg (1.5 mmol) of triethylamine, followed by a solution of 36 mg (0.31 mmol) of methanesulfonyl chloride (Aldrich) in 1.5 mL of dichloromethane. The mixture was stirred overnight at 0 °C. The reaction was quenched with water, and the product was extracted into ether. The ether layer was washed with 2 N HCl and then with water (3 × 5 mL). The ether layer was dried ($MgSO_4$) and concentrated, giving 46 mg (100% yield) of crude [4-^{14}C]cholesteryl mesylate. To the latter were added 384 mg (2.6 mmol) of triethylene glycol (Aldrich, distilled before use; bp 132–135 °C/0.5–0.7 mm) and 1 mL of toluene. After the mixture was heated at 120 °C for 1.5 h, toluene was removed under vacuum; water was added to assist in the azeotropic distillation. The product was

extracted with ether (10 mL, the 3 × 5 mL), and the organic phase was washed with 10% aqueous sodium carbonate and then with water (4 × 5 mL). The ether phase was dried ($MgSO_4$), and the solvent was evaporated to afford 47 mg of the crude product as a yellow residue. Purification by preparative TLC (elution with chloroform–ethyl acetate, 1:1) gave 33 mg (63%, based on cholesterol) of pure product; TLC R_f 0.27 (chloroform–ethyl acetate, 1:1); $[\alpha]_D^{25}$ −26.5° (*c* 1.0, $CHCl_3$); IR (CCl_4) 3460, 1660 cm^{-1}; NMR (200 MHz, $CDCl_3$) δ 5.35 (d, 1 H, C_6-*H*), 3.65 (m, 12 H, CH_2O), 3.18 (m, 1 H, O*H*). It should be noted that Patel et al. (1984) prepared triethoxycholesterol from cholesteryl tosylate and reported $[\alpha]$ −25.3° ($CHCl_3$) and R_f 0.43 (chloroform–ethyl acetate, 1:1) for this product.

[4-^{14}C]Epicholesterylamine (3α-aminocholesterol) was prepared by a modification of the procedure of Loibner and Zbiral (1976). [4-^{14}C]3α-Azido-5-cholestene was prepared in ~55% yield from cholesterol using HN_3, followed by purification by preparative TLC (elution with petroleum ether) as follows: To 37 mg (0.1 mmol) of cholesterol containing 10 μCi of [4-^{14}C]cholesterol in 0.25 mL of benzene were added 52 mg (0.2 mmol) of triphenylphosphine and 5 mL of benzene. Then 0.15 mL of a 5.7% solution of hydrazoic acid in benzene [~8.6 mg (0.2 mmol)] of hydrazoic acid [generated according to Wolff (1946)] was added, followed by dropwise addition of a solution of 35 mg (0.2 mmol) of diethyl azodicarboxylate in 2 mL of benzene. The mixture was stirred at room temperature for 2 h, then purified by preparative TLC; mp 113–114 °C; $[\alpha]$ −3.2° (*c* 1.0, $CHCl_3$); IR (KBr) 2090, 1640 cm^{-1}; NMR (60 MHz, $CDCl_3$) δ 5.44, 3.90. Reduction of 21.5 mg of [4-^{14}C]3α-azido-5-cholestene with 50 mg of lithium aluminum hydride in 5 mL of ether at room temperature for 2 h, followed by workup with wet ether, water, separation of the ether layer, washing with saturated NaCl, and drying (Na_2SO_4), gave 19 mg (97% yield) of [4-^{14}C]3α-amino-5-cholestene; mp 100–101 °C; $[\alpha]_D^{25}$ −42.4° (*c* 1.0, $CHCl_3$); NMR (200 MHz, $CDCl_3$) δ 5.36, 3.16; TLC (C18 plate from Analtech, Newark, DE) R_f 0.25 (elution with 2-propanol–concentrated NH_4OH, 8:1).

[4-^{14}C]Cholesterylamine (3β-aminocholesterol) was prepared in 67% yield from the reaction of 25 mg of [4-^{14}C]-epicholesterol, 34.5 mg of triphenylphosphine, 0.1 mL of hydrazoic acid in benzene, and 23.5 mg of diethyl azodicarboxylate. Purification by preparative TLC gave 17.8 mg of the desired azide; mp 84–85 °C; $[\alpha]_D^{25}$ −19.1° (*c* 1.0, $CHCl_3$); IR (KBr) 2100, 1620 cm^{-1}; NMR (60 MHz, $CDCl_3$) δ 5.40, 3.20. The azide was reduced with excess lithium aluminum hydride as described above to give [4-^{14}C]cholesterylamine in 95% yield; mp 93–94 °C; $[\alpha]_D^{25}$ −29.8° (*c* 1.0, $CHCl_3$); NMR (200 MHz, $CDCl_3$) δ 5.32, 2.62; TLC (C18 plate) R_f 0.16 (elution with 2-propanol–concentrated NH_4OH, 8:1).

[4-^{14}C]Cholestanol was prepared by reduction of [4-^{14}C]-cholesterol with hydrogen for 2 days at room temperature using 10% Pd/C in ethanol or 2-propanol. The reaction mixture was filtered, the solvent was evaporated under reduced pressure, and the residue was purified by preparative TLC. GC/MS *m/z* 388, R_t 31.33 min, identical to authentic sample; cholesterol gave *m/z* 368 (M-18), R_t 34.58 min.

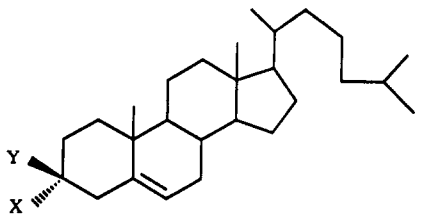
[4-^{14}C]7-Keto- and 7α- and 7β-hydroxycholesterol were prepared by a modification of the procedures of Chicoye et al. (1968) and Parish (1987). Briefly, [4-^{14}C]cholesteryl benzoate was prepared by the reaction of 40 mg of [^{14}C]-cholesterol with benzoyl chloride in pyridine. Excess benzoyl chloride was quenched with ice, and the product was extracted with ether. The crude [^{14}C]cholesteryl benzoate was dissolved in benzene and oxidized with pyridinium chlorochromate in the presence of 3-Å molecular sieves at 80–85 °C for 24 h. The product was extracted with ether, and the organic phase was concentrated and spotted on a preparative TLC plate (elution with ether/petroleum ether, 1:4). [4-^{14}C]7-Ketocholesteryl benzoate (31.3 mg, 0.062 mmol) was dissolved in 2 mL of THF and hydrolyzed at room temperature for 2 days with 2 mL of 5% potassium carbonate in methanol–water (3:1). The solvents were evaporated, and the residue was purified by preparative TLC (elution with ether), giving the product in 73% yield. A mixture of epimeric 7-hydroxycholesterols was obtained by reduction of [^{14}C]7-ketocholesterol with lithium aluminum hydride in ether at room temperature. After the reaction mixture has stirred for 2 h, wet ether was added to decompose the excess of lithium aluminum hydride, followed by water and 6 N sulfuric acid. The organic phase was separated, and the aqueous phase was washed with ether. The combined ether layer was washed with water, dried over $MgSO_4$, and evaporated. The residue was dissolved in chloroform and spotted on a preparative TLC plate. The plate was developed twice; first with cyclohexane–ether (1:9), then with ether. Zones of the desired epimers were visualized using iodine vapors; 7α- and 7β-hydroxycholesterols were scraped from the plates, giving yields of 20% and 66%, respectively.

[^{14}*C*]*Glucose Permeability of Liquid Vesicles.* Lipid films were prepared from 61 mol % DPPC, 24 mol % sterol, and 15 mol % DCP (10 mM total lipid concentration). Aqueous dispersions were prepared at 50 °C with 20 mM sodium phosphate buffer, pH 6.0, containing [^{14}C]glucose (270 000 dpm). Untrapped [^{14}C]glucose was removed by dialysis of the liposomes vs large volumes of the buffer at 4 °C. Aliquots of liposomes were withdrawn from the inside of the dialysis sac during the dialysis and counted. When the ^{14}C dpm reached an approximately constant value, the dialysis sac containing the liposomes was immersed in buffer at 50 °C and incubated in a LabLine shaking water bath. Aliquots (100 μL) were withdrawn at various time intervals and counted after adding 4 mL of Scintiverse II scintillation fluid.

HPLC Capacity Factors. Retention times of the sterols were recorded by reversed-phase HPLC on a model 410 Perkin-Elmer HPLC system equipped with a LC-235 diode-array detector and LCI-100 computer-integrator. The column was C18-Carbosphere, 4.6 × 250 mm, purchased from Phenomenex (Rancho Palos Verdes, CA). The mobile phase was acetonitrile–2-propanol (4:1 v/v) with varying proportions of water (from 0 to 5% by volume). The flow rate was 2.5 mL/min. The capacity factor k' was calculated as shown in the footnote to Table I.

Results

Exchange of Cholestanol, Epicholesterol, and Triethoxycholesterol. The chemical structures of the [^{14}C]sterols we used with modifications at the 3 position are shown in Figure 1. In order to examine the effects of changes in both the nucleus and polar group of cholesterol on the exchange rate, we also synthesized [^{14}C]cholestanol (which lacks the Δ^5 double bond) and a series of oxysterols with different groups at the 7 position. Kinetic data for exchange of sterols differing in nuclear and polar group structure at the 3 position are shown in Figure 2, panels A and 2B, respectively. The rates of exchange of cholesterol and cholestanol between DPPC–sterol vesicles are similar at 24 mol % sterol ($t_{1/2}$ values of 445 and 555 min, respectively); the inset to Figure 2 shows that about 90% of these ^{14}C-labeled sterols undergo exchange in one kinetic pool. Figure 2B shows that the 3α-substituted sterols,



X	Y	
OH	H	epicholesterol
NH_3^+	H	3α-aminocholesterol
H	NH_3^+	3β-aminocholesterol
H	$OCCH{=}N{=}N$	cholesteryl diazoacetate
$O(CH_2)_2O(CH_2)_2O(CH_2)_2OH$	H	triethoxycholesterol

FIGURE 1: Structures of the [4-^{14}C]cholesterol analogues used.

epicholesterol and 3α-triethoxycholesterol, undergo much faster exchange than does cholesterol ($t_{1/2}$ values of 37.8 and 38.6 min, respectively). The inset shows that these synthetic ^{14}C-labeled sterols also undergo exchange as a single pool. The sizes of the exchangeable pool, as calculated using eq 4, are >97–100% for cholesterol, cholestanol, and epicholesterol, and 90% for triethoxycholesterol.

Intervesicle Movement of Aminocholesterol and Cholesteryl Diazoacetate. To test whether the presence of a positive charge at the 3 position of the sterol affects the rate and extent of the exchange between vesicles, we compared the kinetics of [^{14}C]cholesterol and [^{14}C]aminocholesterol exchange. Figure 3 shows that 3α- and 3β-aminocholesterol display biphasic kinetics in the ion-exchange assay, which requires the presence of a negatively charged nonexchangeable lipid in the donor (or the acceptor) vesicles. In order to assure the presence of net negative charge in the donor vesicles, we reduced the sterol content to 6 mol %. The half-times and pool sizes for exchange of the two positively charged sterols are 3α-aminocholesterol, 393.1 min (29.3%) and 16.1 min (33.2%) (Figure 3A); 3β-aminocholesterol, 118.9 h (52.3%) and 9.3 h (20%) (Figure 3B). In contrast, [^{14}C]cholesterol underwent full exchange under the identical conditions as a single pool with a half-time of 256 min (Figure 3A).

We also analyzed the kinetics of [^{14}C]3β-aminocholesterol transfer from egg PC–sterol–glycolipid vesicles to egg PC acceptor vesicles in an assay system that does not involve a separation of donor and acceptor species based on charge. An assay system involving incorporation of glycolipid into the donor vesicles, with subsequent agglutination by addition of a lectin, was used in order to avoid incorporation of DCP (see Materials and Methods). Figure 4 shows that biphasic kinetics were still observed for sterols bearing a charged polar group in this assay method. For comparison, the time course of cholesterol transfer is shown; the relatively rapid half-time for exchange (54 min) in this system compared with the exchange from DPPC vesicles presented above reflects the looser bilayer packing when egg PC is the host phospholipid compared with DPPC. The rapidly exchanging pool of [^{14}C]3β-aminocholesterol ($t_{1/2}$ of ~12 min) shown in Figure 4A represents 58% of the exchangeable pool; the slowly exchanging pool represents 32% of the total sterol and has a $t_{1/2}$ of 155 min. Since 90% of the total [^{14}C]3β-aminocholesterol underwent

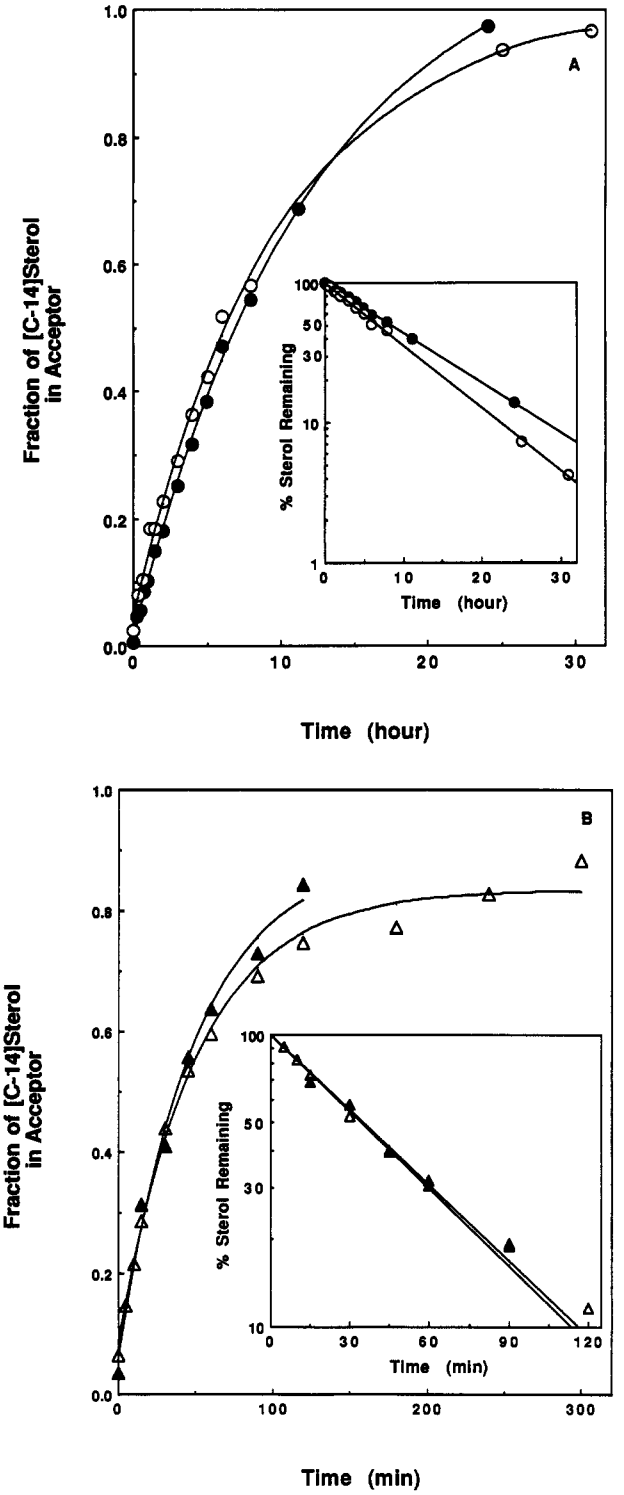


FIGURE 2: Time course of [^{14}C]sterol exchange between DPPC–sterol unilamellar vesicles at 50 °C. The vesicles contained 24 mol % sterol. The incubation medium contained albumin (2% w/v). (Inset) First-order plots of the exchange data. (A) The sterols are cholesterol (○) and cholestanol (●). (B) The sterols are epicholesterol (▲) and triethoxycholesterol (△).

exchange, we conclude that 3β-aminocholesterol molecules are distributed unequally between the two leaflets of the bilayer, with an excess localized in the outer leaflet of these vesicles (diameter >100 nm). Figure 4B shows that [^{14}C]cholesteryl diazoacetate undergoes biphasic transfer kinetics, with half-times and relative pool sizes of 35.4 min (32%) and 2468 min (68%). These results suggest that cholesteryl diazoacetate is localized preferentially in the inner leaflet of the bilayer;

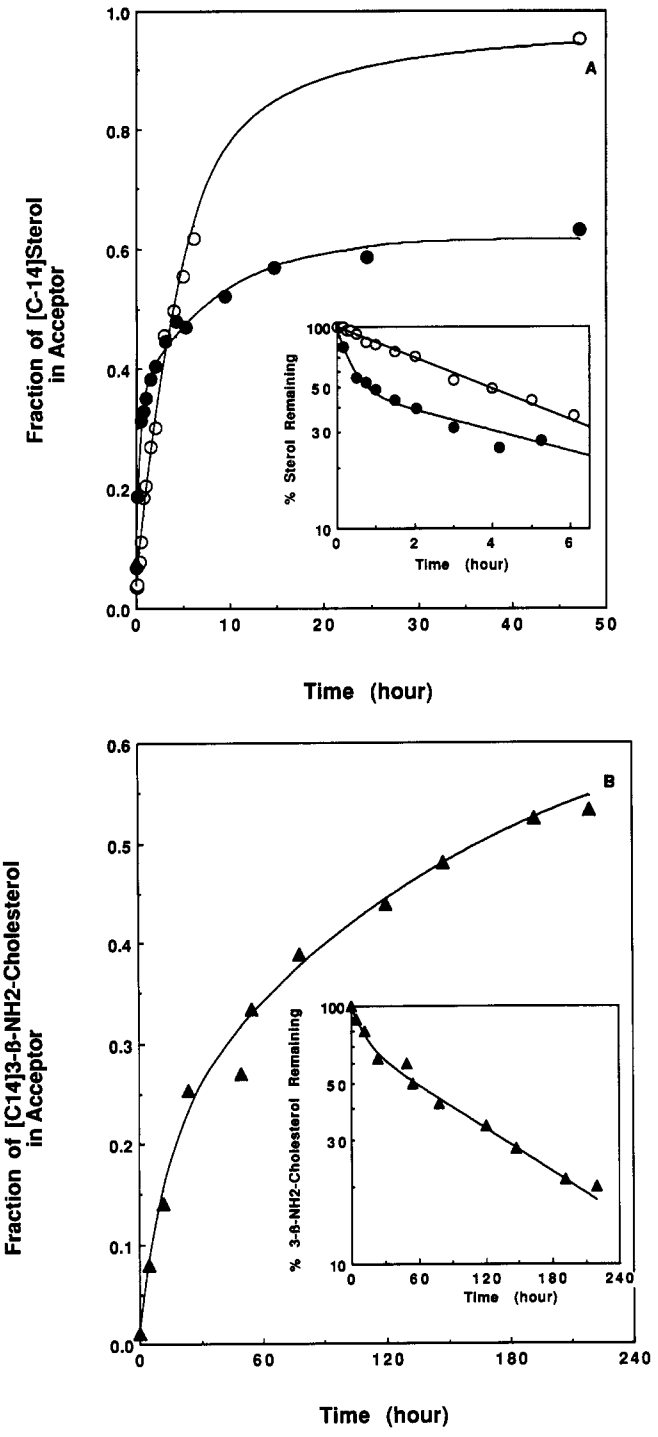


FIGURE 3: Time course of [^{14}C]sterol transfer at 50 °C from DPPC–sterol donor vesicles to DPPC acceptor vesicles using the ion-exchange chromatography assay. The donor vesicles contained 6 mol % sterol. (Inset) First-order plot of the transfer data. The sterols in the donor vesicles are (A) cholesterol (○) and 3α-aminocholesterol (●); (B) 3β-aminocholesterol.

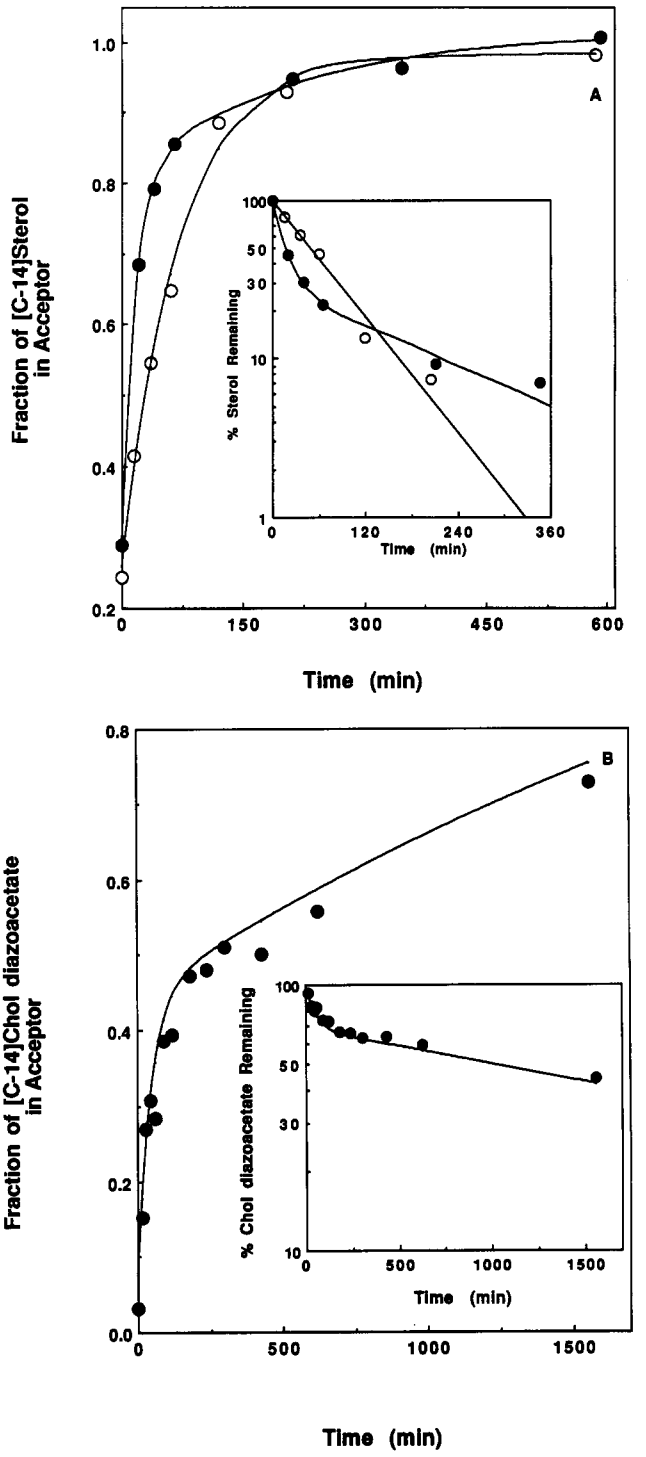


FIGURE 4: Time course of [^{14}C]sterol transfer at 50 °C from egg PC–sterol donor vesicles to egg PC acceptor vesicles using the lectin assay. The donor vesicles contained 24 mol % sterol, 56 mol % egg PC, and 20 mol % *N*-palmitoyldihydrolactocerebroside. (Inset) First-order plot of the transfer data. The sterols in the donor vesicles are (A) cholesterol (○) and 3β-aminocholesterol (●); (B) cholesteryl diazoacetate.

furthermore, the differences observed in exchange kinetics between this probe and cholesterol suggest that cholesteryl diazoacetate may not be incorporated into PC bilayers in the same manner as cholesterol.

7-Oxosterols. Finally, we studied the exchange of labeled sterols that differ from cholesterol in the sterol nucleus by having oxygen-containing functions at the 7 position. The kinetics of exchange was studied at 37 °C instead of at 50 °C because the 7-substituted sterols exchange very rapidly at 50 °C. Figure 5 shows that the 7-oxysterols undergo exchange much more rapidly than cholesterol. The $t_{1/2}$ values shown in Figure 5 are 7-ketocholesterol, 81 min; 7α-hydroxycholesterol, 16 min; 7β-hydroxycholesterol, ~7 min; and cholesterol, 1825 min. These results are consistent with previous studies of oxysterol transfer from monolayers to a subphase containing vesicles or lipoproteins, in which it was found that the initial rates of sterol transfer followed the order 7α-hydroxycholesterol > 7-ketocholesterol > cholesterol (Theunissen et al., 1986; van Amerongen et al., 1989). However, our finding of

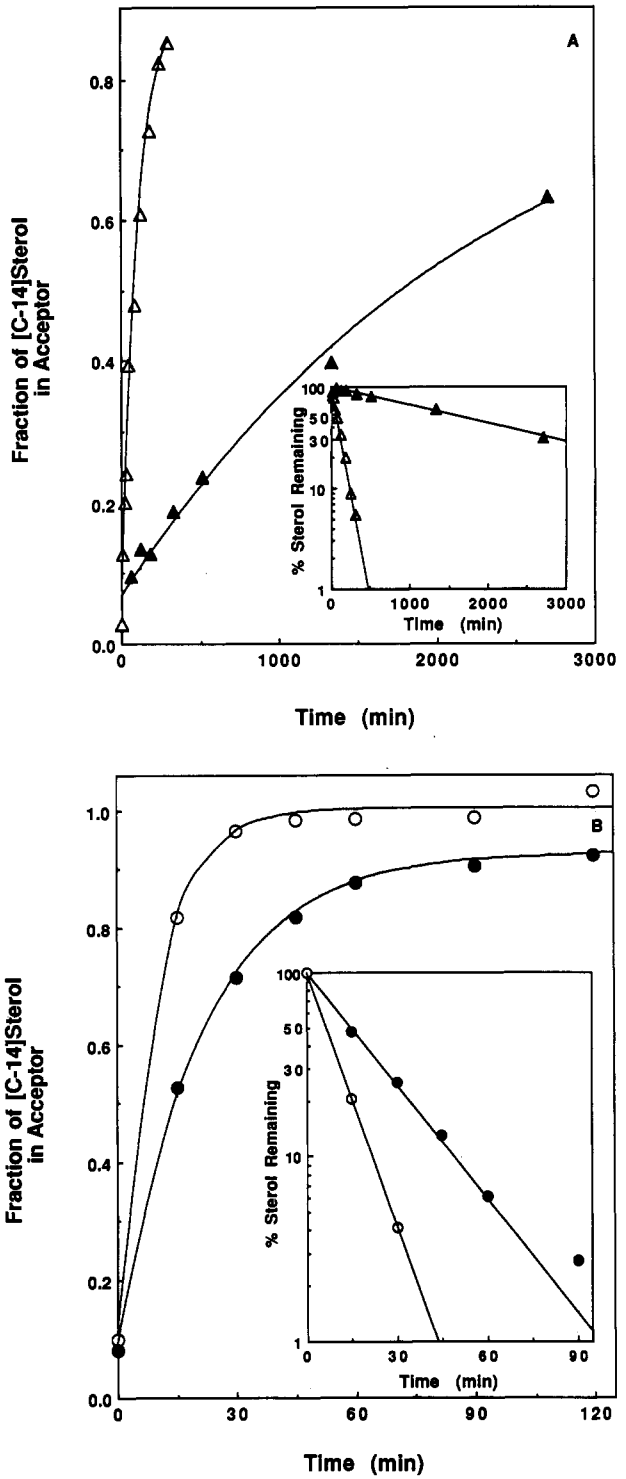


FIGURE 5: Time course of [^{14}C]cholesterol, [^{14}C]-7-ketocholesterol, and [^{14}C]-7α- and 7β-hydroxycholesterol exchange at 37 °C between DPPC–sterol unilamellar vesicles. (Inset) First-order plot of the exchange data. (A) The sterols are cholesterol (▲) and 7-ketocholesterol (△). The donor and acceptor vesicles contained 24 mol % cholesterol of 7-ketocholesterol. (B) The sterols are 7α-hydroxycholesterol (●) and 7β-hydroxycholesterol (○). The donor and acceptor vesicles contained 24 mol % 7α- or 7β-hydroxycholesterol.

full exchangeability of 7β-hydroxycholesterol between vesicles differs from the observation that only 25% of this sterol undergoes exchange from *Mycoplasma capricolum* membranes to vesicles (Lelong et al., 1988).

k′ Values. In a previous analysis of relative sterol hydrophobicities, Armstrong and Carey (1987) obtained the k' values by reversed-phase chromatography using acetonitrile–methanol as the mobile phase. We measured the retention times of the synthetic sterols using acetonitrile–2-propanol (4:1 v/v) mobile phases consisting of 1, 2, 3, 4, and 5% water by volume. A plot of the logarithm of the k' values vs water content was made for each synthetic sterol; Table I presents the logarithm of the k' values extrapolated to 10% water content. Marked differences in reversed-phase HPLC retention behavior are found in mobile phases consisting of acetonitrile–2-propanol (4:1 v/v) with varying proportions of water. Sitosterol displays a slightly higher k' value than cholesterol, and sterols containing an oxygen functionality at the 7 position display lower k' values. Epicholesterol is slightly less hydrophobic than cholesterol, in agreement with the results of Armstrong and Carey (1987). 3α-Triethoxycholesterol has essentially the same mobility as cholesterol in reversed-phase HPLC elution, suggesting that it has the same hydrophobicity as cholesterol.

Table I: Examination of a Relationship between the Relative Retention Times of Sterols on a C18 Carbosphere HPLC Column and Their Half-Times of Desorption from DPPC Vesicles

sterol	log k'[a]	$t_{1/2}$[b] (min)	$t_{1/2}$[c] (min)
7-ketocholesterol[d]	0.80	81	
7α-hydroxycholesterol[d]	0.74	16	
7β-hydroxycholesterol[d]	0.94	7	
epicholesterol	1.31		35 ● 4
triethoxycholesterol	1.41		33 ● 8
cholesterol	1.43	1825	500 ● 78
sitosterol[e]	1.59		1701 ± 165

[a] k' is a normalized HPLC retention value obtained by using the relationship $[(R_{t,sterol}/R_{t,solvent}) - 1]$. The solvent for the sterols was chloroform. The retention times were measured by reversed-phase HPLC as described under Materials and Methods. The log k' values are reported for a mobile phase consisting of acetonitrile–2-propanol (4:1 v/v) with 10% water content. These values were obtained by extrapolation of a plot of log k' vs H_2O content (by volume). [b] Transfer data from Figure 5. The temperature was 37 °C. [c] Exchange data from Figure 2. The temperature was 50 °C. [d] Donor vesicles were sonicated for 40 min in order to obtain similar diameters to those found when dispersions of other sterol–PC mixtures were sonicated for 60 min. [e] Sitosterol was purified by preparative HPLC.

Discussion

In previous studies of the kinetics of cholesterol transfer between membranes, the influence of *phospholipid* structure on cholesterol uptake into or efflux from bilayers has been examined in considerable detail. Preferential interactions between cholesterol and phospholipids, as in the case of saturated glycerophospholipids and sphingomyelin, impede the rate of cholesterol release from the donor surface (Wattenberg & Silbert, 1983; Clejan & Bittman, 1984c; Fugler et al., 1985; Yeagle & Young, 1986; Lund-Katz et al., 1988; Gold & Phillips, 1990; Kan et al., 1991). In addition to the effects of alterations in phospholipid structure and content, membrane cholesterol and protein content and chemical modification of membrane protein by cross-linking on cholesterol exchange rate have been examined [reviewed by Bittman (1988) and Bittman et al. (1985)]. Local domain structure, as influenced by membrane proteins and saturated phospholipid fatty acyl chains, also affect the rate of cholesterol transfer from cell membranes (Clejan & Bittman, 1984a; Bellini et al., 1984).

In contrast to the rather extensive investigation of the effects of phospholipid structure variation on cholesterol transfer between membranes, relatively little has been accomplished in terms of the effects of variation of sterol structure on the kinetics of intermembrane movement. In previous studies from this laboratory, we showed that the half-times for exchange of [^{14}C]sitosterol and [^{14}C]desmosterol from mycoplasma cells

to lipid vesicles are higher than that of [^{14}C]cholesterol (Clejan & Bittman, 1984b) and that [^{14}C]sitosterol undergoes slower exchange between phospholipid vesicles than does [^{14}C]-cholesterol (Kan & Bittman, 1990, 1991). Rujanavech and Silbert (1986) found that sitosterol and campesterol partitioned about equally into DPPC and egg PC vesicles at 37 °C, whereas cholesterol showed a preference for partitioning into DPPC vesicles. The initial rate of transfer of 4-cholesten-3-one from vesicles to erythrocytes was higher than that of cholesterol (Bruckdorfer & Sherry, 1984), and 7-ketocholesterol was taken up by erythrocytes faster than cholesterol (Wharton & Green, 1982).

The mechanism by which cholesterol undergoes spontaneous intermembrane movement is not fully established. Strong evidence has been presented in favor of the aqueous diffusion pathway, in which the rate-determining step is the release of cholesterol from the surface of the donor particle [see Pownall et al. (1983), Phillips et al. (1987), Dawidowicz (1987), and Bittman (1988)]; however, a collisional complex between donor and acceptor particles has been postulated in some systems (Steck et al., 1988). Nevertheless, it is clear from studies of cholesterol movement between a variety of membrane structures, such as vesicles, erythrocytes, mycoplasmas, lipoproteins, and mammalian cells in tissue culture, that both van der Waals attractive interactions with phospholipids in the donor membrane and aqueous phase solubility are important factors that determine the ease with which sterols undergo efficient intermembrane exchange. Because of the limited number of commercially available radiolabeled sterols, it was not possible to attempt a distinction between these two factors without undertaking the required syntheses. For further elucidation of the factors that are involved in the spontaneous distribution of cholesterol between membranes, we synthesized a series of ^{14}C-labeled sterols that differ in either the nuclear or polar head group portion of cholesterol and used them in the kinetic studies of exchange or transfer between vesicles reported in this paper.

Sterols Modified at the 7 Position. Figure 5 shows that the rate of sterol exchange between DPPC–sterol vesicles is enhanced when polar groups are introduced at the 7 position. The half-time of exchange of [^{14}C]7-ketocholesterol between DPPC–sterol vesicles at 50 °C is about 25-fold lower than that of [^{14}C]cholesterol (Figure 5A); [^{14}C]7α-hydroxycholesterol undergoes exchange between DPPC/sterol vesicles at 37 °C about 115 times faster than does [^{14}C]cholesterol, and the rate of exchange of [^{14}C]7β-hydroxycholesterol is even higher (Figure 5B). The interactions between PC and 7-ketocholesterol or 7α- and 7β-hydroxycholesterol are weaker than those between PC and cholesterol, as estimated by the condensing effect in monolayers and permeability of liposomes (Bruckdorfer et al., 1968; Nakagawa et al., 1980; Demel et al., 1972a,b; Theunissen et al., 1986). In addition, vibrational studies indicated a looser packing of DPPC bilayers in the presence of 7-keto- and 7α-hydroxycholesterol compared with cholesterol (Rooney et al., 1986).

Relationship between Log k' Values and Half-Times. Since previous studies suggested that aqueous monomeric sterol solubilities may play a role in the rate of sterol transfer between vesicles (Bruckdorfer & Green, 1967; Quarfordt & Hilderman, 1970; Bruckdorfer & Sherry, 1984; Clejan & Bittman, 1984; Vlahcevic et al., 1990), we examined whether differences in hydrophobicity of the synthetic sterols are correlated with their rates of intervesicle exchange. The decreased hydrophobicity caused by introducing a polar oxygen functionality into 7-keto- and 7α- and 7β-hydroxycholesterol is reflected in increased mobility, relative to cholesterol, on reversed-phase HPLC. Although these three sterols have similar k' values, the relative ease with which they desorb from DPPC–sterol vesicles differs significantly (Table I). The observation that sterol molecules with similar hydrophobicities undergo exchange with significantly different rates suggests that differences exist in the extent of packing of these sterols with DPPC. It should also be noted that 3α- and 3β-aminocholesterol have similar k' values (data not shown) but different rate constants for the slow and fast exchange processes. Moreover, cholesteryl diazoacetate and 3β-aminocholesterol have similar k' values but different kinetic behavior (Figure 4). Thus, there is no apparent correlation between the log k' value and the half-time of sterol exchange.

Sitosterol has a slightly higher k' value than does cholesterol, indicative of more hydrophobic character owing to the insertion of an ethyl group at C-24 in place of a hydrogen atom. However, the ratio of the sitosterol–cholesterol exchange half-times (Kan & Bittman, 1991) is much greater than the difference in their HPLC mobilities. Relatively small differences in the extent of interaction of these sterols with phospholipids have been noted in bilayers (Bittman et al., 1981) and monolayers (Ghosh & Tinoco, 1972). Additional van der Waals interactions involving the hydrophobic sterol side chain and phospholipid acyl chains may be more important in determining off-rate constants from lipid–water interfaces than in determining condensing effects in monolayers and efflux of trapped markers from bilayer vesicles. Indeed, a model of the transition state for cholesterol desorption proposes that the sterol is held to the surface of the donor species by its side chain (Phillips et al., 1987).

Sterols Modified at the 3 Position. Inversion of the configuration at the 3 position and alkylation with a hydrophilic chain also led to a dramatic enhancement in exchange rate. The half-times of exchange of [^{14}C]epicholesterol and [^{14}C]-3α-triethoxycholesterol between DPPC–sterol vesicles at 50 °C are about 15-fold lower than that of [^{14}C]cholesterol (Figure 2B). It is well known that epicholesterol does not condense phospholipids in monolayers and reduce the permeability of liposomes to a similar extent to that of cholesterol (Bittman & Blau, 1972; Demel et al., 1972a,b; Demel & DeKruijff, 1976; Clejan et al., 1979); indeed, a model based on ^{2}H NMR studies has been proposed to explain the failure of epicholesterol to interact tightly with PC because the 3α-hydroxy group is oriented toward the phospholipid acyl chains (Murari et al., 1986). 3-Oxyethylene derivatives of cholesterol may alter the hydration capacity of lipids at the lipid–water interface and may also impose steric constraints that modify lipid packing. Triethoxycholesterol did not reduce the phase transition temperature of DPPC as a function of sterol content to the same extent as cholesterol; in addition, triethoxycholesterol did not order dioleoyl-PC bilayers to as large an extent as did cholesterol (Goodrich et al., 1988). We estimated the capacity of these sterols to inhibit the initial rate of [^{14}C]glucose efflux from DPPC vesicles at 50 °C. We found that triethoxycholesterol and epicholesterol were much less effective in reducing the initial rate of glucose efflux compared with cholesterol (data not shown), in agreement with previous studies of the packing of these sterols in PC bilayers.

Cholestanol. Catalytic hydrogenation of the double bond in the sterol nucleus gave [^{14}C]cholestanol (dihydrocholesterol). Figure 2 shows that the half-time of exchange is not affected significantly by removing the double bond from the sterol nucleus. Cholestanol is slightly more mobile than cholesterol in reversed-phase HPLC in mobile phases of acetonitrile–

methanol (Armstrong & Carey, 1987) and acetonitrile–2-propanol (data not shown), suggesting a slightly more hydrophilic character than cholesterol. In previous comparisons of the properties of the binding of the two sterols with phospholipids in membranes, cholestanol was found to be somewhat less effective than cholesterol in raising the membrane order parameter in egg PC liposomes as determined with 12-doxylstearic acid and was also less effective than cholesterol in inhibiting release of trapped glucose from egg PC–sterol liposomes at 33 mol % sterol (Ranadive & Lala, 1987). Cholestanol showed no preference for partitioning into DPPC vs egg PC vesicles at 37 °C, whereas cholesterol partitioned preferentially into DPPC over egg PC vesicles (Rujanavech & Silbert, 1986).

Biphasic Exchange Kinetics. The data for the exchange of [^{14}C]3α- and 3β-aminocholesterol are fitted to a two-exponential function, with a fast and slow phase (Figures 3 and 4), whereas only one kinetic phase was found for exchange of all of the other ^{14}C-labeled sterols we investigated. 3-Aminocholesterol is the only sterol analogue we used that bears a net positive charge at the pH of the exchange experiments. To evaluate whether the two phases arise because a pool of 3-aminocholesterol interacts tightly with dicetyl phosphate and consequently would be impeded with respect to its movement across the bilayer and desorption from the external surface, we changed our assay system to avoid the use of dicetyl phosphate. Figure 4 shows that two kinetic pools are still observed in the lectin assay system. In contrast, [^{14}C]cholesterol undergoes exchange in one kinetic pool under the identical assay conditions. The slow phase of [^{14}C]3α- and 3β-aminocholesterol exchange probably arises from the high energy required for the inner to outer leaflet movement of the positively charged sterol. Transverse movement of sterol molecules from the inner to outer leaflet of the bilayer (from where desorption and transfer can occur) requires at least transient disruption of lipid packing and unfavorable interactions between the sterol polar group and phospholipid acyl chains. Nevertheless, only one kinetic pool has been observed for exchange of radiolabeled cholesterol between vesicles; this observation indicates that transbilayer movement of cholesterol is not rate limiting for desorption from the surface of the donor particle.[2] Our observation that the exchange or transfer data for [^{14}C]3α- and 3β-aminocholesterol between vesicles are best fit by a two-exponential function in two different assay systems (Figures 3 and 4) suggests that transbilayer movement of the amino compound becomes rate limiting relative to desorption. In agreement with the hypothesis that sterols bearing a charged polar head group disrupt membrane packing during transverse migration to a greater extent that does cholesterol, we found that cholesteryl diazoacetate (which is zwitterionic) also undergoes biphasic exchange kinetics (Figure 4B).

In conclusion, decreasing the affinity of cholesterol for phospholipids in the donor membranes appears to stimulate the rate of movement of cholesterol between membranes to a greater extent than increasing the sterol's aqueous solubility. Introduction of a zwitterionic or ionic group into the sterol molecule results in a change in the kinetics of exchange, with two kinetic pools observed. Our finding of full sterol exchangeability is in full agreement with many earlier reports of cholesterol exchange [e.g., Phillips et al. (1987)] but differs from the conclusions of some other investigators who detected a sizeable nonexchangeable pool of cholesterol in vesicles prepared with palmitoyloleoyl-PC (Bar et al., 1986; Nemecz et al., 1988), dimyristoyl-PC (Bar et al., 1987), and sphingomyelin (Bar et al., 1987). The possible reasons for the discrepancy are discussed elsewhere (Kan & Bittman, 1991).

[2] In contrast, the finding of two kinetic pools for cholesterol exchange from mycoplasma and acholeplasma cells to lipid vesicles indicates that transbilayer migration across the membranes of these cells is slow relative to exchange from erythrocyte and other biological membranes that display only one kinetic pool of cholesterol [see Phillips et al. (1987)]. The slowly exchanging cholesterol pool in mycoplasma and acholeplasma membranes may arise from contacts with membrane proteins and/or phospholipids (Bittman, 1988; Rottem & Davis, 1986).

References

Armstrong, M. J., & Carey, M. C. (1987) *J. Lipid Res. 28*, 1144–1155.

Backer, J. M., & Dawidowicz, E. A. (1981) *Biochim. Biophys. Acta 551*, 260–270.

Bar, K. L., Barenholz, Y., & Thompson, T. E. (1986) *Biochemistry 25*, 6701–6705.

Bar, K. L., Barenholz, Y., & Thompson, T. E. (1987) *Biochemistry 26*, 5460–5465.

Bellini, F., Phillips, M. C., Pickell, C., & Rothblat, G. H. (1984) *Biochim. Biophys. Acta 777*, 209–215.

Bittman, R. (1988) in *Biology of Cholesterol* (Yeagle, P. L., Ed.) pp 173–195, CRC Press, Boca Raton, FL.

Bittman, R., & Blau, L. (1972) *Biochemistry 11*, 4831–4839.

Bittman, R., Clejan, S., Robinson, B. P., & Witzke, N. M. (1985) *Biochemistry 24*, 1403–1409.

Bittman, R., Leventhal, A. M., Karp, S., Blau, L., Tremblay, P.-A., & Kates, M. (1981) *Chem. Phys. Lipids 28*, 323–335.

Bruckdorfer, K. R., & Green, C. (1967) *Biochem. J. 104*, 270–277.

Bruckdorfer, K. R., & Sherry, M. K. (1984) *Biochim. Biophys. Acta 769*, 187–196.

Bruckdorfer, K. R., Graham, J. M., & Green, C. (1968) *Eur. J. Biochem. 4*, 512–518.

Chicoye, E., Powrie, W. D., & Fennema, O. R. (1968) *Lipids 3*, 551–556.

Clejan, S., & Bittman, R. (1984a) *J. Biol. Chem. 259*, 441–448.

Clejan, S., & Bittman, R. (1984b) *J. Biol. Chem. 259*, 449–455.

Clejan, S., & Bittman, R. (1984c) *J. Biol. Chem. 259*, 10823–10826.

Clejan, S., Bittman, R., Deroo, P. W., Isaacson, Y. A., & Rosenthal, A. F. (1979) *Biochemistry 18*, 2118–2125.

Clejan, S., Bittman, R., & Rottem, S. (1981) *Biochemistry 20*, 2200–2204.

Curatolo, W., Yau, A. O., Small, D. M., & Sears, B. (1978) *Biochemistry 17*, 5740–5744.

Dawidowicz, E. A. (1987) *Curr. Top. Membr. Transp. 29*, 175–202.

Demel, R. A., Bruckdorfer, K. R., & van Deenen, L. L. M. (1972a) *Biochim. Biophys. Acta 255*, 311–320.

Demel, R. A., Bruckdorfer, K. R., & van Deenen, L. L. M. (1972b) *Biochim. Biophys. Acta 255*, 321–330.

Fugler, L., Clejan, S., & Bittman, R. (1985) *J. Biol. Chem. 260*, 4098–4102.

Ghosh, D., & Tinoco, J. (1972) *Biochim. Biophys. Acta 266*, 41–49.

Gold, J., & Phillips, M. C. (1990) *Biochim. Biophys. Acta 1027*, 85–92.

Goodrich, R. P., Handel, T. M., & Baldeschwieler, J. D. (1988) *Biochim. Biophys. Acta 738*, 143–154.

Hwang, J. S., & Cummins, H. Z. (1982) *J. Chem. Phys. 77*, 616–621.

Kan, C.-C., & Bittman, R. (1990) *J. Am. Chem. Soc. 112*, 884–886.

Kan, C.-C., & Bittman, R. (1991) *J. Am. Chem. Soc. 113*, 6650–6656.
Kan, C.-C., Bittman, R., & Hajdu, J. (1991) *Biochim. Biophys. Acta 1066*, 95–101.
Kasper, A. M., & Helmkamp, G. M., Jr. (1981) *Biochemistry 20*, 146–151.
Lelong, I., Luu, B., Mersel, M., & Rottem, S. (1988) *FEBS Lett. 232*, 354–358.
Loibner, H., & Zbiral, E. (1976) *Helv. Chim. Acta 59*, 2100–2113.
Lund-Katz, S., Laboda, H. M., McLean, L. R., & Phillips, M. C. (1988) *Biochemistry 27*, 3416–3423.
McLean, L. R., & Phillips, M. C. (1984) *Biochim. Biophys. Acta 776*, 21–26.
Middlemas, D. S., & Raftery, M. A. (1987) *Biochemistry 26*, 1219–1223.
Murari, R., Murari, P., & Baumann, W. J. (1986) *Biochemistry 25*, 1062–1067.
Nakagawa, Y., Nojima, S., & Inoue, K. (1980) *J. Biochem. 87*, 497–502.
Nelder, J. A., & Mead, R. (1965) *Comput. J. 7*, 308.
Nemecz, G., Fontaine, R. N., & Schroeder, F. (1988) *Biochim. Biophys. Acta 943*, 511–521.
Noggle, J. H. (1985) in *Physical Chemistry on a Microcomputer*, pp 145–165, Little, Brown, and Co., Boston.
Parish, E. J. (1987) *Lipids 22*, 760–761.
Patel, K. R., Li, M. P., Schuh, J. R., & Baldeschwieler, J. D. (1984) *Biochim. Biophys. Acta 797*, 20–26.
Phillips, M. C., Johnson, W. J., & Rothblat, G. H. (1987) *Biochim. Biophys. Acta 906*, 223–276.
Pownall, H. J., Hickson, D. L., & Smith, L. C. (1983) *J. Am. Chem. Soc. 105*, 2440–2445.
Press, W. H., Flannery, B. P., Tevkolsky, S. A., & Vetterling, W. T. (1986) in *Numerical Recipes*, Cambridge University Press, Cambridge.
Quarfordt, S. H., & Hilderman, H. L. (1970) *J. Lipid Res. 11*, 528–535.
Ranadive, G. N., & Lala, A. K. (1987) *Biochemistry 26*, 2426–2431.
Rooney, M., Tamura-Lis, W., Lis, L. J., Yachnin, S., Kucuk, O., & Kauffman, J. W. (1986) *Chem. Phys. Lipids 41*, 81–92.
Rottem, S., & Davis, P. J. (1986) in *Enzymes of Lipid Metabolism* (Freysz, L., Dreyfus, H., Massarelli, R., & Gatt, S., Eds.) Part II, pp 421–428, Plenum Press, New York.
Rujanavech, C., & Silbert, D. F. (1986) *J. Biol. Chem. 261*, 7215–7219.
Steck, T. L., Kezdy, F. J., & Lange, Y. (1988) *J. Biol. Chem. 263*, 3023–3031.
Theunissen, J. J. H., Jackson, R. L., Kempen, H. J. M., & Demel, R. A. (1986) *Biochim. Biophys. Acta* 860, 66–74.
van Amerongen, A., Demel, R. A., Westerman, J., & Wirtz, K. W. A. (1989) *Biochim. Biophys. Acta* 1004, 36–43.
Vlahcevic, Z. R., Gurley, E. C., Heuman, D. M., & Hylemon, P. B. (1990) *J. Lipid Res. 31*, 1063–1071.
Wattenberg, B. W., & Silbert, D. F. (1983) *J. Biol. Chem. 258*, 2284–2289.
Wharton, S. A., & Green, C. (1982) *Biochim. Biophys. Acta 711*, 398–402.
Wolff, H. (1946) *Org. React. (NY) 3*, 307–336.
Yan, J., & Bittman, R. (1990) *J. Lipid Res. 31*, 160–162.
Yeagle, P. L., & Young, J. E. (1986) *J. Biol. Chem. 261*, 8175–8181.