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Kan, Chu-Cheng, Ph.D.

City University of New York, 1990

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STUDIES OF THE KINETICS OF STEROL EXCHANGE BETWEEN VESICLES

by

CHU-CHENG KAN

A dissertation submitted to the Graduate Faculty in Biochemistry in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York.

1990

This manuscript has been read and accepted for the Graduate Faculty in Biochemistry in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

Sept. 25, 1990
date

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Sept. 26, 1990
date

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Abstract

Studies of the Kinetics of Sterol Exchange between Vesicles

by

Chu-Cheng Kan

Advisor: Professor Robert Bittman

The extent of sterol-phospholipid interaction between sterols and phospholipids can be investigated by using kinetic studies of radiolabeled sterol desorption from lipid/water interfaces. Two model systems of lipid-water dispersions were used in the studies described in this dissertation: I, unilamellar vesicles, and II, biosurfactant dispersions. Kinetic measurements were carried out using assays based on ion-exchange chromatography or by lectin agglutination. Kinetic data were treated with a nonlinear least-squares analysis using a simplex algorithm to estimate the initial and infinity values and to obtain the psuedo first-order rate constant.

The rates of desorption of β-sitosterol (24α-ethylcholesterol) from donor species are lower than those of cholesterol by factors of 3 to 9. It is concluded that the 24α-ethyl group contributes to impeding spontaneous transfer of sitosterol between lipid surfaces. The half-times for cholesterol exchange from vesicles prepared with 3-methoxy- or 3-deoxy-*N*-C18-SPM were only slightly faster than that found using vesicles prepared from *N*-C18-SPM. Vesicles containing *O*-ethyl-*N*-C18-SPM and *O*-THP egg SPM gave rate enhancements of ~14 and 35, compared with the rates observed in vesicles made from *N*-C18- and egg SPM, respectively. These

measurements indicate that hydrogen bonding of the hydroxyl group of SPM with cholesterol does not contribute to the slow desorption of cholesterol. Cholesterol exchange using synthetic PC analogs in vesicles was studied to explore the role of interlipid hydrogen bonding on the rate of cholesterol desorption. The half-times for cholesterol exchange from vesicles of synthetic PCs containing a *sn*-2 *N*-linked chain are not very different from that obtained using DPPC vesicles, although the exchange rate was much slower in *N*-C16-SPM than in DPPC vesicles. The rates of cholesterol exchange from vesicles prepared from PCs containing a *sn*-2 *S*-linked chain are also similar to the rate of cholesterol exchange from DPPC vesicles. This indicates that the interlipid hydrogen-bonding between cholesterol and PC does not play a major role in determining the rate of cholesterol desorption.

Synthetic [4-^{14}C]cholesterol analogs were used to reveal the effect of structural modifications on sterol movement. [^{14}C]Epicholesterol and [^{14}C]triethoxycholesterol undergo exchange at rates much faster than cholesterol. The movement of [^{14}C]3-aminocholesterol and [^{14}C]cholesteryl diazoacetate is biphasic, indicating the existence of two kinetic pools of these sterol derivatives in the lipid matrix of the donor species. The slow kinetic phase may reflect the slow transbilayer migration of these charged cholesterol analogs from the inner to outer monolayer. 7-Ketocholesterol and 7-α- or 7β-hydroxycholesterol undergo exchange much more rapidly than cholesterol, probably because of the weak lipophilic interactions between oxysterols and DPPC. [^{14}C]5α-Cholestan-3β-ol undergoes desorption only slightly more slowly than that does cholesterol. The effect of sterol mol % on the rate of sterol exchange was investigated. It seems that the rate of exchange is sensitive to the size of the sterol-rich domain in vesicles.

Acknowledgments

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Abbreviations

BSA, bovine serum albumin

C18-lyso-PC, 1-stearoyl-*sn*-glycero-phosphocholine

DCP, dicetyl phosphoric acid

DEAE-Sepharose, diethylaminoethyl-Sepharose

DMPC, dimyristoylphosphatidylcholine

DPPC, dipalmitoylphosphatidylcholine

DSC, differential scanning calorimetry

GC/MS, gas chromatography/mass spectroscopy

HPLC, high performance liquid chromatography

N-C16-SPM, *N*-palmitoyl-SPM

N-C18-SPM, *N*-stearoyl-SPM

O-THP-egg SPM, *O*-tetrahydropyranyl-egg SPM

PA, phosphatidic acid

PC, phosphatidylcholine

PCS, photon correlation spectroscopy

PG, phosphatidylglycerol

SPM, sphingomyelin

TLC, thin-layer chromatography

Introduction

Sterol molecules undergo spontaneous movement from donor vesicles to an excess of acceptor vesicles. This process follows first-order kinetics, and the rate-limiting step in sterol movement is desorption of the sterol from the donor species (McLean and Phillips, 1981; Backer and Dawidowicz, 1981; reviews, Phillips et al., 1987; Dawidowicz, 1987; Bittman, 1988). The cholesterol desorption rate is sensitive to interactions between cholesterol and its nearest neighbor molecules in the membrane (Phillips et al., 1987; Dawidowicz, 1987; Bittman, 1988). Kinetic studies of sterol movement between lipid surfaces may increase our understanding of the interaction between sterol and phospholipid at the molecular level.

The predominant plant sterol β-sitosterol (referred to as sitosterol below) differs from cholesterol only by the presence of a 24α-ethyl group. This structural difference results in a much less efficient intestinal absorption of sitosterol than that of cholesterol and a preferential excretion of sitosterol in bile. It has been estimated that about 50% of the daily dose of ingested cholesterol is absorbed, whereas less than 5% of dietary sitosterol is absorbed; campesterol and stigmasterol, which are the other characteristic sterols of plants, are also absorbed less efficiently than cholesterol (Salen et al., 1970; Vahouny et al., 1983; Bhattacharyya, 1981; Ikeda et al., 1988a). As a result of these processes, the level of sitosterol and other plant sterols normally found in mammalian tissues and plasma is very low (<1% of total plasma sterols) (Gould et al., 1969; Salen et al., 1970; Bhattacharyya, 1981). Furthermore, plant sterols inhibit the intestinal absorption of cholesterol (Salen et al., 1970; Vahouny et al., 1983; Ikeda and Sugano, 1983; Ikeda et al., 1988b; Chijiiwa, 1987). A loss of sterol recognition capacity occurs in the inherited lipid-storage disease called sitosterolemia and xanthomatosis. This

disorder is characterized by the abnormal hyperabsorption of ingested dietary sitosterol (Bhattacharyya and Connor, 1974) and shellfish sterols (Gregg et al., 1986). with a concomitant increase in the concentrations of plant and shellfish sterols (Bhattacharyya and Connor, 1974; Gregg et al., 1986) and 5α-saturated stanols (Salen et al., 1985) in the plasma (Gregg et al., 1986).

Cholesterol is thought to be transported into the lymphatic circulation by a passive diffusion process that depends on bile salts for absorption (Treadwell and Vahouny, 1968; Westergaard and Dietschy, 1976). The mechanisms that govern cell discrimination between cholesterol and the structurally related phytosterols are not clear, nor is it understood how phytosterols lower plasma cholesterol. A number of processes have been considered to be involved in the ability of normal intestinal cells to absorb cholesterol preferentially to sitosterol during lipid digestion, including differences in (a) solubility of the sterols in the aqueous intestinal contents as a mixture of mixed micelles and unilamellar vesicles (Borgström, 1968; Armstrong and Carey, 1987; Ikeda et al., 1988b), (b) partitioning of sterols between micelles and membrane lipids and interactions with individual phospholipids (Rujanavech and Silbert, 1986; Child and Kuksis, 1986; Ikeda et al., 1988b), (c) recognition by mucosal plasma membrane proteins (Mayer et al., 1985; Ikeda et al., 1988b) or other proteins that mediate sterol uptake into the intestinal brush-border membranes (Chow and Hollander, 1978; Ikeda and Sugano, 1983; Bloj and Zilversmit, 1982), (d) intracellular reactions with membrane-bound enzymes, such as differential rates of esterification by acyl-CoA cholesterol acyltransferase (Tavani et al., 1982; Field and Mathur, 1983) or hydroxylation by cholesterol 7α-hydroxylase (Aringer and Eneroth, 1973; Boyd et al., 1974; Shefer et al., 1988), and (e) partitioning between subcellular membranes (Child and Kuksis, 1980) and uptake into

chylomicrons or other lipoproteins (Glover and Green, 1957). Uptake of sterol from solutions containing bile salts into cells is complicated by many factors, including dependence of micelle structure on lipid structure and concentration (Westergaard and Dietschy, 1976; Rampone and Machida, 1981), perturbation of membrane structure by physiological concentrations of bile salts, interactions with many membrane proteins and lipids, and subsequent metabolic events.

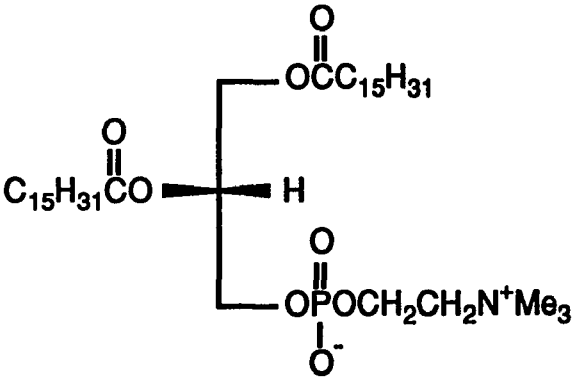
Cholesterol and sitosterol exchange between vesicles were investigated as described in Chapter 1 of this dissertation in order to determine whether the structure of the sterol side chain influences the rate of sterol exchange between phospholipid membranes and whether the greater extent of uptake of cholesterol by intestinal cells observed in normal individuals is correlated with a difference in the spontaneous exchange rate. The rates of exchange of [4-^{14}C]sitosterol and [4-^{14}C]cholesterol between unilamellar vesicles prepared with different phospholipids and different sterol to phospholipid molar ratios were estimated (Kan and Bittman, 1990). The results shown here indicate that the presence of the 24α-ethyl group impedes the rate of movement of sterol but not the extent of exchange at equilibrium between bilayers prepared with egg PC and DPPC over a wide range of sterol to phospholipid ratios. The exchange rate of sitosterol between egg SPM bilayers is also lower than that of cholesterol. These results indicate that sitosterol transport between membranes is impeded because of the additional interactions of its side chain with saturated and unsaturated acyl chains of phospholipids. In addition, the hydrophobicity of the ethyl group may reduce the aqueous solubility of sitosterol. Although enzymes and other proteins that have specific binding sites for the isooctyl side chain of cholesterol may contribute to the ability of intestinal cells to take

up exogenous cholesterol selectively from the micellar phase in vivo, the results suggest that the C_{24}-alkyl substituent of sitosterol has a significant effect on the distribution of the sterol between micelles and plasma membranes of intestinal cells and that simple competition between micelles and plasma membranes may contribute significantly to the early events in the absorptive discrimination of sterols observed in vivo during the interaction of digested sterols with the surface of enterocytes.

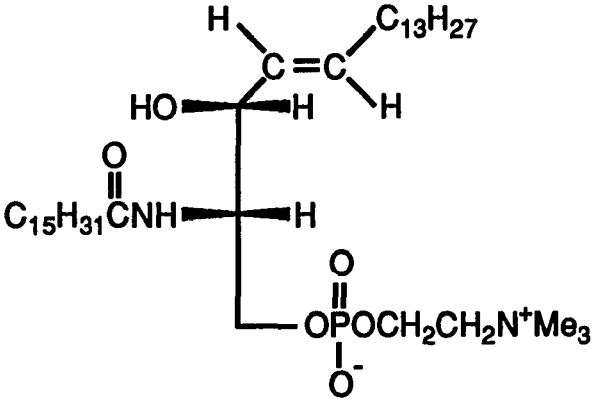
Cholesterol and SPM are very important constituents of biological membrane. Cholesterol is a principal component of the plasma membrane of many mammalian cells. Among the major functions of cholesterol in plasma membranes is the modulation of the molecular motion of phospholipid acyl chains (Stockton and Smith, 1976). In biological membranes and plasma lipoproteins, SPM is one of the most abundant components (Barenholz and Thompson, 1980). It has been suggested that SPM forms stable complexes with cholesterol (van Dijck, 1979; Demel et al., 1977) and strong intermolecular hydrogen bonds with other phospholipids (Boggs, 1980, 1987), although the specific function of SPM in biological membranes is not well understood. Kinetic studies of radiolabeled cholesterol exchange between membranes (Clejan and Bittman, 1984b; Fugler et al., 1985; Bar et al., 1987) have shown that the cholesterol molecules exchange more slowly in membranes containing SPM than in those lacking SPM, indicating that cholesterol interfacial packing is tighter in SPM than in PC bilayers (Phillips et al., 1987). In a binary mixture showing phase separation, DSC studies indicated that SPM, either isolated from erythrocytes or with a chemically defined (*N*-palmitoyl) chain, interacted with cholesterol to a greater degree than did various synthetic PCs (van Dijck, 1979). Fluorescence polarization measurements on phospholipid-cholesterol bilayers also indicated a higher

degree of structural order in membranes from egg SPM than from various synthetic PCs (van Blitterswijk et al., 1987). Studies of the surface pressure-molecular area isotherms of mixed monolayers of cholesterol and SPM revealed that cholesterol has the capacity to condense bovine brain SPM to a greater extent than PCs (Lund-Katz et al., 1988; Grönberg and Slotte, 1990). The resistance of oxidation of cholesterol by cholesterol oxidase in monolayers is also related to strong SPM/cholesterol interactions (Grönberg and Slotte, 1990). Hence, there is evidence that the interaction of cholesterol with various naturally occurring (egg or bovine) or synthetic SPM is greater than that with egg PC or DPPC in bilayer membranes.

Insights into the molecular basis for the higher apparent affinity of cholesterol for SPM have been lacking, although greater opportunities for van der Waals interactions (Lund-Katz et al., 1988) and hydrogen bonding (Barenholz and Thompson, 1980; Boggs, 1980, 1987) have been postulated as a stabilizing force between the 3β-hydroxyl group of cholesterol and the amide oxygen of sphingolipids. Although the crystal structures of sphingolipids indicate that intermolecular hydrogen bonding takes place in the crystalline state (Pascher, 1976; Pascher and Sundell, 1977; Abrahamsson et al.,1977), there is no direct evidence for a greater extent of hydrogen bonding between cholesterol and SPM vs. glycerophospholipids in membranes. However, the hydroxy and amide groups may afford an important inter- and intramolecular hydrogen bond capability (Thompson and Huang, 1980). Figure 1 shows the structures of SPM and PC, drawn to emphasize their differences, i. e., the presence in SPM of a free hydroxy group, a long-chain amide group, and a *trans* double bond in the sphingosine unit. A comparison of the structures of PC and SPM reveals that the allylic hydroxy group and amide group of SPM are likely sites at which hydrogen-



DPPC



N-Palmitoyl-SPM

Figure 1. The Structures of DPPC and *N*-Palmitoyl-SPM

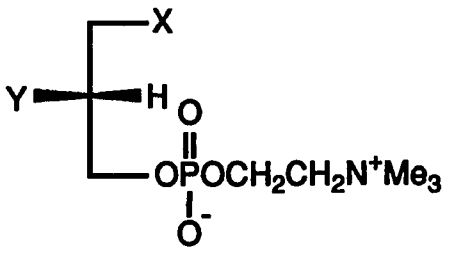
bonding interactions may take place between SPM and cholesterol; these sites are absent in PC. SPM analogs were synthesized in this laboratory in which the hydroxy group has been replaced by other groups, with the amide linkage maintained. In Capter 2 of this dissertation, the rates of [4-^{14}C]cholesterol exchange between vesicles containing these SPM analogs were compared. Since the rate constant for cholesterol desorption is sensitive to differences in cholesterol-phospholipid interaction energy in bilayer membranes (Lund-Katz et al., 1988), the results reveal new insight into the role of the SPM-hydroxy group on SPM-cholesterol interactions.

Sulfur- and nitrogen-substituted PCs are synthetic phospholipid analogs that have attracted a great deal of attention recently because of their interesting biophysical and pharmacological properties (Agarwal et al., 1986; Yu et al., 1990). These lipids have acyl or alkyl chains linked to the glycerol backbone via nitrogen or sulfur instead of oxygen. Thioester phospholipids provide chromogenic phospholipase substrates that permit spectrophotometric assay of lipolytic enzymes (Balet et al., 1988; Farooqui et al., 1984; Kucera et al., 1988). Phospholipids that have a *sn*-1 thioether or a hexadecanamido chain in place of the *sn*-1 *O*-alkyl chain and a *sn*-2 *O*-methyl group have antitumor properties against various leukemic cells (Marx et al., 1988). Acylaminodeoxy phospholipids that have a RCONH group at the *sn*-2 position are resistant to phospholipase A_2 catalyzed degradation and are potential phospholipase inhibitors and potential drug carriers. Derivatives of PC in which a glycerol oxygen atom has been replaced by a sulfur or nitrogen atom have been used in Chapter 3 of this dissertation to study the kinetics of cholesterol exchange between vesicles. These experiments are aimed at understanding the role of interlipid hydrogen bonding in sterol desorption and at an evaluation of the possible alterations that may be introduced in vesicles

by introducing *N*- and *S*-linked phospholipids.

Previous studies showed that the rate of intervesicle cholesterol exchange is sensitive to the degree of fatty acid saturation and phospholipid head-group structure, in addition to SPM content (reviews, see Phillips et al., 1987; Bittman, 1988); however, the hypothesis that "hydrogen-bonded belts" (Brockerhoff, 1974) influence cholesterol exchange or transfer between membranes has not been tested. In order to assess the role of interlipid hydrogen-bonding capability on the rate of intervesicle cholesterol exchange vesicles were prepared from two PC analogs having a *sn*-2-acylaminodeoxy chain in place of the ester chain (compounds **1** and **2**, see Figure 2). The kinetics of cholesterol exchange from vesicles containing carbamoyl-PC (compound **3**, see Figure 2) were also measured. Compound **3** is another nitrogen-bearing PC analog; the ROCONH group of **3** replaces the RCONH group of the amido-PCs **1** and **2**. Since the N-H group of acylaminodeoxy-PCs and carbamoyl-PC may interact with the cholesterol hydroxy group by hydrogen bonding, analogs **1-3** may be considered as glycerolipid analogs of SPM, which also possesses a NHCOR group.

In order to further assess the role of hydrogen-bonding capability on cholesterol desorption the rates of cholesterol movement between vesicles prepared from thio-containing PCs (structures, see Figure 2) have also been measured. Since sulfur is less electronegative than oxygen, such analogs permit a further estimate of the importance of hydrogen-bonding groups in PCs on the rate of cholesterol transfer between bilayers. In compound **4**, the glycerol oxygen atom at the *sn*-2 position of DPPC has been replaced with sulfur. Compound **5** has a *sn*-2 thiopalmitoyl chain and a *sn*-1 *O*-hexadecyl chain. The studies described in Chapter 3 of this dissertation address the topic of the influence of modifications in the hydrogen-bonding



Compound	X	Y
1, 1-ether-2-amide-PC	$OC_{16}H_{33}$	$NHCC_{15}H_{31}$ (O)
2, 1-thioether-2-amide-PC	$SC_{16}H_{33}$	$NHCC_{15}H_{31}$ (O)
3, 1-thioether-2-carbamoyl-PC	$SC_{16}H_{33}$	$NHCOC_{14}H_{29}$ (O)
4, 1-ester-2-thioester-PC	$OCC_{15}H_{31}$ (O)	$SCC_{15}H_{31}$ (O)
5, 1-ether-2-thioester-PC	$OC_{16}H_{33}$	$SCC_{15}H_{31}$ (O)

Figure 2. The Structures of Synthetic PC Analogs Used

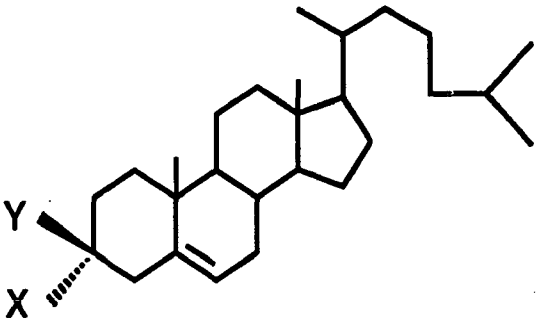
capability of PCs in the donor vesicles on the rate of spontaneous cholesterol desorption from the vesicle surface.

Studies of the rates of cholesterol movement between membranes composed of different phospholipids have indicated an inverse relationship between the unidirectional or bidirectional (exchange) transfer rate and the degree of molecular packing of cholesterol with phospholipids (Fugler et al., 1985; Yeagle and Young, 1986; 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 and Bittman, 1984c), bile salts (Vlahcevic et al., 1990) and organic solvents (Bruckdorfer and Green, 1967; Quarfordt and Hilderman, 1970; Bruckdorfer and Sherry, 1984). Recent data suggest 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 and Bittman, 1990). In Chapter 4 of this dissertation, the results of studies of the rates of intervesicular movement of various radiolabeled synthetic analogs of cholesterol (structures, see Figure 3) are reported. These studies were undertaken in order to evaluate the effects of structural modifications in cholesterol on the movement of sterols between vesicles. The cholesterol photoaffinity probe, cholesteryl diazoacetate, has been also used to determine whether this probe behaves analogously to cholesterol with respect to exchange, and thus with respect to interactions with donor phospholipids.

There are indications that the cholesterol exchange rate from donor vesicles to acceptor particles is not sensitive to cholesterol concentration,

since no significant change in rate was found between 1 and 40 mol % cholesterol (McLean and Phillips, 1982; Schroeder et al., 1987). These studies agree with a previous finding of similar % transfer of cholesterol after 6 h of incubation when 0, 33, and 50 mol % of cholesterol was used in the vesicles (Nakagawa et al., 1979). On the other hand, an increase in cholesterol exchange rate between vesicles and erythrocyte ghosts was reported when the cholesterol content was raised in the donor and acceptor species (Poznansky and Czekanski, 1979).

A slowly exchanging sterol (cholesterol, dehydroergosterol, and cholestatrienol) pool was found in PC/cholesterol vesicles at high mol % of sterol; this pool may represent sterol molecules that dissociate from sterol-sterol complexes (Nemecz et al., 1988). Exchange between *Mycoplasma gallisepticum* cell membranes and lipid vesicles was studied as a function of cholesterol content; it was found that the exchange rate decreased as the cholesterol contents increased from 20 to 26 mol %, then leveled off as the cholesterol content was further increased (Clejan and Bittman, 1984c). It was suggested that the rapid exchange between 1 and 20 mol % cholesterol reflects the weaker van der Waals attractive interactions that are present in these bilayers. When van der Waals forces become approximately constant, as at ≥20-30 mol % cholesterol, the exchange rate does not vary appreciably. In order to resolve the conflicting information about the dependence of exchange rate on bilayer cholesterol content, a study of the kinetics of [^{14}C]cholesterol exchange between vesicles prepared from egg PC and DPPC at 50 °C is presented in Chapter 5. Other sterols (sitosterol, cholestanol) also have been investigated to study the dependence of exchange rate on sterol content in the bilayer.



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 3. The Structures of Cholesterol Analogs with Structural Modifications at the 3 Position

Experimental Section

Materials. [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 purchased from DuPont New England Nuclear (Boston, MA), and [4-^{14}C]sitosterol (specific activity 56 mCi/mmol) was obtained from Amersham Corp. (Arlington Heights, IL). Egg PC, egg PG, DPPC, egg SPM, *N*-C16-SPM, DCP, *N*-palmitoyldihydrolactocerebroside, cholesterol, sitosterol, 5α-cholestan-3β-ol (cholestanol), epicholesterol, 7-ketocholesterol, 7α-hydroxycholesterol, bovine serum albumin (BSA, Fraction V, fatty acid poor), and DEAE-Sepharose CL-6B were purchased from Sigma Chemical Co. (St. Louis, MO). C18-Lyso-PC was obtained from Avanti Polar Lipids (Pelham, AL) and from Sigma. *Ricinus communis* agglutinin I (RCA120, referred to as lectin below) was from Vector Laboratories (Burlingame, CA). Synthetic PC analogs were prepared as described in the following publications: compounds **1**, Chandrakumar and Hajdu, 1983; compounds **2** and **3**, Bhatia and Hajdu, 1988; compound **4**, Bhatia and Hajdu, 1987; compound **5**, Bhatia and Hajdu, 1989; structures, see p. 9. Synthetic SPM analogs were prepared by Z-s. Ruan (Ruan, 1990) in this laboratory. [4-^{14}C]-Epicholesterol was prepared as described by Yan and Bittman (1990). [4-^{14}C]Sterols (cholestanol, 3α-triethoxycholesterol, 3α- and 3β-aminocholesterol, cholesteryl diazoacetate, 7-ketocholesterol, and 7α- and 7β-hydroxycholesterol) and nonradiolabeled 7β-hydroxycholesterol were prepared by J. Yan in Dr. Bittman's laboratory.

Cholesterol and sitosterol were recrystallized twice before use from ethanol-acetone (95:5 v/v). Lipid purities were assayed by thin-layer chromatography on silica gel G plates (Analtech, Newark, DE) in the following solvent systems (v/v): PCs, chloroform-methanol-water, 65:25:4 (Clejan and

Bittman, 1981); C18-lyso-PC, chloroform-methanol-water, 65:35:8; SPMs, chloroform-methanol-ammonium hydroxide-water, 65:30:3:3; cholesterol, sitosterol, and cholestanol, ether-petroleum ether, 3:1 (Clejan and Bittman, 1984a); epicholesterol, ether-petroleum ether, 1:1; triethoxycholesterol, chloroform-ethyl acetate, 1:1; 3α- and 3β-aminocholesterol, 2-propanol-ammonium hydroxide, 8:1 (on a C18 reverse-phase TLC plate); cholesteryl diazoacetate, ether-petroleum ether, 1:7; 7-ketocholesterol, 7α- and 7β-hydroxycholesterol, ether. The sterols, PCs, and SPMs migrated as single spots when visualized by spraying with H_2SO_4 solution (10% in ethanol) and charring (Witzke and Bittman, 1985). The purities of the synthetic cholesterol analogs were also assayed by reverse-phase HPLC on a C18 Carbosphere column (25 cm x 4.6 mm, Phenomenex, Rancho Palos Verdes, CA); sterols were eluted with acetonitrile-2-propanol 4:1 (v/v) at a flow rate of 2 mL/min.

The purity of nonlabeled sitosterol was checked by reverse-phase HPLC detection at 210 nm and by gas chromatography (30 m x 0.25 mm i.d., 0.25 μm DB-5 bonded phase fused silica capillary column, J & W Scientific, Folsom, CA). Sitosterol was found to be contaminated by campesterol (24α-methylcholesterol), which was not separated from sitosterol on TLC. Most preparations were found to consist of about 55% sitosterol (HPLC: R_t 24.2 min; GC: R_t 24.8 min, MS: m/z 414.45) and 45% campesterol (HPLC: R_t 21.2 min; GC: R_t 20.8 min, MS: m/z 400.30). Sitosterol was purified by semi-preparative reverse-phase HPLC (Phenomenex C18 Carbosphere, 10 x 250 mm, elution as described above); analysis on the analytical HPLC column indicated that the purified sitosterol contained only 8% of campesterol as the sole contaminant. The HPLC-purified sitosterol was used where indicated.

The purities of the radiolabeled sterols were determined by TLC

(elution with ethyl acetate-benzene, 3:2 v/v); zones were cut at positions corresponding to standards and counted in 5 mL of scintillation cocktail (Ecoscint, National Diagnostics, Highland Park, NJ). It was determined that the purities of [4-^{14}C]cholesterol and [4-^{14}C]sitosterol were ≥98%, with ~0.3% of each 7-ketosterol present. In some of the studies described in Chapters 1, 3, and 5 SPM was purified from egg yolk. This SPM is highly enriched in saturated fatty acids (24:0, 22:0, and 16:0) (van Blitterswijk et al., 1987)

Methods

Preparation and Characterization of Vesicles. The vesicles were prepared as described previously (Fugler et al., 1985) with a slight modification. The desired aliquots of lipid solutions were mixed and then dried under a nitrogen stream to make a lipid film. The lipid film was further dried under vacuum in a desiccator. Donor (1.0 mM total lipid) and acceptor (10.0 mM total lipid) vesicles contained the same phospholipid (i.e., egg PC, DPPC, or egg SPM) in order to minimize phospholipid exchange during the prolonged incubation periods required to approach equilibrium. When the synthetic PCs or synthetic SPM were used in the donor vesicles, DPPC or egg SPM was contained in the acceptor vesicles. The molar ratio of sterol to total lipid was the same in the donor and acceptor for bidirectional exchange experiments, whereas there is no sterol in acceptors for unidirectional transfer measurements. Donor vesicles contained 15 mol % DCP to confer negative charge (to permit ion-exchange chromatographiy assay) or 20 mol % *N*-palmitoyl-dihydrolactocerebroside (to permit lectin assay) and a trace (0.08 μCi) of radiolabeled sterol. The neutral acceptor vesicles contained a trace (0.046 μCi) of [^{3}H]triolein as a nonexchangeable marker to monitor their recovery. Aqueous dispersions of the lipids in 20 mM sodium phosphate

buffer containing 1 mM EDTA and 0.05% sodium azide, pH 6.0, were incubated for 10 min in a water bath maintained at ~10 °C above the phospholipid phase transition temperature and were then sonicated at room temperature in a Heat Systems Ultrasonics (Farmingdale, NY) Model W375 sonicator. The donor vesicles were sonicated using a cup-horn for a continuous period of 1 h, and the acceptor vesicles were sonicated using a microtip at 50% duty cycle for 1 h. The sonicated lipid dispersions were centrifuged for 10 min at 10,000 rpm in a DuPont Sorvall centrifuge Model RC5C (Sorvall Instruments, Wilmington, DE) with a SS-34 rotor. Pellets containing undispersed lipids, multilamellar liposomes, and titanium fragments were discarded.

The sizes and homogeneities of the donor vesicles were estimated by by dynamic light scattering using PCS at 50 °C and 90° scattering angle using a 15 mW He-Ne laser (632.8 nm) equipped with a Brookhaven Instruments Corp. (Holtsville, NY) Model Bi-2030AT 128-channel digital correlator or at 20 °C and 90° scattering angle using a argon ion laser (488 nm) as described by Hwang and Cummins (1982) and Aurora et al. (1985). Briefly, light from the laser was focused onto the vesicle sample in a glass cuvette or tube maintained at 50 ° ± 0.1 °C or 20 ° ± 0.1 °C in a temperature-controlled cell holder. The intensity of the scattered light was detected at a 90° angle to the incident beam. The values of the refractive index and viscosity of water were used in calculations of vesicle diameters. The sizes of the acceptor vesicles were not measured because their curvature does not influence the rate of sterol exchange between vesicles (Fugler et al., 1985). Polystyrene spheres were used as size standards (e. g., diameter 129.2 ± 1.3 nm as measured by argon ion laser). Vesicles were prepared as described above except that the buffer was prefiltered five times through Millipore GS 0.2-μm filters (Millipore

Corp., Bedford, MA). The total lipid concentration was usually 1 mM. Some preparations were diluted to 0.1 mM, without significant change in size, indicating that intervesicular interactions did not influence the measurements. The diameters of the donor vesicles (index of polydispersity in parentheses) were described as follows. Vesicles prepared with DPPC, 15 mol % DCP, and 1, 10, and 50 mol % cholesterol had diameters in the range of 188 to 264 nm (0.19-0.29); DPPC vesicles with the corresponding mol % sitosterol had diameters in the range of 177-221 nm (0.17-0.28). Vesicles prepared from egg PC and sterols had a similar diameter, 156-175 nm (0.17- 0.19). SPM-containing vesicles with cholesterol or sitosterol and egg PC had diameters in the range of 200-240 nm (Kan and Bittman, 1990). The diameters of vesicles containing 10 mol % cholesterol and 15 mol % DCP were 96.0 nm (0.23) for egg SPM and 85.6 nm (0.34) for *O*-THP-egg SPM, respectively. Vesicles prepared with *N*-C18-SPM analogs, 10 mol % cholesterol, and 15 mol % DCP had diameters in the range of 177-231 nm (0.24-0.40). Donor vesicles containing synthetic PC analogs (see p. 9), 6 mol % cholesterol, and 15 mol % DCP had diameters in the range of 128-227 nm (0.18-0.23). Vesicles prepared with DPPC, 15 mol % DCP, and 24 mol % sterol had diameters as follows: cholesterol, 141 nm (0.18); cholestanol, 156 nm (0.25); epicholesterol, 131 nm (0.19); triethoxycholesterol, 123 nm (0.26); 7-ketocholesterol, 118 nm (0.09); 7α- and 7β-hydroxycholesterol, 184 and 194 nm (0.14 and 0.14). Vesicles with DPPC, 15 mol % DCP, and 6 mol % cholesterol, 3α- or 3β-aminocholesterol had diameters of 128 nm (0.18), 237 nm (0.26), and 188 nm (0.18), respectively. The diameter of *N*-palmitoyldihydrolactocerebroside-containing vesicles with 56 mol % egg PC and 24 mol % 3β-aminocholesterol was 166 nm (0.23). It should be noted that the diameters of the sonicated vesicles appear larger than expected,

probably because ultrasonic radiation generated by using the cup horn is significantly lower than that from a standard microtip. The sizes of the vesicles employed in this study (range of ~118-264-nm diameter) are larger than the sizes of vesicles found to display a dependence between the rate of cholesterol efflux and vesicle curvature (Thomas and Poznansky, 1988a). Thus, the differences we found in sterol exchange rates between vesicles of different composition do not arise from a marked difference in vesicle size.

Preparation of a Biosurfactant Model System. The donor and acceptor particles contained 50 mol % C18-lyso-PC. This model system resembles the egg lyso-PC/egg PC/cholesterol mixed lipid system used by Thurnhofer and Hauser (1990) as donors of cholesterol to brush border vesicles as acceptors; these investigators referred to the mixture as mixed micelles, but the term dispersions is used to refer to the surfactant-containing mixtures. Donors contained 15 mol % of negatively charged lipids (egg PG) to impart a charge to allow separation from neutral acceptors on ion-exchange columns, whereas the acceptors were neutral. Sterol, egg PC, and egg PG (when present) in chloroform and C18-lyso-PC in chloroform/methanol 2:1 were mixed and dried under nitrogen and then under vacuum overnight. The donor preparation (total lipid concentration, 2 mM) consisted of 1.5 mol % sterol (with a trace of [^{14}C]sterol), 15 mol % egg PG, 33.5 mol % egg PC, and 50 mol % C18-lyso-PC. The acceptor preparation (total lipid concentration, 20 mM) contained 1.5 mol % sterol, 48.5 mol % egg PC, 50 mol % C18-lyso-PC, and a trace of [^{3}H]triolein as a nonexchangeable marker. The lipid film was dispersed in 20 mM sodium phosphate buffer, pH 7.4, containing 0.05% w/v sodium azide, without sonication. After incubation at 37 °C for 30 min, donors and acceptors were shaken vigorously by vortexing until the dispersions were clear. Quasi-elastic light scattering was used to assess the size and

polydispersity of the donor particles as described above. The average mean diameters of the cholesterol-containing donors (0.7-2.0 mM total lipid concentration) were 165-180 nm, with a polydispersity of 0.15-0.22; sitosterol-containing preparations had average mean diameters of 210-220 nm and a polydispersity of 0.20.

Efflux of Trapped [^{14}C]Glucose from Surfactant Dispersions. A trace of D-[U-^{14}C]glucose (1.5 x 10^6 dpm), specific activity 290 Ci/mol (Research Products International), was added to 6 mL of 5 mM glucose, 50 mM NaCl solution. A portion (1.5 mL) of this solution was added to a dry film of lipids (1.5 mol % sterol, 15 mol % egg PG, 33.5 mol % egg PC, 50 mol % C18-lyso-PC; 10 mM total lipid). After the lipid film was dispersed as described above, the dispersions were placed in dialysis sacs and untrapped glucose was removed by extensive dialysis at 4 °C against large volumes of 50 mM NaCl solution, with frequent changes of the dialysate. It was found that <1% of the glucose was trapped; under similar conditions, liposomes prepared from DPPC and sterol trapped >4% of the total [^{14}C]glucose at 4 °C. Thus, the mixed C18-lyso-PC dispersions have a very small trapped aqueous volume.

Measurement of [^{14}C]Sterol Movement between Vesicles. Acceptor vesicles with 4% albumin (w/v, where indicated) and donor vesicles without albumin were preincubated separately at 50 °C in a Labline shaking water bath. Exchange was initiated by mixing and vortexing equal volumes of donor and acceptor vesicles. Incubations were carried out at 50 °C or 37 °C. Aliquots (200 µL) of the incubation mixture were applied to DEAE-Sepharose CL-6B columns (0.6 x 3 cm) at room temperature; the columns were prewashed with the buffer. To begin the collection of the eluate, the column was washed with 300 µL of buffer as soon as the aliquot had entered

completely into the column. An additional 1 mL of buffer was applied to the column and the eluate was collected in the same liquid scintillation vial. To the eluate was added 8 mL of ScintiVerse II (Fisher Scientific Co.) or 5 mL of Ecoscint scintillation cocktail (National Diagnostics, Highland Park, NJ). After the mixture was shaken by vortex for about 30 s, the vials were counted in a Packard 2000CA liquid scintillation counter (Packard Instrument Co., Downers Grove, IL) for 10 min. Data were corrected for spillover (typically, 2.5% spillover of ^{3}H dpm into the ^{14}C channel, 0% spillover of ^{14}C dpm into the ^{3}H channel). The recovery of the neutral acceptor vesicles in the eluate was approximately 85-90%, and <0.5% of the negatively charged donor vesicles were eluted (Fugler et al., 1985).

When the donor vesicles contained *N*-palmitoyl-dihydrolacto-cerebroside (20 mol %), separation of donor and acceptor vesicles was achieved by addition of 7.5-30 μg of lectin to a 200- 600-μL aliquot of the incubation mixture in an Eppendorf centrifuge tube, followed by centrifugation at room temperature for 2 min in Eppendorf model 5412 Centrifuge (Brinkmann Instruments, Westbury, NY). The supernatant was transferred to a scintillation vial, 5 mL of Ecoscint 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 of the acceptor vesicles estimated from the ^{3}H dpm in the supernatant was >98%. The efficiency of agglutination was assayed by using the donor vesicles labeled with ^{3}H-triolein instead of ^{14}C-cholesterol and by comparing the dpm count in the supernatant; only about 4% of donor vesicles were in the supernatant.

Measurement of [^{14}C]Sterol Movement between C18-lyso-PC-Containing Dispersions. Donor and acceptor dispersions were incubated at 37 °C, and the exchange was initiated by mixing equal volumes of donors and

acceptors. Aliquots (200 µL) were withdrawn at the desired time intervals, placed on Pasteur pipets containing DEAE-Sepharose CL-6B or DEAE-Sephacel (Sigma), and eluted by washing the column first with 100 µL of buffer (after the incubation mixture had entered into the column completely) and then with an additional 1 mL of buffer. The retention of ^{3}H dpm in the eluate (which consists of the neutral C18-lyso-PC dispersions) as a function of time of incubation indicated that the recovery of acceptor micelles was efficient (>80%).

Analysis of Sterol Oxidation. The extent of cholesterol oxidation in vesicles subjected to 4-day incubation periods at 50 °C was examined by using two methods. The total lipids were extracted with chloroform-methanol (1:1, v/v). In a gas chromatographic method of analysis, an aliquot of the extracted lipids was injected onto a 30 m x 0.25 mm DB-5 fused silica capillary column (J & W Scientific) in a Hewlett-Packard Model 5988A GC/MS equipped with a H-P 1000 data system. Under the conditions used (1.0 mL/min flow rate), the R_f values for cholesterol, 7-ketocholesterol, 7α- and 7β-hydroxycholesterol were 16.8, 20.0, 10.8, and 10.7 min, respectively. No oxidized sterol was detected in the lipid extracts, indicating that 1 mM EDTA is an effective agent in protection of cholesterol from oxidation (Lijana et al., 1986). Since underivatized oxysterols may decompose at the high temperatures required for GC/MS analysis, the lipid extracts were also examined by preparative thin-layer chromatography on silica gel GF plates (1000 µm, Analtech). The total dpm of ^{14}C applied to the plates was ≥200,000. The plates were eluted twice using ethyl acetate-benzene (3:2, v/v), which resolved the sterols with the following R_f values: cholesterol, 0.92; 7-ketocholesterol, 0.71; 7α-hydroxycholesterol, 0.49; and 7β-hydroxy-cholesterol, 0.57. Zones corresponding to these standards were cut and

eluted with chloroform-methanol (2:1, v/v). Analysis of the residues obtained after evaporation of the solvents was by liquid scintillation counting in 5 mL of Ecoscint. Only 1.2% of the total [4-^{14}C]cholesterol was converted to oxidized sterols, of which 92% was 7-ketocholesterol, 6% was 7β-hydroxycholesterol, and 2% was 7α-hydroxycholesterol. The polar lipid fraction at the origin of the plate was found to contain no ^{14}C dpm.

Analysis of Rates and Extents of Sterol Movement. The fraction of labeled sterol undergoing exchange at time t is defined as α_t and 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 or lyso-PC-containing dispersions without separation, respectively. The α_t values at times zero and infinity are α_0 and α_∞, respectively. These values were estimated by using a simplex iterative least-squares program (Noggle, 1985). In this analysis, all of the kinetic data (α_t), including experimental points obtained at times approaching the equilibrium value, were fit to eq 1:

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

The rate constant and half-time were calculated using the following relationships:

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

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

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

$$X_{xch} = \{[(\alpha_\infty - \alpha_0) / (1 - \alpha_0)] \times 100\%\} \times 1.1 \quad (4)$$

The factor of 1.1 used in eq 2 is to correct the rate constant of the unidirectional rate of ^{14}C-sterol movement from donor to acceptor species for

back exchange of label. In eq 4, the factor of 1.1 is used to normalize the apparent size of kinetic pool, i. e., $(\alpha_\infty - \alpha_0) / (1 - \alpha_0)$ to 100%, since 90.9% of ^{14}C-sterol in acceptor species at equilibrium (acceptor/donor, 10:1) represents that the size of the kinetic pool of ^{14}C-sterol in the donor species is 100%, where 100% = 90.9% x 1.1. The curves in the plots of the fraction of ^{14}C-sterol in acceptor vesicles vs. time were obtained by an interpolative curve fit of a plot of y_t vs. t; y_t is the fraction of ^{14}C-sterol in acceptor at time t as calculated by eq 1. In the semilog plots, the solid lines were obtained by an exponential curve fit of a plot of $[1 - (X_t / X_\infty)]$ vs. t The term $1 - (X_t / X_\infty)$ represents the % ^{14}C-sterol remaining in donor species as calculated from eq 5:

$$1 - (X_t / X_\infty) = \exp(\text{slope} \times t) \qquad (5)$$

Here $X_t = y_t - \alpha_0$ and $X_\infty = (\alpha_\infty - \alpha_0)$. When the semilog plots obtained by eq 5 were biphasic, kinetic data were analyzed by eq 6:

$$F(t) = [\alpha_{\infty f} + (\alpha_{0f} - \alpha_{\infty f})\exp(\text{slope}_f \times t)] + [\alpha_{\infty S} + (\alpha_{0S} - \alpha_{\infty S})\exp(\text{slope}_S \times t)] \qquad (6)$$

or by eq 7, assuming that $\alpha_{0f} = \alpha_{0S} = \alpha'_0$:

$$F(t) = \alpha_{\infty \text{total}} + [(\alpha'_0 - \alpha_{\infty f})\exp(\text{slope}_f \times t)] + [(\alpha'_0 - \alpha_{\infty S})\exp(\text{slope}_S \times t)] \qquad (7)$$

where s, f, and total represent the slow, fast, and total phases. The curves in the fraction of ^{14}C-sterol in acceptors vs. time biphasic plots were obtained as described above except that eq 6 or 7 was used to calculate the y_t values. In the biphasic semilog plots (Figure 4-2 and 4-3, insets), the solid lines were obtained by an interpolative curve fit of a plot of z_{total} vs. t. z_{total} is the % ^{14}C-sterol remaining in donor particles as calculated by eq 8:

$$z_{\text{total}} = f + s \qquad (8)$$

where $f = \{1 / [1 + (\alpha_{0S} - \alpha_{\infty S}) / (\alpha_{0f} - \alpha_{\infty f})]\}\exp(\text{slope}_f \times t)$ and

$$s = \{1 / [1 + (\alpha_{0f} - \alpha_{\infty f}) / (\alpha_{0S} - \alpha_{\infty S})]\}\exp(\text{slope}_S \times t)$$

The rate constant, half-time, and size of the kinetic pool were calculated as

described above. In the lectin assay, the kinetic analysis was basically the same as described above except that $[^{14}C/^{3}H]_t$ and $[^{14}C/^{3}H]_{mix}$ represent the values in the supernatant obtained by addition of lectin to the donor-acceptor aliquots withdrawn at time *t* and in the supernatant obtained without the addition of lectin, respectively.

Results and Discussion

Chapter 1. Sitosterol and Cholesterol Exchange between Phospholipid Vesicles and Lysophospholipid Dispersions

Kinetics of Sitosterol Exchange between DPPC, Egg PC, or Egg PC/egg SPM Vesicles. The kinetics of sterol exchange between DPPC vesicles was examined over a prolonged period of time, appearing to reach equilibrium after about 60 h (Figure 1-1A). Exchange of radiolabeled sterol from negatively charged donor vesicles to a 10-fold excess of neutral acceptor vesicles was monitored as described previously (Fugler et al., 1985). The half-times for sterol exchange are more than 4-fold higher for sitosterol than for cholesterol in DPPC bilayers (Table 1-1). In egg PC bilayers (Figure 1-1B), the rates of sterol exchange are much faster than in DPPC bilayers; this has been observed for cholesterol by several investigators (McLean and Phillips, 1981; Phillips et al., 1987; Fugler et al., 1985; Lund-Katz et al., 1988) and is considered to reflect the increased lateral packing density at the lipid-water interface in saturated relative to unsaturated PC bilayers. Table I shows that the half-time for sitosterol exchange is about 7 times higher than that for cholesterol exchange in egg PC bilayers. Incorporation of SPM into egg PC bilayers (Figure 1-2) results in a decrease in the rate of sterol exchange, with sitosterol undergoing exchange 6 fold more slowly than cholesterol. The ability of SPM to lower the cholesterol exchange rate in mixed PC-SPM and SPM bilayers has been reported previously (Fugler et al., 1985; Lund-Katz et al., 1988; Bhuvaneswaran and Mitropoulos, 1986) and is explained by the greater lateral packing density in the lipid-water interface, which decreases the rate of cholesterol desorption. The magnitude of the difference in exchange rates between sitosterol and cholesterol was unexpected, since only a small difference in the promotion of acyl chain order was noted in

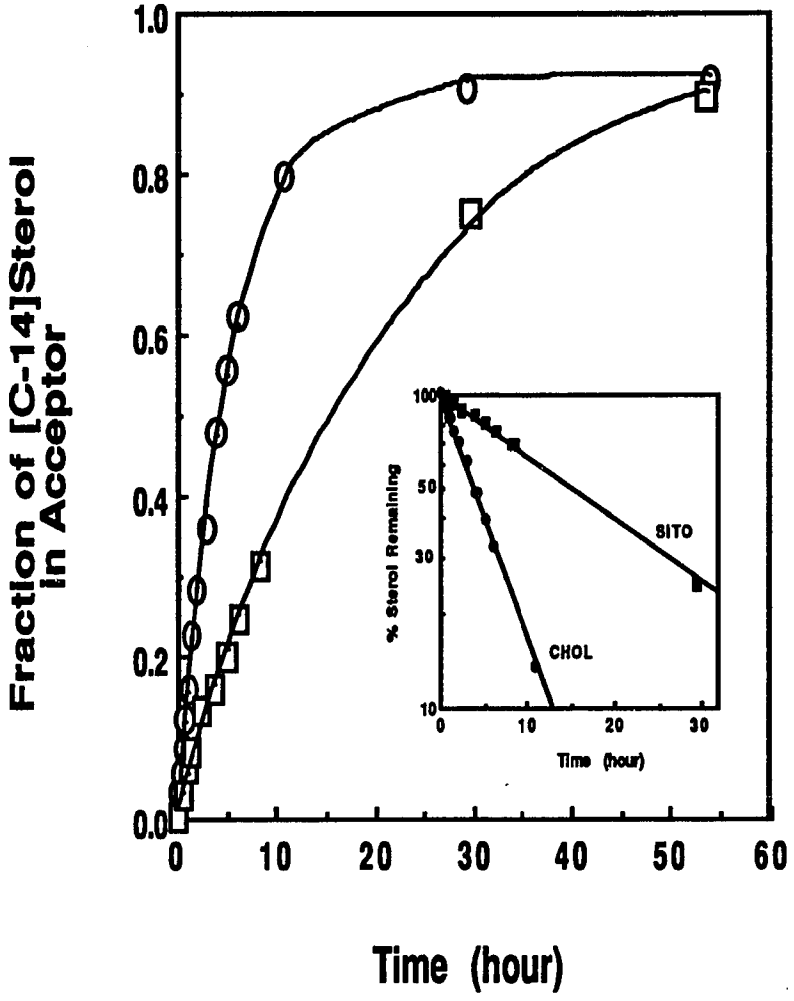


Figure 1-1A: Kinetics of exchange of [4-^{14}C]cholesterol (o) and [4-^{14}C]sitosterol (□) between unilamellar vesicles containing 1 mol % sterol at 50 °C. Donor and acceptor vesicles were prepared from DPPC. *Inset:* Semilog plot of the exchange data. Albumin (2% w/v) was present in the incubation medium.

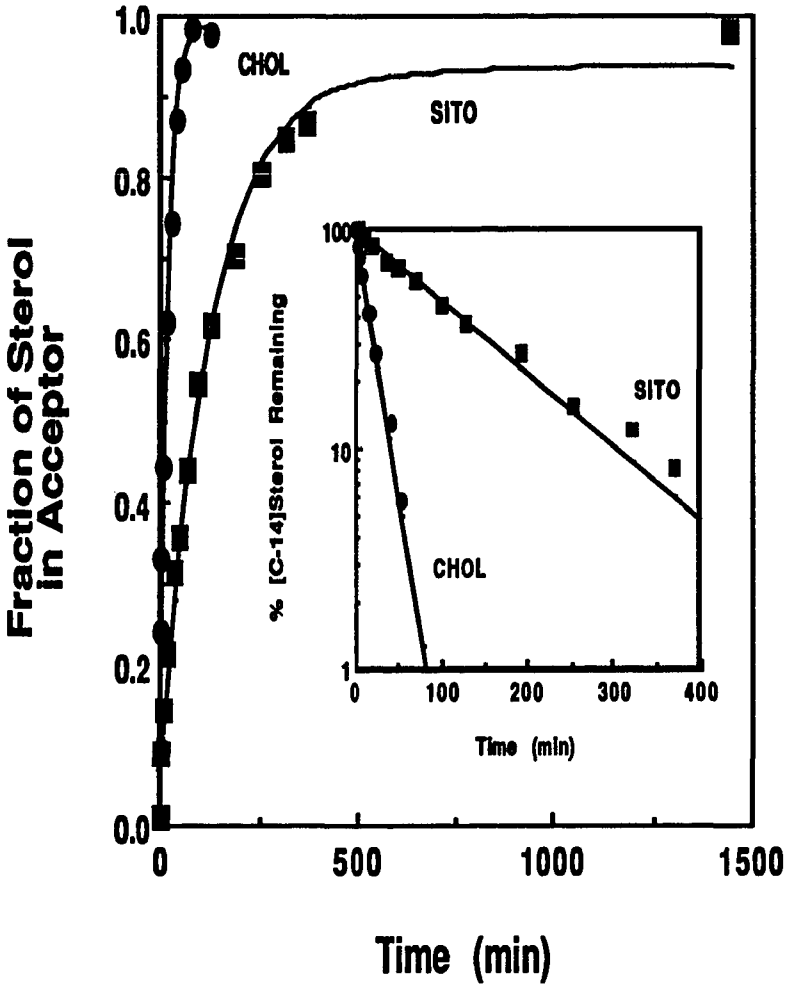


Figure 1-1B: Kinetics of exchange of [4-^{14}C]cholesterol (•) and [4-^{14}C]sitosterol (■) between unilamellar vesicles containing 1 mol % sterol at 50 °C. Donor and acceptor vesicles were prepared from egg PC. *Inset:* Semilog plot of the exchange data. Albumin (2% w/v) was present in the incubation medium.

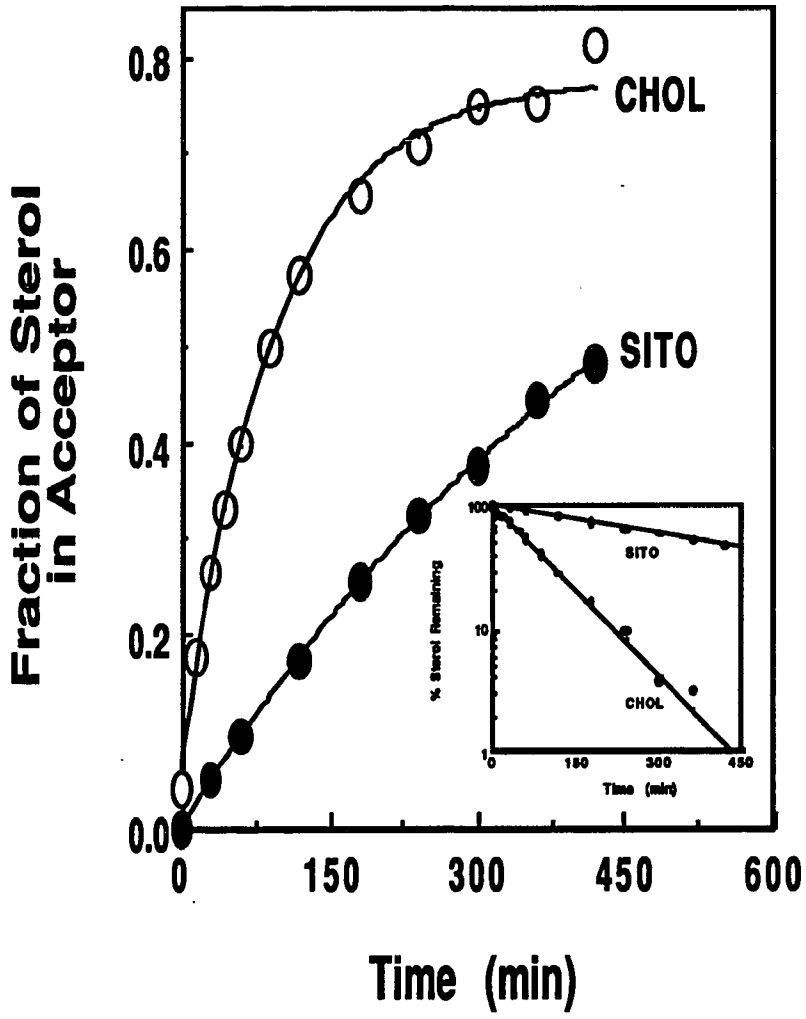


Figure 1-2: Kinetics of [4-^{14}C]cholesterol (o) and [4-^{14}C]sitosterol (•) movement between unilamellar vesicles containing 6 mol % sterol at 45 °C. Donor vesicles were prepared from egg PC (44 mol %) and egg sphingomyelin (35 mol %), and acceptor vesicles contained 94 mol % egg PC and 6 mol % sitosterol. *Inset:* Semilog plots of the exchange data.

previous comparisons of their effects on phospholipid bilayers and monolayers (Bittman et al., 1981; Ghosh and Tinoco, 1972); furthermore, a membrane-disordering effect of sitosterol has been proposed in yeast (Bloch, 1983).

To account for some of the 5- to 10-fold lower rate of absorption of sitosterol by intestinal mucosal cells, the constraint in the intermembrane movement of sitosterol compared with cholesterol observed in the studies of exchange rates may contribute to the different absorption. The results reported in Figures 1-1 and 1-2 support the model of the transition state postulated for cholesterol transfer between membranes, in which the cholesterol molecule is proposed to be attached by the tip of its hydrophobic side chain (Phillips et al., 1987). The additional van der Waals interactions between the sitosterol side chain and acyl chains of phospholipids appear to contribute significantly to the basis of discrimination in absorption of cholesterol and sitosterol.

Kinetics of Exchange of Purified Sitosterol between DPPC and Egg PC Vesicles. To compare the rates of intermembrane movement of sitosterol and cholesterol the time course of sterol exchange from donor to acceptor unilamellar vesicles of different PC acyl chain compositions at 50 °C were measured. Figure 1-3 gives the time course of exchange between vesicles containing 24 mol % sterol, and the insets show semilog plots of labeled sterol remaining in the donor vesicles as a function of time. Unlabeled sitosterol was purified from the commercially available, naturally occurring mixture of sitosterol/campesterol by HPLC (see p. 14). Both sterols undergo exchange between DPPC vesicles at a much slower rate than between egg PC vesicles. In order to approach equilibrium in the DPPC vesicle system it was necessary to carry out exchange measurements over a 5-day period. Nevertheless, the recovery of acceptor vesicles remained very high as judged

Table 1-1. Comparison of Half-times for [^{14}C]Cholesterol and [^{14}C]Sitosterol Exchange between Vesicles Prepared with Different Phospholipid Composition[a]

Phospholipid composition	$t_{1/2}$ (min) cholesterol	sitosterol
DPPC	222.3 ± 43.2	990.8 ± 76.0
egg PC	14.9 ± 1.3	109.1± 12.7
egg PC/egg SPM	71.6	419.2

[a] The exchange experiments were carried out at 50 °C for DPPC and egg-PC vesicles and at 45 °C for egg PC (44 mol %)/egg SPM (35 mol %) vesicles. The sterol content was 1 mol % for DPPC and egg PC vesicles and 6 mol % for egg PC/egg SPM vesicles. At least two different vesicle preparations were used in the experiments with DPPC and egg PC. PSC was used to estimate donor vesicle sizes at 50 °C as described on p. 16. DSC (Hart Scientific, Provo, UT) was used at a scanning rate of 15 °C/h to determine the phase transition temperature of aqueous dispersions of DPPC with 1 mol % sterol; no difference was found in the sitosterol- and cholesterol-containing preparations.

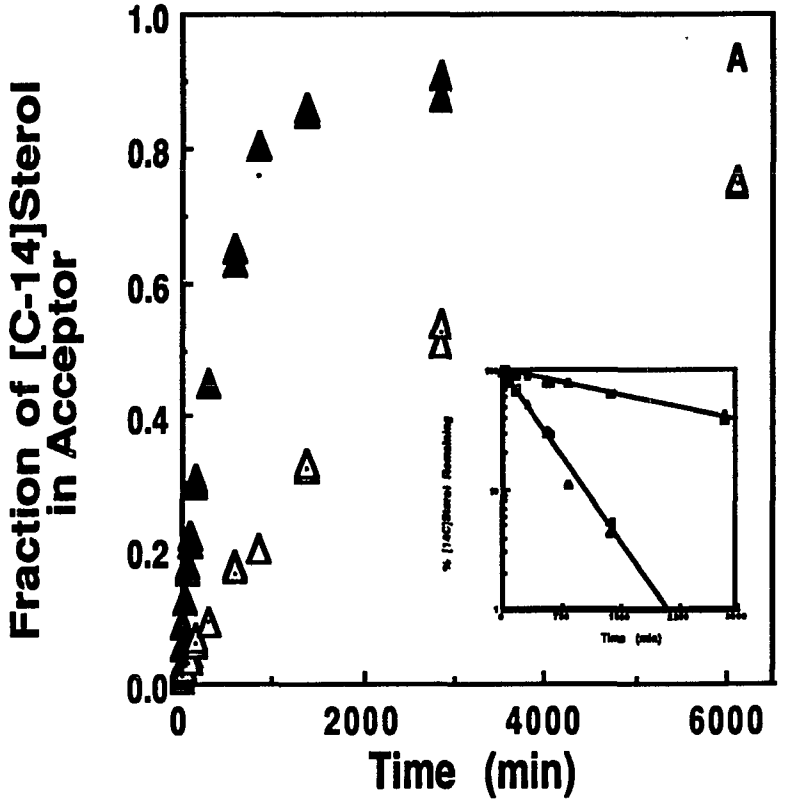


Figure 1-3: Time course of [^{14}C]cholesterol (▲) and [^{14}C]sitosterol (△) exchange between PC/sterol unilamellar vesicles at 50 °C. The vesicles contained 24 mol % sterol; sitosterol was purified as described on p. 14. Albumin was omitted in the incubation media. The host phospholipid in both donor and acceptor vesicles was DPPC. *Inset*, first-order plot of the exchange data; (▲) $t_{1/2}$ 349.8 min (X_{xch} = 98%), (△) $t_{1/2}$ 2337.5 min (X_{xch} = 95%).

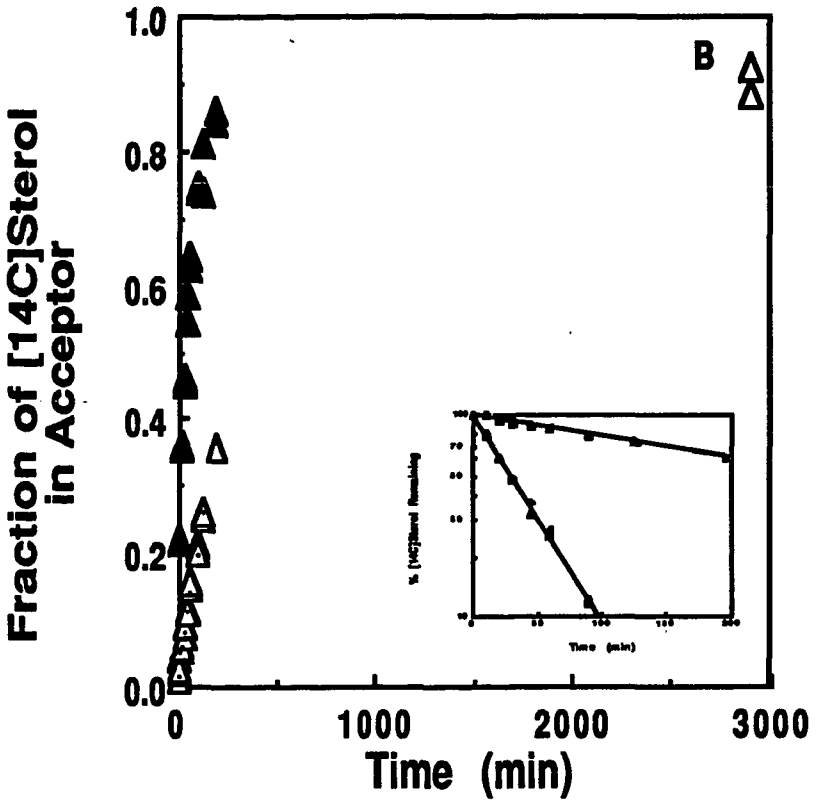


Figure 1-3: Time course of [^{14}C]cholesterol (▲) and [^{14}C]sitosterol (△) exchange between PC/sterol unilamellar vesicles at 50 °C. The vesicles contained 24 mol % sterol; sitosterol was purified as described on p. 14. Albumin was omitted in the incubation medium. The host phospholipid in both donor and acceptor vesicles was egg PC. *Inset*, first-order plot of the exchange data; (▲) $t_{1/2}$ 32.3 min (X_{xch} = 99%), (△) $t_{1/2}$ 296 min (X_{xch} = 91%).

by the retention of [^{3}H]triolein, indicating that fusion between donor and acceptor vesicles did not increase during the prolonged incubation; also, oxidation of cholesterol could not be detected (see "Methods"). Exchange of both sterols between egg PC vesicles was relatively fast (Figure 1-3B), reaching equilibrium within a 7-48-h period for cholesterol and 50-100-h period for sitosterol. The extent of sterol exchange at equilibrium (X_{xch}) was similar for both sterols, and essentially all of the sterol was fully exchangeable. The inset to Figure 1-3 shows that the semilog plots are linear (correlation coefficients >0.99), indicating that both sterols undergo exchange in one kinetic pool. The rate of sterol exchange from PC bilayers containing unsaturated acyl chains is much faster than that from saturated PC bilayers; the half-times in egg PC bilayers are 296 min for sitosterol and 32 min for cholesterol exchange, compared with 2338 min for sitosterol and 350 min for cholesterol exchange in DPPC bilayers. These half-times for cholesterol exchange are consistent with data obtained in other studies of cholesterol exchange by several investigators (reviewed by Phillips et al., 1987; Dawidowicz, 1987; Bittman, 1988). The data are explained by reduced packing constraints in membranes prepared with phospholipids having unsaturated acyl chains.

Comparison of Cholesterol and Sitosterol/Campesterol Exchange Kinetics at Various Mol %. The previous study showed that even when present at only 1 mol % of the membrane lipid, sitosterol undergoes exchange between DPPC vesicles and between egg PC vesicles more slowly than does cholesterol in the presence of albumin (2% w/v) (Kan & Bittman, 1990); in this study, the naturally occurring mixture of sitosterol and campesterol was used to prepare the vesicles. Table 1-2 compares the half-times for sterol exchange at various molar ratios of sitosterol and cholesterol in DPPC and

Table 1-2: Kinetics of Cholesterol and Sitosterol/Campesterol Exchange between DPPC and Egg PC Vesicles at 50 °C[a]

mol %	DPPC vesicles			egg PC vesicles		
of	cholesterol	sitosterol[b]	rate	cholesterol	sitosterol	rate
sterol	$t_{1/2}$[c] (min)	$t_{1/2}$ (min)	ratio	$t_{1/2}$ (min)	$t_{1/2}$ (min)	ratio
1[d]	222.3 ± 43.2	990.8 ± 76.0	4.4	14.9 ± 1.3	109.1 ± 12.7	7.3
6				23.5 ± 10.6	136.6 ± 29.9	5.8
10	293.0 ± 48.1	1183 ± 60.2	4.0			
24				26.1 ± 12.7	104.9 ± 19.8	4.0
50	60.7 ± 15.3	171.5 ± 20.7	2.8	31.2 ± 0.1	176.3 ± 12.6	5.7

[a] Vesicles were prepared with a constant total lipid concentration (donor vesicles, 1.0 mM; acceptor vesicles, 10.0 mM) and various concentrations of sterol and phospholipid. Albumin (2%, w/v) was present in the incubation medium. [b] A naturally occurring mixture obtained from Sigma was used; HPLC analysis indicated that it consisted of 55 % sitosterol and 45 % campesterol (see p. 14). [c] The $t_{1/2}$ values are the means ± SE of at least three different vesicle preparations. [d] Data from Kan & Bittman (1990).

egg PC bilayers. For both sterols the half-times increase on increasing the sterol content in egg PC bilayers from 1 to 6 mol %, and then increase slightly at higher sterol content. In DPPC vesicles, sterol exchange is slower at 7.5:1 than at the other DPPC:sitosterol molar ratios used. With both sterols the increase in rate is very pronounced as an equimolar ratio of DPPC to sterol is approached. In agreement with Figure 1-3, which shows that the [^{14}C]sitosterol exchange rate from PC vesicles prepared using purified sitosterol is slow relative to [^{14}C]cholesterol exchange in the absence of albumin, the data in Table I-2 show that [^{14}C]sitosterol undergoes slow exchange between DPPC and egg PC vesicles prepared with sitosterol and campesterol.

Kinetics of Sterol Exchange between Egg SPM Vesicles. The exchange of sterols between SPM bilayers in the presence or absence of albumin was also investigated. The very slow rate of cholesterol exchange from egg SPM bilayers (Figure 1-4) has been observed previously over a limited range of cholesterol concentrations in mixed glycerolipid-SPM bilayers (Clejan & Bittman, 1984b; Fugler et al.,1985; Yeagle & Young, 1986; Bhuvaneswaran & Mitropoulos, 1986; Thomas & Poznansky, 1988b; Lund-Katz et al., 1988). An incubation period of about ~7 days was used in order to allow exchange to approach the equilibrium position. During this period there was no visible flocculation or coalescence of vesicles, and the fusion of unilamellar vesicles and oxidation of cholesterol were minimal based on the recovery of ^{3}H dpm and TLC/GC analysis of the extracted lipids. Figure 1-4 shows that sitosterol at 10 mol % undergoes exchange more slowly than cholesterol at the same concentration, both in the presence and absence of albumin. The half-times for exchange of both sterols are much higher in SPM than in DPPC bilayers (Table 1-3), which reflects the high lateral packing density of SPM

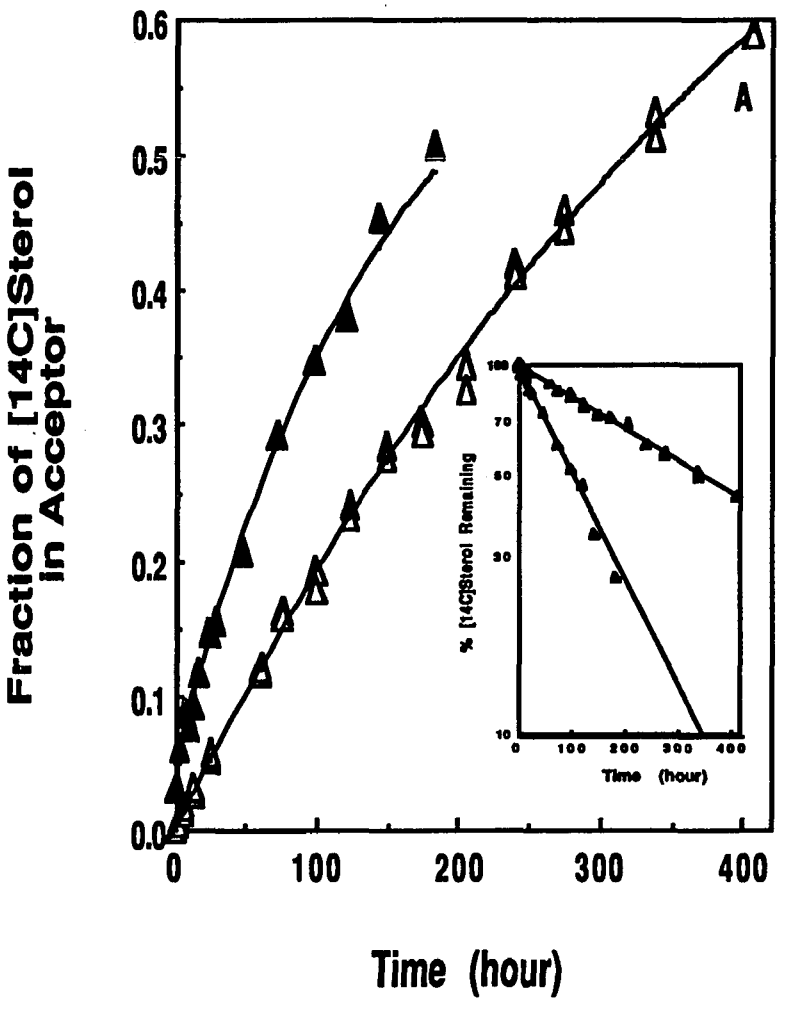


Figure 1-4A: Time course of [^{14}C]cholesterol (▲) and [^{14}C]sitosterol (△) exchange at 50 °C between egg SPM unilamellar vesicles. The vesicles contained 10 mol % sterol. Sitosterol from Sigma contained 45 mol % campesterol and was used without further purification. Exchange experiments were run with albumin (2% w/v). *Inset,* first-order plot of the exchange data; *A,* (▲) $t_{1/2}$ 116 h (X_{xch} = 73%), (△) $t_{1/2}$ 380 h (X_{xch} = 100%).

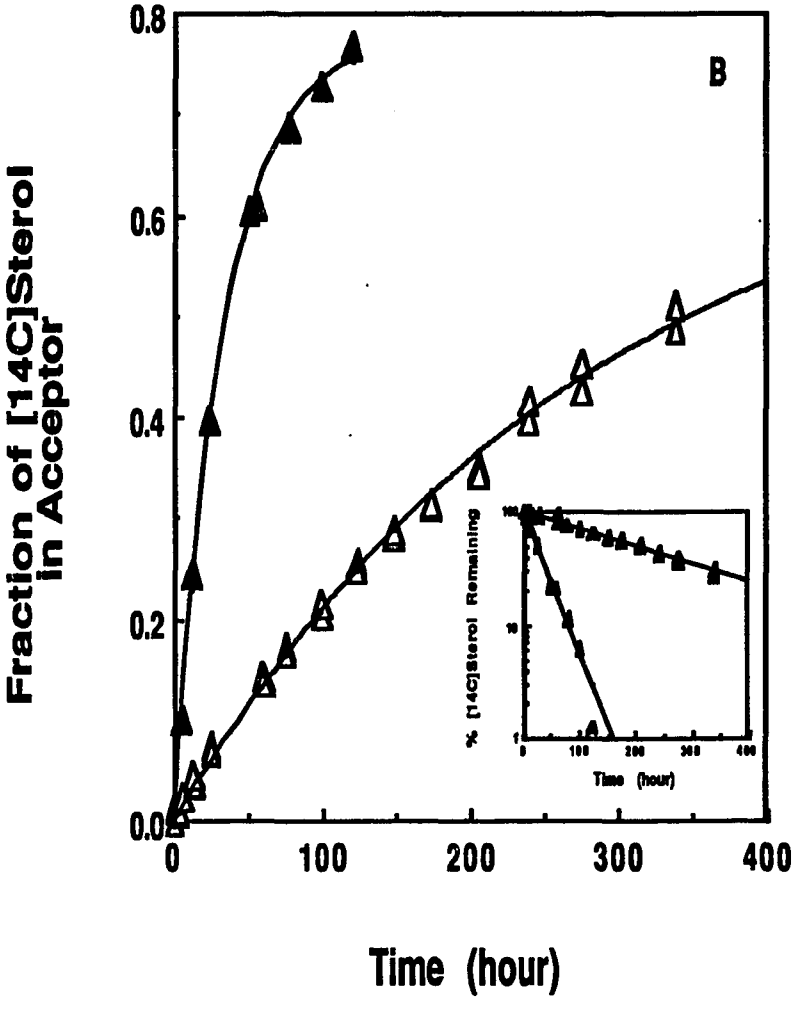


Figure 1-4B: Time course of [^{14}C]cholesterol (▲) and [^{14}C]sitosterol (△) exchange at 50 °C between egg SPM unilamellar vesicles. The vesicles contained 10 mol % sterol. Sitosterol from Sigma contained 45 mol % campesterol and was used without further purification. Exchange experiments were run without albumin (*B*). *Inset,* first-order plot of the exchange data; *B,* (▲) $t_{1/2}$ 26 h (X_{xch} = 86%), (△) $t_{1/2}$ 216 h (X_{xch} = 78%).

molecules relative to PC molecules (Lund-Katz et al., 1988). The extent of exchange of cholesterol between egg SPM bilayers is markedly lower than in DPPC bilayers at 10 mol % sterol, whereas sitosterol underwent essentially complete exchange. The nonexchangeable pool in SPM bilayers prepared with 10 mol % cholesterol amounts to 27% of the total cholesterol; other investigators have observed a much larger nonexchangeable pool at 50 °C (discussed on p. 44). It is apparent that the rates of exchange of both sterols are decreased in the presence of 2% w/v albumin (Figure 1-4A vs. B); a small increase in the X_{xch} value of cholesterol and a small decrease in the X_{xch} value of sitosterol was also found on addition of albumin. In contrast to these effects of albumin, previous reports indicated that addition of albumin (2% w/v) caused a marked enhancement of the cholesterol exchange rate between mycoplasma cell membranes and PC vesicles as acceptors without affecting membrane structure (Rottem et al., 1981; Clejan & Bittman, 1984a).

Kinetics of Sitosterol Exchange between C18-lyso-PC Aqueous Dispersions. In order to determine whether the rates of sitosterol and cholesterol exchange differ in surfactant-containing preparations, a new experimental system containing 50 mol % C18-lyso-PC was used. Figure 1-5 shows that both sterols undergo first-order exchange from donor dispersions to an excess of acceptor dispersions at 37 °C. As in PC and SPM vesicles, sitosterol underwent exchange between the C18-lyso-PC-containing-dispersions much more slowly than cholesterol. The average $t_{1/2}$ values for sitosterol and cholesterol exchange are 438 ± 64 min and 105 ± 11 min, respectively, indicating that the 24α-ethyl group of sitosterol decreases the ease of sterol desorption from the lipid-water interfaces of these surfactant-enriched surfaces by a factor of 4. As observed in Figure 1-3, both sterols are fully exchangeable in a single kinetic pool.

Table 1-3: Comparison of Cholesterol and Sitosterol/Campesterol Exchange Kinetics between Egg SPM Vesicles and between DPPC Vesicles at 50°C[a]

	Egg SPM		DPPC	
sterol (10 mol %)	half-time (h)	X_{xch}[b]	half-time (h)	X_{xch}
cholesterol	126 ± 15	73%	4.9 ± 0.8	100%
sitosterol	306 ± 104	96%	19.7 ± 1.0	100%

[a]Albumin (2% w/v) was present in the donor-acceptor incubation medium. [b]The fraction of exchangeable ^{14}C-sterol, X_{xch}, at equilibrium was calculated as described on p. 22.

Previous Comparisons of Sitosterol and Cholesterol Uptake. The studies described in this Chapter were carried out in order to determine the effects of alkylation at C_{24} of the sterol molecule on the kinetics of sterol exchange between phospholipid/sterol membranes and lysophospholipid/phospholipid/sterol dispersions. The influence of structural modifications in the sterol side chain on dynamic processes involving sterol movement between membranes or across membrane leaflets has not been studied extensively, but several studies have demonstrated significant differences in intermembrane rates of sitosterol and cholesterol transfer. The rates of exchange of ^{14}C-labeled sterols from mycoplasma membranes to an excess of acceptor lipid vesicles were used previously to study the influence of sterol side chain structure on the transbilayer distribution of sterols (Clejan et al., 1981; Clejan & Bittman, 1984a). Sterols with alkyl groups at C_{24} were found to accumulate in the outer half of the bilayer, possibly because they experience steric interference during translocation from the outer to inner leaflet. Initial rates of transfer of cholesterol analogs between vesicles were also used to estimate the effects of changes in sterol side chain structure on partitioning between DPPC and egg PC bilayers (Rujanavech & Silbert, 1986). These investigators found that cholesterol partitioned preferentially into DPPC vesicles vs. egg PC vesicles at 37 °C, whereas sitosterol and campesterol partitioned about equally into these phospholipids at this temperature. Cholesterol was incorporated into erythrocytes more readily than sitosterol, and the classes of phospholipids present in the erythrocyte membranes or in the micellar solution appeared to affect the degree of selectivity of sterol uptake (Edwards & Green, 1972; Child & Kuksis, 1982). A four-fold greater uptake of 7-dehydrocholesterol over 7-dehydrositosterol into brush border membrane vesicles and rat erythrocyte

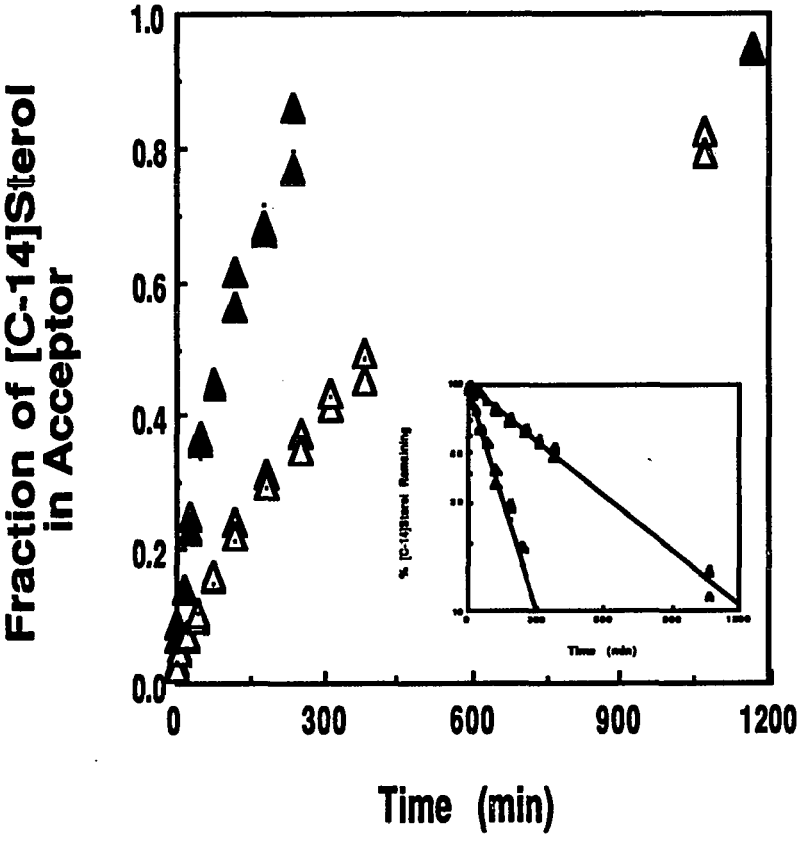


Figure 1-5: Time course of [^{14}C]cholesterol (▲) and [^{14}C]sitosterol (△) exchange at 37 °C between C18-lyso-PC-containing dispersions. Both donor and acceptor dispersions contained egg PC and 1.5 mol % sterol. Sitosterol was purified as described under Experimental Procedures. Albumin was not used in the experiments. *Inset*, first-order plot of the exchange data; (▲) $t_{1/2}$ 100 min (X_{xch} = 100%); (△) $t_{1/2}$ 409.6 min (X_{xch} = 100%).

membranes from bile salt-egg PC solution was observed (Child & Kuksis, 1983). The extent of cholesterol transfer between erythrocytes and plasma was greater than that of sitosterol, which was considered to be related to the higher rate of esterification of cholesterol by plasma lecithin:cholesterol acyltransferase (Sugano & Kida, 1980).

Summary of Results. Comparison of the exchange data presented in Figures 1-3 and 1-4 indicates that the rate of sterol movement from saturated and unsaturated PC- or SPM-water interfaces is sensitive to the presence of the 24α-ethyl group of sitosterol. The very slow rate of sterol exchange between SPM bilayers indicates that both of these sterols pack very tightly with SPM. The ratio of the half-times for sitosterol/cholesterol exchange half-times at 10 mol % sterol is about 4 in DPPC and about 2.4 in egg SPM bilayers (Table 1-3). This is consistent with our observation of a sitosterol/cholesterol $t_{1/2}$ ratio of ~6 from vesicles prepared from 6 mol % sterol, 44 mol % egg PC, 35 mol % egg SPM, and 15 mol % DCP (Kan & Bittman, 1990). At 24 mol % sterol, the sitosterol/cholesterol $t_{1/2}$ ratio is 9.2 in egg PC vesicles and 6.7 in DPPC vesicles (Figure 1-3). The lower rate of sitosterol movement between vesicles prepared from DPPC, egg PC, and egg SPM (Tables 1-2 and 1-3) indicates that membrane phospholipid composition and extent of saturation of fatty acyl chains did not dictate the sitosterol/cholesterol exchange rate ratio. This ratio of $t_{1/2}$ values is 4 in aqueous dispersions containing 50 mol % C18-lyso-PC (Figure 1-5). The marked differences that have been observed in $t_{1/2}$ values for sitosterol and cholesterol reflect the differences in cholesterol-phopsholipid and sitosterol-phospholipid interaction energies in the donor particles.

These results differ from previous studies that showed a loss of selectivity of cholesterol vs. sitosterol uptake into rat erythrocytes and upper

villus cells when DPPC and egg SPM were added to the micellar incubation medium in place of egg PC (Child & Kuksis, 1982; Child & Kuksis, 1986). The results shown here indicate that the substitution of an ethyl group for a hydrogen at C-24 impedes the slow dissolution of the lipid from the surface of PC and SPM vesicles by increasing the molecular packing in the bilayer. The results shown in this Chapter support the model postulated for the transition state in cholesterol transfer between vesicles (Phillips et al., 1987), in which the cholesterol molecule is considered to be attached to the surface of the donor species by the tip of its hydrophobic side chain. Additional van der Waals interactions are possible between phospholipid hydrocarbon chains and the sitosterol side chain compared with the cholesterol side chain; these interactions may raise the energy of activation of the desorption process.

The rates of sitosterol and cholesterol exchange between DPPC vesicles are much faster at 50 mol % sterol than at 1-10 mol % sterol (Table 1-2). In egg PC vesicles, however, the sterol exchange rates did not increase on increasing the content of sterol from 1-24 mol % to 50 mol %. The increase in exchange rate at high sterol content in DPPC vesicles suggests that a region of sterol-rich clusters exists which facilitates sterol desorption. It is well known that cholesterol is clustered in domains above ~33.3 mol % sterol (reviewed by Hui, 1988). McLean and Phillips (1982) proposed that cholesterol desorbs from equimolar cholesterol-PC clusters in the bilayer, whereas Nemecz et al. (1988) proposed that escape of cholesterol from cholesterol-rich clusters gives raise to a very slow exchange rate. Since we observe only one kinetic pool of cholesterol and sitosterol at both low (Kan and Bittman, 1990) and high sterol content, the results indicate that the enhanced rates of sterol desorption at 50 mol % sterol in the donor DPPC vesicles (sterol/PC molar ratio of 1.4) may arise from interfacial regions

between sterol-rich and sterol-PC phases, with rapid movement of sterol between the phases accounting for the detection of only one kinetic pool. The absence of a similar rate enhancement at high sterol content in egg PC vesicles suggests that phase boundaries may not be present at 50 °C in this system. It should be noted that McLean and Phillips (1982) also observed an increase in the rate of [^{14}C]cholesterol efflux at 37 °C from DPPC vesicles at very high sterol content (cholesterol/PC molar ratio of 3.4) compared with <50 mol % cholesterol. The rate enhancement at high cholesterol content was attributed to altered cholesterol-nearest-neighbor interactions in the bilayer.

Extent of Sterol Exchange. All of the radiolabeled sitosterol and cholesterol underwent exchange between vesicles prepared from DPPC and egg PC in single kinetic pools. Thus the rate of transbilayer movement of sitosterol and cholesterol from the inner to outer leaflet of the donor vesicles must exceed that of exchange. The observation that all of the cholesterol is exchangeable from vesicles prepared with saturated and unsaturated PCs is consistent with many earlier reports (reviewed by Phillips et al., 1987), but does not agree with recent studies in which a significant nonexchangeable pool of cholesterol was found in 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine vesicles at 37 °C (Bar et al., 1986) and at 24 °C (Nemecz et al., 1988) and in DMPC vesicles at 50 °C (Bar et al., 1987). Although a sizeable (27%) nonexchangeable pool of cholesterol in egg SPM bilayers containing 10 mol % cholesterol was observed (Table 1-3), the results presented here differ significantly from those of Bar et al. (1987) who reported that 64% of the total [^{3}H]cholesterol in egg SPM vesicles containing 10 mol % cholesterol was not exchanged at 50 °C and from those of Bar et al. (1989) who found that 47% of the cholesterol probe dehydroergosterol was nonexchangeable in bovine-brain SPM vesicles at 50 °C. Furthermore, near

full exchangeability of cholesterol at other cholesterol/SPM molar ratios was found (Kan and Bittman, unpublished results). Since the results are not strictly comparable to those reported by Bar et al. (1986, 1987, 1989) and Nemecz et al. (1988) because of differences in experimental design, the following experiments were carried to examine possible sources of the discrepancy in the exchangeable cholesterol pool size. First, the experiments were designed to measure radiolabeled cholesterol exchange from neutral donor vesicles containing SPM to acceptors containing egg PG or egg PA and SPM (as was done by Bar et al., 1986, 1987), but were unsuccessful because of low recovery of the neutral vesicles. However, the discrepancy is unlikely to arise from the location of the negatively charged lipid since neutral and charged donor vesicles of PC gave similar rates of cholesterol exchange (McLean & Phillips, 1984; Bar et al., 1986). Another difference in experimental design that may affect exchangeability involves the presence of albumin, which was present in the exchange measurements shown here (Figure 1-4A) but not in those of Bar et al. (1987) and Nemecz et al. (1988); however, when exchange measurements were conducted without albumin (Figure 1-4B) X_{xch} values of 86% and 78% for cholesterol and sitosterol were observed, respectively. Third, the discrepancy in exchangeable pool size could arise from differences in methods of data analysis. However, some of the data sets were kindly analyzed by Dr. K. L. Bar with the iterative nonlinear least-squares program used by Bar et al. (1986, 1987); the values of $t_{1/2}$ and X_{xch} were quite similar to those obtained with the program used here. Finally, it was considered the possibility that a difference in the time frame of the experiments affects the values of $t_{1/2}$ and X_{xch}. In the studies at 37 °C (Bar et al., 1986) and 50 °C (Bar et al., 1987) and at 24 °C (Nemecz et al., 1988), exchange measurements were halted at about 8 h, whereas in the present studies

aliquots were taken until equilibrium was approached and the endpoint was then estimated using the simplex program. Lower values of X_{xch} and faster rates of exchange were obtained when data gathered before one half-time were used to compute these values. For example, by using the data obtained between 0 and 16.5 hours for [^{14}C]cholesterol exchange from SPM bilayers the values were obtained as following: X_{xch} = 10% and $t_{1/2}$ = 6.7 h; for data points between 0 and 47.5 h, X_{xch} = 15% and $t_{1/2}$ = 11.1 h; for data points between 0 and 97.6 h, X_{xch} = 28% and $t_{1/2}$ = 23.0 h; for all of the experimental points (between 0 and 182 h) X_{xch} = 73% and $t_{1/2}$ = 116 h. Therefore it is concluded that the apparent nonexchangeable pool size is increased when measurements are not extended to times that approach the equilibrium position. Other factors, presently unresolved, may also contribute to the differences in X_{xch} values determined in the present study compared with previous results.

Conclusions. The preferential uptake of exogenous cholesterol by small-intestinal cells observed in vivo is presumably a complicated process and may involve important contributions from interactions with plasma membrane proteins that have specific binding sites for the isooctyl side chain of cholesterol (Mayer et al., 1985; Chow & Hollander, 1978; Ikeda & Sugano, 1983; Bloj & Zilversmit, 1982). Differences in the solubility of sterols in the aqueous intestinal contents (Borgström, 1968; Armstrong & Carey, 1987; Ikeda et al., 1988) and intracellular reactions with membrane-bound enzymes could also contribute to preferential absorption of cholesterol. Nevertheless, the results with sterol/phospholipid bilayers and sterol/lyso-PC/phospholipid dispersions have implications for the mechanism(s) by which dietary cholesterol is transferred preferentially into the outer leaflets of intestinal cell membranes compared with ingested phytosterols. Although the physical form

of sterols in the aqueous intestinal contents has not been fully established, it appears that sterols are present as a mixture of mixed bile salt micelles and unilamellar vesicles (Carey et al., 1983; Peled et al., 1989; Staggers et al., 1990; Hernell et al., 1990). Since PC and SPM are present in high amounts in many intestinal cell membranes (Forstner et al., 1968; Douglas et al., 1972; Hauser et al. 1980; Child & Kuksis, 1983; Chapelle & Gilles-Baillien, 1983; Pind & Kuksis, 1987), these phospholipids have been chosen to form the vesicles for experiments. The slower rates of desorption of sitosterol that have been observed from PC- and SPM-water interfaces over a wide range of sterol to phospholipid molar ratios suggests that slow spontaneous efflux of sitosterol relative to cholesterol contributes to the lower rate of sitosterol absorption by intestinal mucosal cells.

There is, of course, a possibility that cholesterol-binding protein(s) may be present in at least some intestinal cell membranes, as observed in brush border membranes from the small intestine of cholesterol-fed rabbits (Bloj & Zilversmit, 1982). It may therefore be speculated that membrane proteins that bind cholesterol preferentially (see section IX of Bittman, 1988) may have a physiological role in enhancing the extent of intestinal discrimination of sterol absorption. Protein-mediated transfer of cholesterol into brush border vesicles derived from rabbit small intestine has recently been demonstrated (Thurnhofer and Hauser, 1990). However, the finding that the membranes of red blood cells and brush borders showed similar preferential uptake of cholesterol over sitosterol (Child & Kuksis, 1982, 1983) casts some degree of doubt about the role of cholesterol-binding proteins in the absorptive selectivity of sterols. The exchange data presented here suggest that simple competition between micelles and cell membranes can be an important factor in determining the relative rates of intestinal uptake of sterols; desorption of

sitosterol from an "activated" micelle into an aqueous region may be significantly slower than desorption of cholesterol. It will be interesting to extend these studies to lipid mixtures containing glycolipids, which are also present in high concentration in intestinal epithelial membranes.

Chapter 2. Cholesterol Exchange from Synthetic SPMs

Effect of Steric Bulk at the 3 Position of SPM on the Cholesterol Exchange Rate. Figure 2-1A shows that cholesterol undergoes slow exchange from donor egg SPM vesicles to acceptor egg SPM vesicles in only one kinetic pool at 50 °C. Figure 2-1B shows that the rate of cholesterol exchange from vesicles containing *O*-THP-egg SPM is much faster than from vesicles containing egg SPM ($t_{1/2}$ values of 44.6 min in *O*-THP-egg SPM vesicles and 26.1 h in egg SPM vesicles). The size of the exchangeable pool is similar in these vesicles, i.e., 92% in *O*-THP-egg SPM vesicles and 86% in egg SPM vesicles. Since the THP group is bulky compared with the hydroxyl group, steric hindrance may alter the molecular packing of *O*-THP-egg SPM in the vesicle bilayer and impede tight contacts with cholesterol, thus accounting for the faster rate of cholesterol desorption from *O*-THP-egg SPM bilayers (see Table 2-1). Calorimetric measurements indicate that the T_m value of the gel to liquid-crystalline phase transition is about 10 °C lower in *O*-THP-egg SPM bilayers compared with egg SPM bilayers (the endotherms are broad because of the mixed chains present in SPM purified from natural sources; T_m: ~27 °C vs. ~37 °C). In order to further explore the role of hydroxyl group, SPM analogs bearing substituents with sizes more similar to that of the hydroxy group were tested.

Effects of Modifications at the 3 Position of N-C18-SPM on Cholesterol Exchange Rates between Vesicles. The time course and semilog plots of [^{14}C]cholesterol exchange from vesicles containing *N*-C18-SPM, 3-*O*-methyl-, 3-*O*-ethyl-, and 3-deoxy-*N*-C18-SPM are shown in Figure 2-2. The rate of exchange from *O*-ethyl-SPM vesicles is much faster than the rates measured in the other vesicles, whereas similar rates are found for vesicles prepared with *N*-C18-SPM, 3-deoxy-, and 3-*O*-methyl-*N*-C18-SPM. The size of

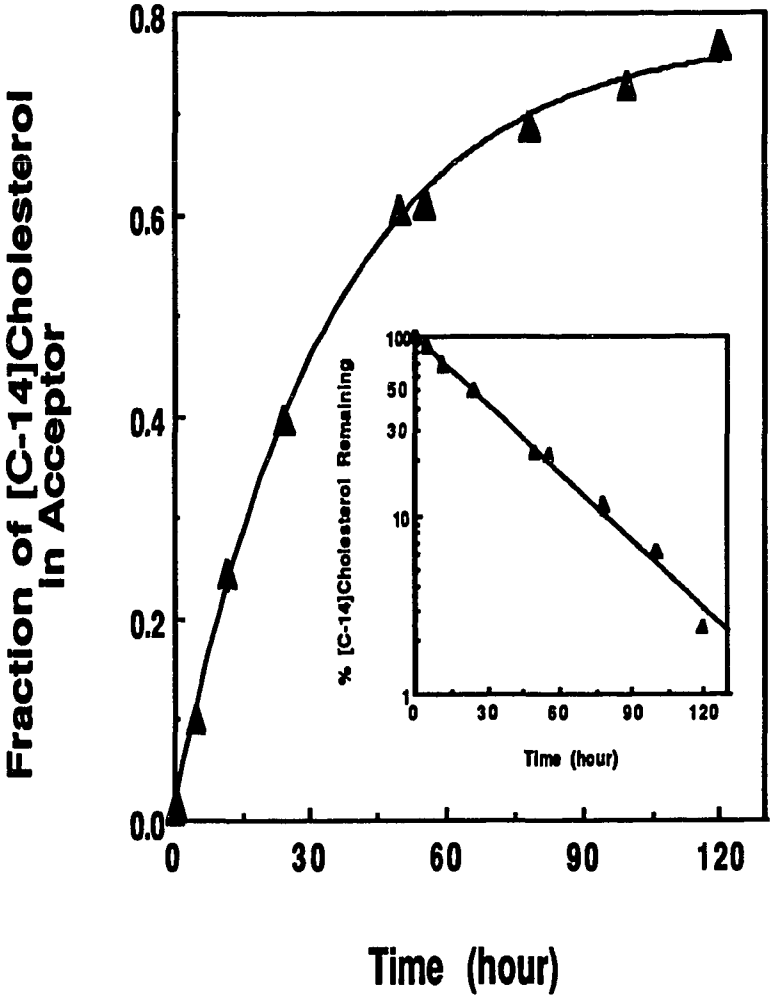


Figure 2-1A. Kinetics of [^{14}C]cholesterol exchange from vesicles containing egg SPM and 10 mol % cholesterol.

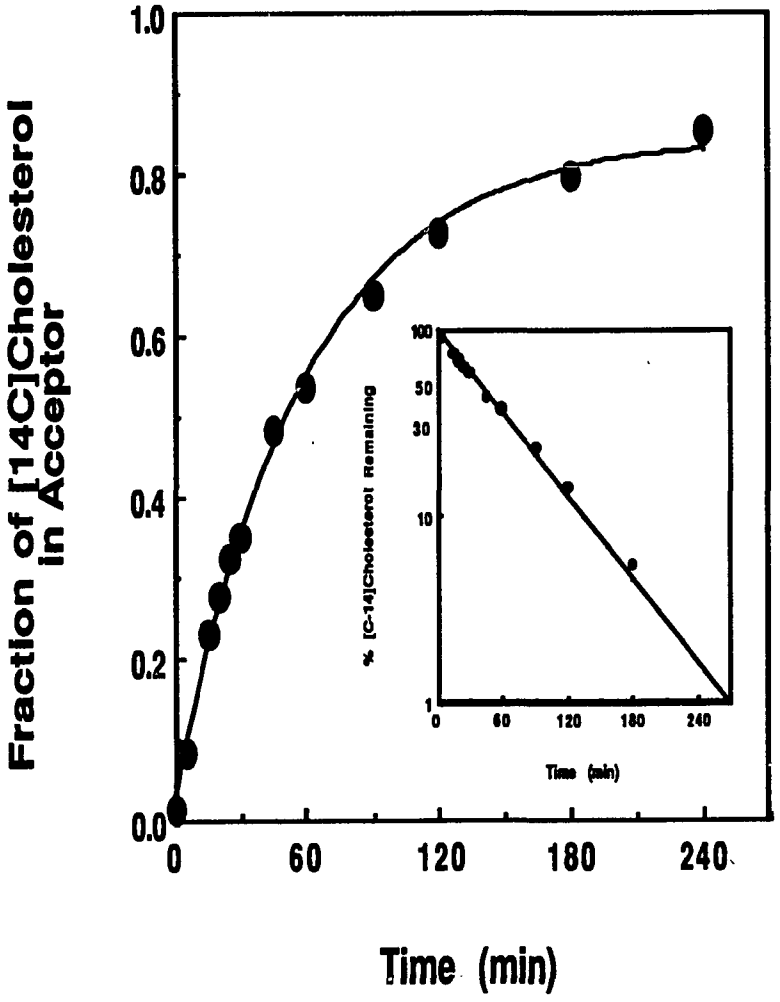


Figure 2-1B. Kinetics of [^{14}C]cholesterol exchange from vesicles containing *O*-THP-egg SPM and 10 mol % cholesterol.

Table 2-1: Effect of Steric Bulk at the 3 Position of Egg SPM on the Half-times for Cholesterol Exchange between SPM Vesicles at 50 °C

egg SPM analog in the donor vesicles	$t_{1/2}$, (h)
egg SPM	23.6 ± 3.6 (2)
O-THP-egg SPM	0.69 ± 0.08 (2)

The number in parentheses indicates the number of separate vesicle preparations that were run.

exchangeable pool is >84% for all of the vesicles with the exception of *N*-C18-SPM vesicles, in which about 73% of the total labeled cholesterol was exchangeable. Again, only one kinetic pool of cholesterol is found (insets, Figure 2-2A-D). The half-times for cholesterol exchange from vesicles containing *N*-C18-SPM and the various synthetic *N*-C18-SPM analogs are listed in Table 2-2. Cholesterol undergoes exchange at the same rate from vesicles prepared from 3-deoxy- and 3-*O*-methyl-*N*-C18-SPM ($t_{1/2}$ values of 22.9 ± 4.3 and 23.0 ± 2.1 h, respectively). These half-times are not markedly different from that found using *N*-C18-SPM vesicles ($t_{1/2}$ = 33.8 ± 0.8 h). These results suggest that the hydroxy group of SPM is not required for the slow rate of cholesterol desorption.

The ease of desorption from the donor particle is dependent on the molecular packing in the membrane bilayer. The molecular basis for the dependence of cholesterol exchange rate on the presence of SPM is not clear; the activation energy required to reach the transition-state complex from which cholesterol molecules desorb is considered to increase when phospholipid-cholesterol interactions are stengthened (Phillips et al., 1987). The present data show that the rate of cholesterol exchange from vesicles containing 3-deoxy-*N*-C18-SPM is the same as that from vesicles containing 3-*O*-methyl-*N*-C18-SPM, and is not markedly faster than the rate measured with *N*-C18-SPM itself. Since 3-deoxy-*N*-C18-SPM has neither hydrogen-bond donating nor accepting property at the 3 position, but still displays a strong affinity for cholesterol, as reflected by a rate of desorption of cholesterol molecules that is nearly as slow as that from *N*-C18-SPM-water interfaces, i t is concluded that the hydroxy group of SPM is not necessary for the strong interaction with cholesterol.

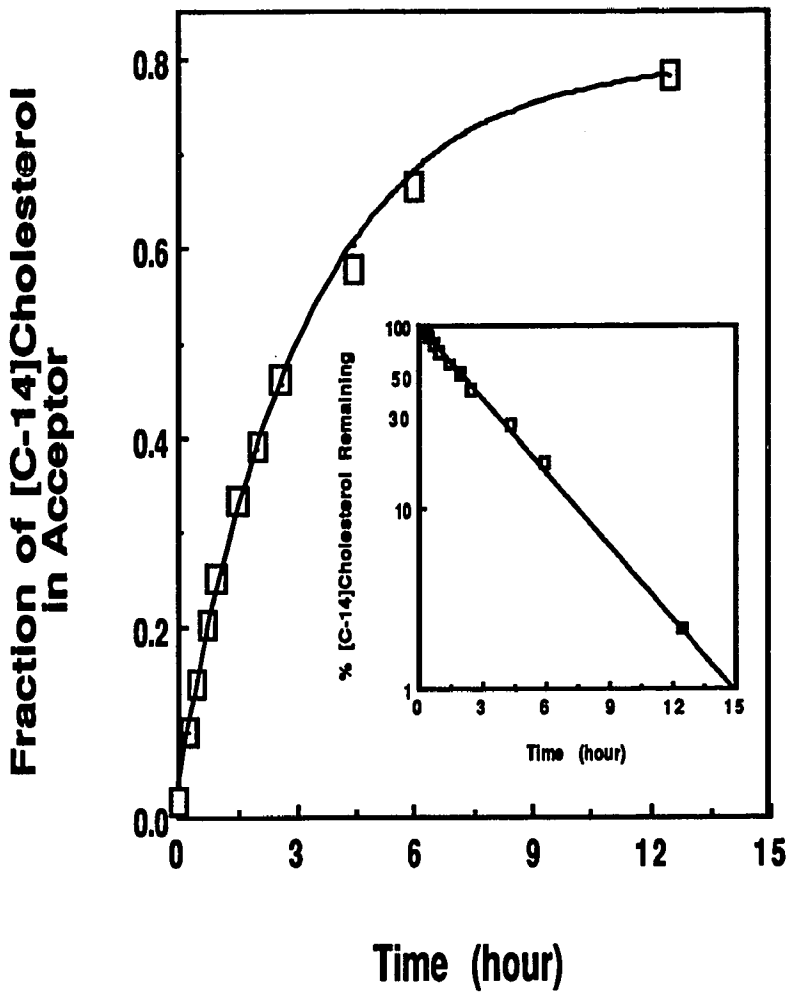


Figure 2-2A. Kinetics of [^{14}C]cholesterol exchange from vesicles containing *3-O*-ethyl-*N*-C18-SPM.

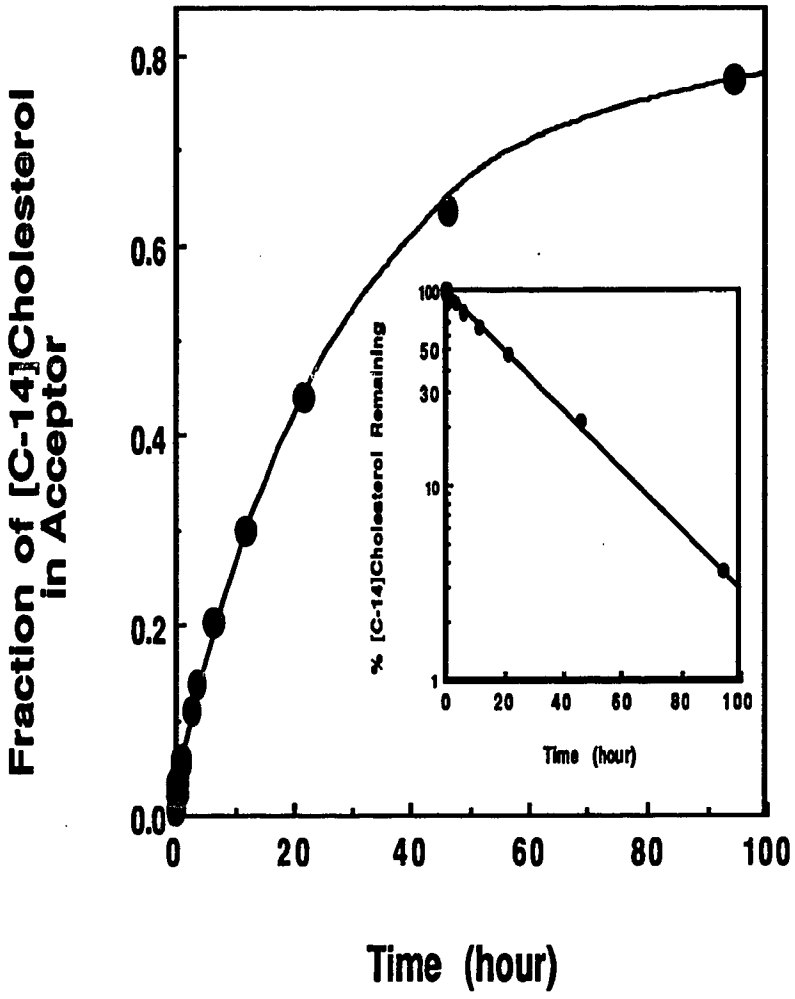


Figure 2-2B. Kinetics of [^{14}C]cholesterol exchange from vesicles containing 3-*O*-methyl-*N*-C18-SPM.

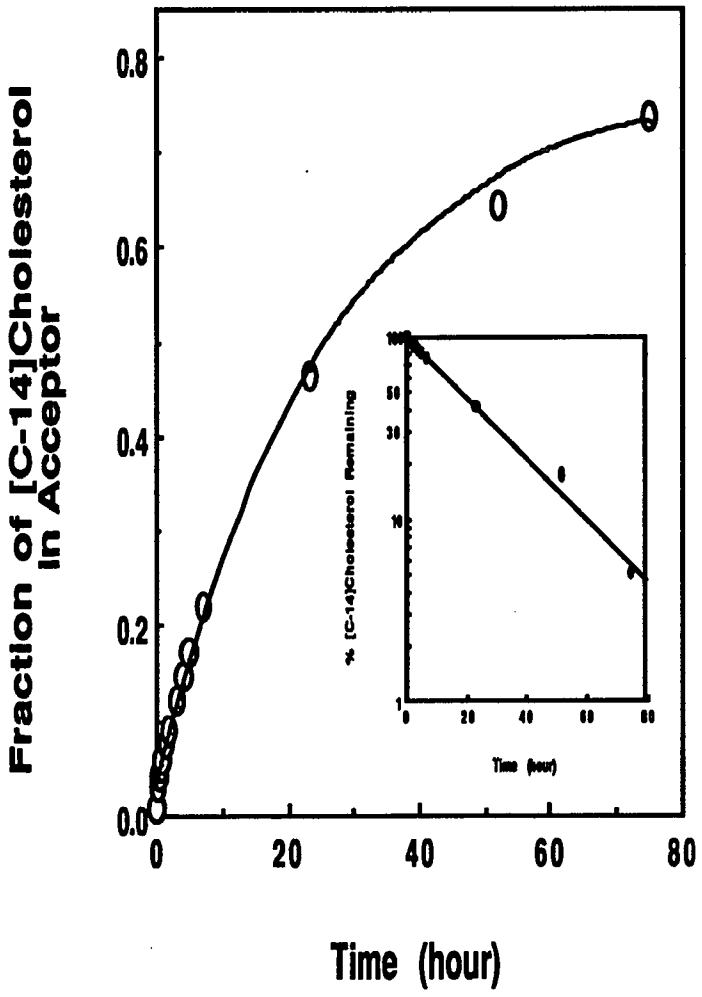


Figure 2-2C. Kinetics of [^{14}C]cholesterol exchange from vesicles containing 3-deoxy-*N*-C18-SPM.

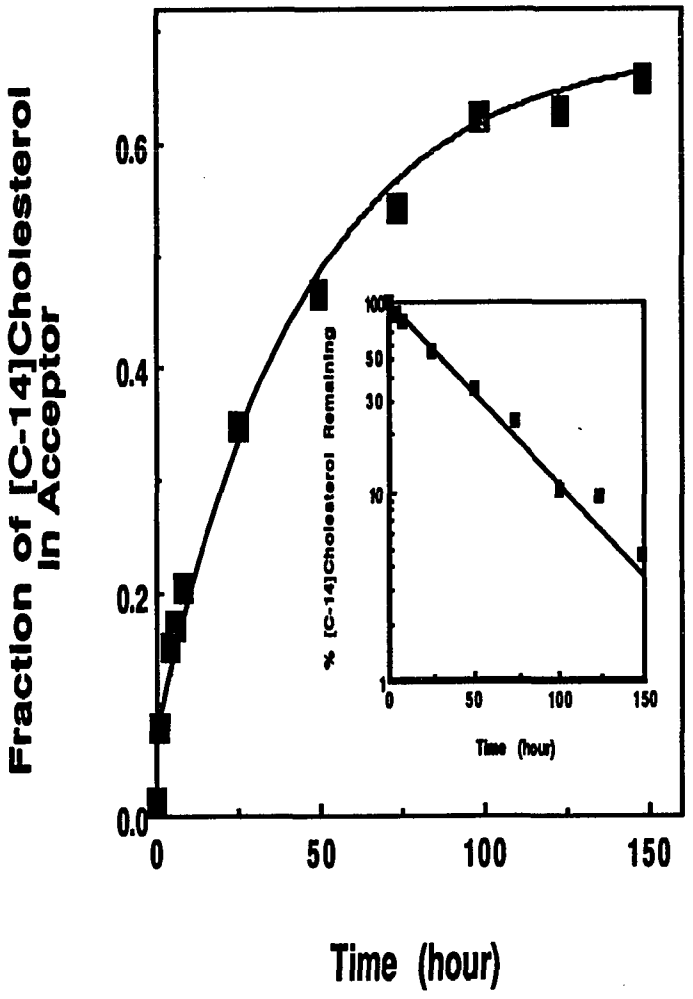


Figure 2-2D. Kinetics of [^{14}C]cholesterol exchange from vesicles containing *N*-C18-SPM.

Table 2-2: Effects of Modifications at the 3 Position of *N*-C18-SPM on the Half-times for Cholesterol Exchange Between SPM Vesicles at 50 °C

N-C18-SPM analog in the donor vesicles	$t_{1/2}$, h
N-C18-SPM	33.8 ± 0.78 (2)
3-deoxy-*N*-C18-SPM	22.9 ± 4.3 (2)
3-*O*-methyl-*N*-C18-SPM	23.0 ± 2.1 (2)
3-*O*-ethyl-*N*-C18-SPM	2.4 ± 0.2 (2)

The number in parentheses indicates the number of separate vesicle preparations that were run. The donor vesicles contained 15 mol % DCP, 10 mol % cholesterol, and 75 mol % synthetic SPM; acceptor vesicles contained 10 mol % cholesterol and 90 mol % egg SPM.

Cholesterol undergoes exchange faster from smaller vesicles than from large vesicles (Fugler et al., 1985; McLean and Phillips, 1984). The faster rate of exchange from 3-*O*-ethyl-*N*-C18-SPM vesicles does not arise from a significant difference in vesicle size since the vesicles prepared from the various *N*-C18-SPM analogs have similar sizes (see p. 17). It is likely that steric hindrance introduced by the ethyl group induces loose packing, which enhances the rate of exchange.

The experiments described in this Chapter were designed to explore the role of the hydroxy group of SPM in binding to cholesterol. The results presented here suggest that the hydroxy group of SPM is not an important site with respect to interaction with cholesterol. The results of cholesterol desorption from vesicles incorporating *N*-C18-SPM, which has both hydroxy and amide groups, seems to display that the intramolecular hydrogen-bonding does not affect the desorption of cholesterol molecules to a great extent since exchange rates using 3-deoxy- and 3-*O*-methyl-*N*-C18-SPM are similar to that using *N*-C18-SPM.

Surface pressure-molecular area isotherms for SPM and PC monolayers indicate that the molecular area of SPM is smaller than that of PC (Lund-Katz et al., 1988; Grönberg and Slotte, 1990), indicating that molecular packing in the lipid-water interface is tighter in monolayers prepared from cholesterol and SPM than from cholesterol and PC; this phenomenon correlates with a slower rate of cholesterol exchange (Lund-Katz et al., 1988). This is probably due to the high van der Waals attraction between SPM and cholesterol relative to PC and cholesterol.

Chapter 3. Cholesterol Exchange from Synthetic PCs

The half-time for cholesterol exchange from donor *N*-C16-SPM unilamellar vesicles is about 9-fold higher than that from donor DPPC unilamellar vesicles at 50 °C (Table 3-1). This rate difference does not arise from a difference in donor vesicle size since the two donor vesicles have similar diameters (128 and 175 nm for DPPC and *N*-C16-SPM vesicles, respectively). Cholesterol undergoes exchange from egg PC bilayers containing 35 mol % egg SPM about 5-fold more slowly than from egg PC vesicles (Kan and Bittman, 1990). These observations are consistent with other reports of a SPM-induced reduction of cholesterol transfer between membranes (Clejan and Bittman, 1984b; Fugler et al., 1985; Yeagle and Young, 1986); Bhuvaneswaran and Mitropoulos, 1986; Thomas and Poznansky, 1988b; Lund-Katz et al., 1988).

It has been postulated that the amide and/or allylic hydroxy group of SPM interact with cholesterol via hydrogen bonding (Boggs, 1987). In an attempt to test the role of the amide group of SPM in the interaction with cholesterol, cholesterol exchange using vesicles from PCs containing *sn*-2 *N*-linked chains (structures, see p. 9) was studied. Figure 3-1 shows the exchange of cholesterol from donor vesicles prepared from PCs containing *sn*-2 *N*-linked chains, i.e., compounds **1**, **2**, and **3**. The kinetic measurements were taken until the fraction of [^{14}C]cholesterol in the acceptor vesicles increased only slightly with time, i.e. until the exchange process approached equilibrium. [^{14}C]Cholesterol was fully exchangeable in donors prepared from compounds **2** and **3**, and about 80% of the total [^{14}C]cholesterol underwent exchange from donor vesicles prepared with compound **1**. The semi-log plots (inset, Figure 3-1) indicate that only one kinetic pool of [^{14}C]cholesterol was present in the donor vesicles. The

Table 3-1. Comparison of Half-times for [^{14}C]Cholesterol Exchange from DPPC and *N*-C16-SPM Vesicles at 50 °C

Phospholipid in donor vesicles	$t_{1/2}$, min
DPPC	185 ± 20 (3)
N-C16-SPM	1626 ± 48 (2)

Donor vesicles contained 6 mol % cholesterol, 15 mol % DCP, and 79 mol % DPPC or *N*-C16-SPM (total lipid concentration, 1.0 mM). Acceptor vesicles contained 6 mol % cholesterol and 94 mol % DPPC (total lipid concentration, 10.0 mM). Kinetic measurements were carried out in the presence of BSA (2 % w/v). The number in parentheses indicates the number of different vesicle preparations used. The exchangeable pool of [^{14}C]cholesterol was 98% in DPPC and 83% in *N*-C16-SPM donor vesicles.

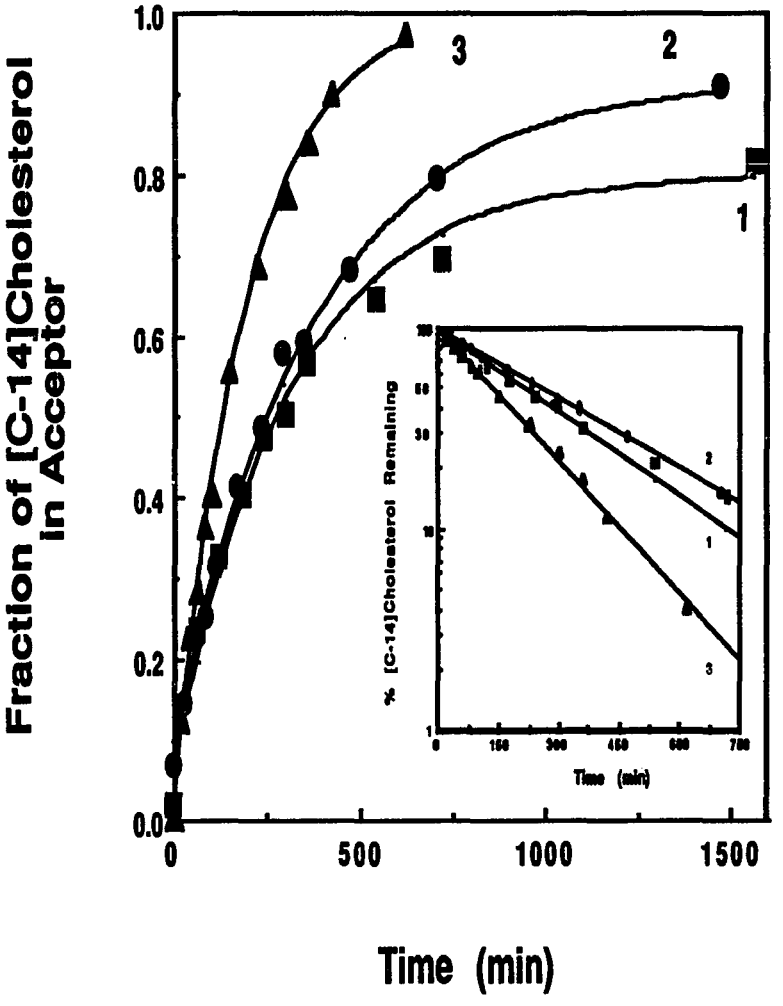


Figure 3-1: Time course of [^{14}C]cholesterol exchange between PC/cholesterol unilamellar vesicles at 50 °C in the presence of BSA (2 % w/v). The vesicles contained 6 mol % cholesterol. The host phospholipid in the acceptor vesicles was DPPC. Inset, first-order plot of the exchange data. The PCs in the donor vesicles are:

1, (■) compound **1**, 1-ether-2-amide PC;

2, (●) compound **2**, 1-thioether-2-amide PC;

3, (▲) compound **3**, 1-thioether-2-carbamoyl PC

half-times for cholesterol exchange from vesicles of these PCs (compounds **1-3**) are not very different from the half-time obtained using DPPC vesicles ($t_{1/2}$, 217.7, 270.5, and 175.6 min for compounds **1**, **2**, and **3**, respectively) (Table 2-2). The sizes of these donor vesicles are similar to each other (diameters of 163, 227, and 178 nm for vesicles from compounds **1**, **2** and **3**, respectively). The difference in rates of cholesterol exchange between vesicles from DPPC and from compounds **1-3** is not as significant as that between DPPC and *N*-C16-SPM vesicles, although the N-H group is present in *N*-C16-SPM and in compounds **1-3**. Since replacement of the glycerol oxygen atom at the *sn*-2 position of DPPC with a N-H group does not affect the rate of cholesterol exchange to a significant extent, the presence ofthe amide group in SPM does not appear to be primarily responsible for the very slow rate of exchange of cholesterol from the SPM vesicles.

To further estimate the importance of hydrogen-bonding groups in PC on the rate of cholesterol exchange, vesicles were prepared from thio-containing PCs. Figure 3-2 shows the exchange of [^{14}C]cholesterol from donor vesicles containing PCs with *sn*-2 *S*-linked chains. The exchange of [^{14}C]cholesterol reached equilibrium after about 6 h of incubation. Again, [^{14}C]cholesterol in the donor vesicles was completely exchangeable and only one kinetic pool was found in donor vesicles (inset, Figure 3-2). The half-times of cholesterol exchange from vesicles prepared with compounds **4** and **5** are very similar to each other ($t_{1/2}$, 115 and 112 min for vesicles from compounds **4** and **5**, respectively) (Table 3-3). The rates of cholesterol exchange from donor vesicles prepared from compounds **4** and **5** are faster than that from DPPC vesicles by a factor of only 1.6. The difference in rates is not due to a difference in donor vesicle size (diameters, 183 and 189 nm for vesicles from compounds **4** and **5**, respectively; diameter of DPPC

Table 3-2. Half-times for [^{14}C]Cholesterol Exchange from Vesicles Prepared from PCs with *sn*-2-Containing *N*-Linked Chains

Compound	X	Y	$t_{1/2}$, min
1	$OC_{16}H_{33}$	$NHCOC_{15}H_{31}$	218 ± 29 (2)
2	$SC_{16}H_{33}$	$NHCOC_{15}H_{31}$	271 ± 20 (2)
3	$SC_{16}H_{33}$	$NHC(O)OC_{14}H_{29}$	176 ± 22 (3)

Donor vesicles contained 6 mol % cholesterol, 15 mol % DCP, and 79 mol % PC analog (total lipid concentration, 1.0 mM). Acceptor vesicles contained 6 mol % cholesterol and 94 mol % DPPC (total lipid concentration, 10.0 mM). BSA (2% w/v) was present in the incubation media. The sizes of the exchangeable pool of [^{14}C]cholesterol in the donor vesicles prepared from compounds **1**, **2**, and **3** were 78%, 100%, and 100%, respectively. See p. 9 for the structures of compounds **1-3**.

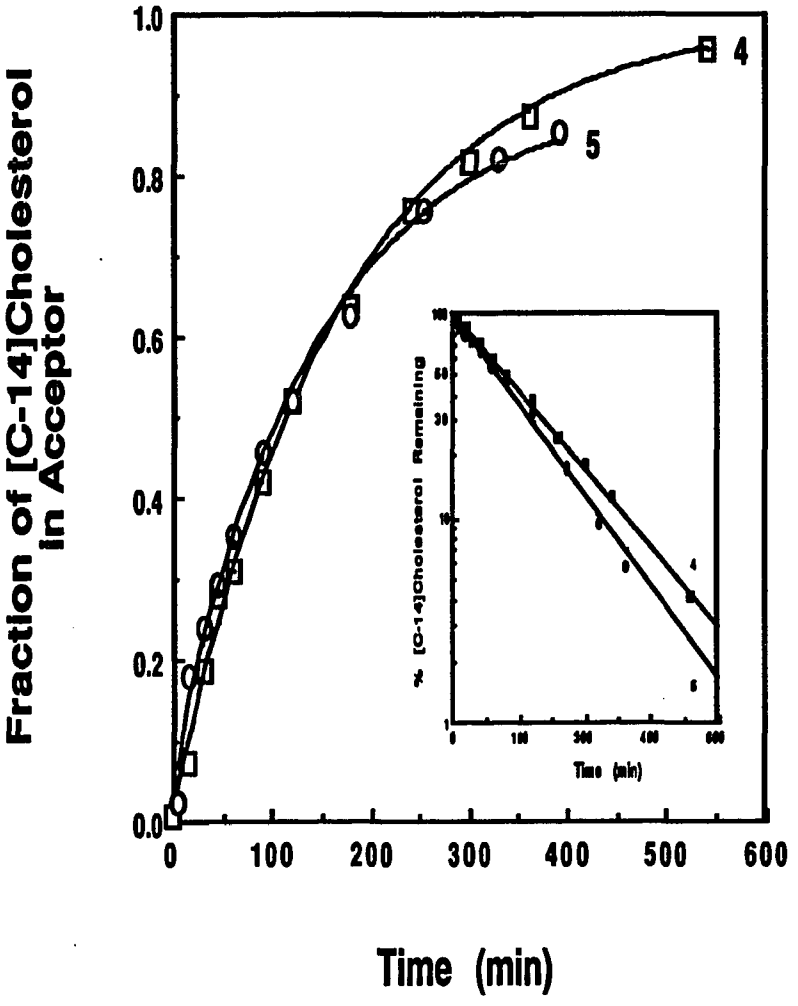


Figure 3-2: Time course of [^{14}C]cholesterol exchange at 50° C between PC/cholesterol unilamellar vesicles. BSA was present in the incubation media. The vesicles contained 6 mol % cholesterol. The host phospholipid in the acceptor vesicles was DPPC. Inset, first-order plot of the exchange data. The PCs in the donor vesicles are:

4, (▫) compound **4**, 1-ester-2-thioester PC;

5, (o) compound **5**, 1-ether-2-thioester PC

vesicles, 128 nm). The replacement of the oxygen atom at the *sn*-2 position of DPPC with sulfur results in only a slight increase in the rate of cholesterol exchange. In fact, a comparison of the rates of cholesterol exchange from vesicles prepared from compounds **1** and **5** (which have the largest difference in hydrogen-bonding capability of the five synthetic PCs we studied) indicates that only a 1.9-fold difference in rate exists.

In conclusion, the results suggest that hydrogen bonding between cholesterol and PC does not play an important role with respect to the spontaneous movement of cholesterol between vesicles. The data presented with PCs containing *N*- and *S*-linked chains indicate that the much slower rate of cholesterol exchange from vesicles containing SPM does not arise because the -NHCOR group of SPM causes its carbonyl oxygen to be more electron-rich than the corresponding carbonyl in acyl-PC or because the N-H group is capable of stronger hydrogen-bonding interactions than the glyceryl-linked oxygen of the acyl group of PC. Hence, interlipid hydrogen bonding between cholesterol and the N-H group of SPM does not appear to exert a large influence on the rate of cholesterol desorption from the SPM vesicle surface. Since synthetic PCs **1**-**5** gave vesicles that showed cholesterol exchange kinetics similar to that found in DPPC vesicles, it is suggested that these compounds can be incorporated into model membrane systems for studies of lipolytic enzyme behavior without significantly affecting membrane properties.

Table 3-3. Half-times for [^{14}C]Cholesterol Exchange from Vesicles Prepared from PCs with *sn*-2-Containing *S*-Linked Chains at 50 °C

Compound	X	Y	$t_{1/2}$, min
4	$OC(O)C_{15}H_{33}$	$SC(O)C_{15}H_{31}$	115 ± 20 (2)
5	$OC_{16}H_{33}$	$SC(O)C_{15}H_{31}$	112 ± 1 (2)

Donor vesicles contained 6 mol % cholesterol, 15 mol % DCP, and 79 mol % PC analog (total lipid concentration, 1.0 mM). Acceptor vesicles contained 6 mol % cholesterol and 94 mol % DPPC (total lipid concentration, 10.0 mM). BSA (2% w/v) was present in the incubation media. The sizes of the exchangeable pool of [^{14}C]cholesterol were 100% and 99% in donor vesicles containing compounds **4** and **5**, respectively. See p. 9 for the structures of compounds **4** and **5**.

Chapter 4. Movement of Synthetic Choleserol Analogs between Vesicles

The chemical structures of the [^{14}C]sterols used with modifications at the 3 position are shown in Fig. 3 (see p. 12). In order to examine the effects of changes in both the nucleus and polar group of cholesterol on the exchange rate, [^{14}C]cholestanol (which lacks the Δ^5 double bond) and a series of sterols with different groups at the 3 or 7 position were synthesized. Kinetic data for exchange of sterols differing in nuclear and polar group structure are shown in Fig. 4-1A and B, respectively. The rates of exchange of cholesterol and cholestanol between DPPC/sterol vesicles are very similar at 24 mol % sterol; the inset to Fig. 4-1 shows that about 90% of these ^{14}C-labeled sterols undergoes exchange in one kinetic pool. Fig. 4-1B shows that the 3α-substituted sterols, epicholesterol and 3α-triethoxycholesterol, undergo much faster exchange than does cholesterol ($t_{1/2}$, cholesterol, 445 min; cholestanol, 555 min; epicholesterol, 38 min; triethoxycholesterol, 39 min). The inset shows that these sterols undergo exchange as a single kinetic pool. The sizes of the exchangeable pool, calculated as described on p. 22, are cholesterol and cholestanol, 100%; epicholesterol, 97%; and triethoxycholesterol, 90%.

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, the kinetics of [^{14}C]cholesterol and [^{14}C]3-aminocholesterol exchange were compared. Fig. 4-2 shows that 3α- and 3β-aminocholesterol display biphasic kinetics in the ion-exchange assay, which requires the presence of a negatively charged nonexchangeable lipid (DCP) in the donor (or the acceptor) vesicles. In order to assure the presence of net negative charge in the donor vesicles and to avoid the fusion between donor and acceptor caused by charge, the sterol content was reduced to 6 mol % in donor

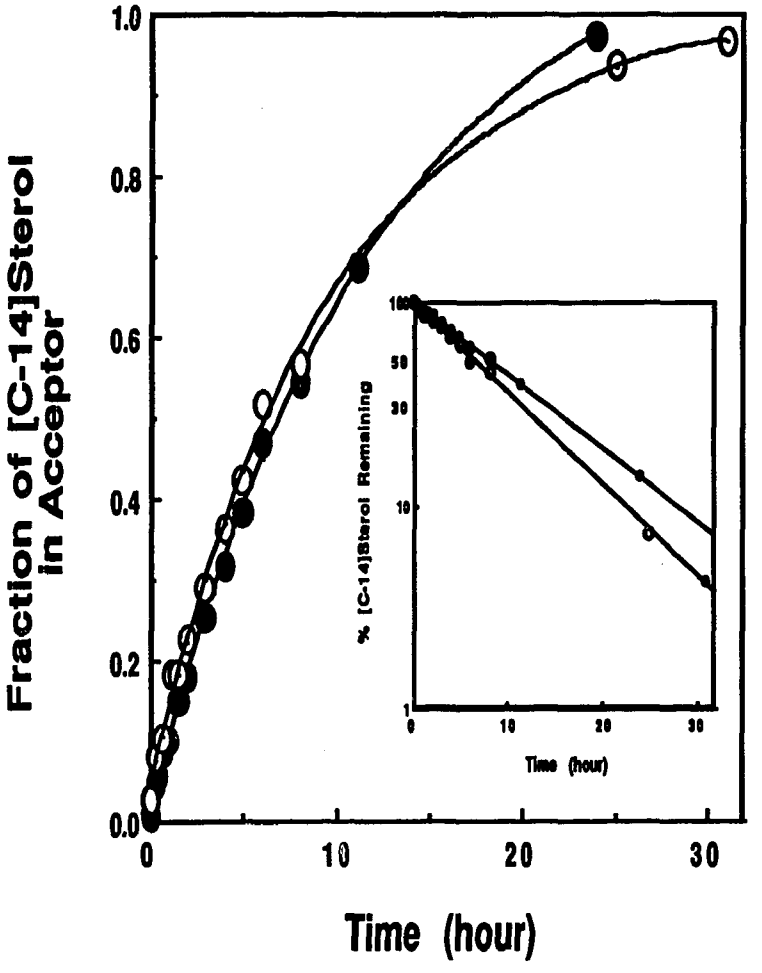


Figure 4-1A. Time course of [^{14}C]sterol exchange between DPPC/sterol unilamellar vesicles at 50 °C. The vesicles contained 24 mol % sterol. Inset, first-order plots of the exchange data. The sterols are cholesterol (o) and cholestanol (•). The incubation medium contained BSA (2 % w/v).

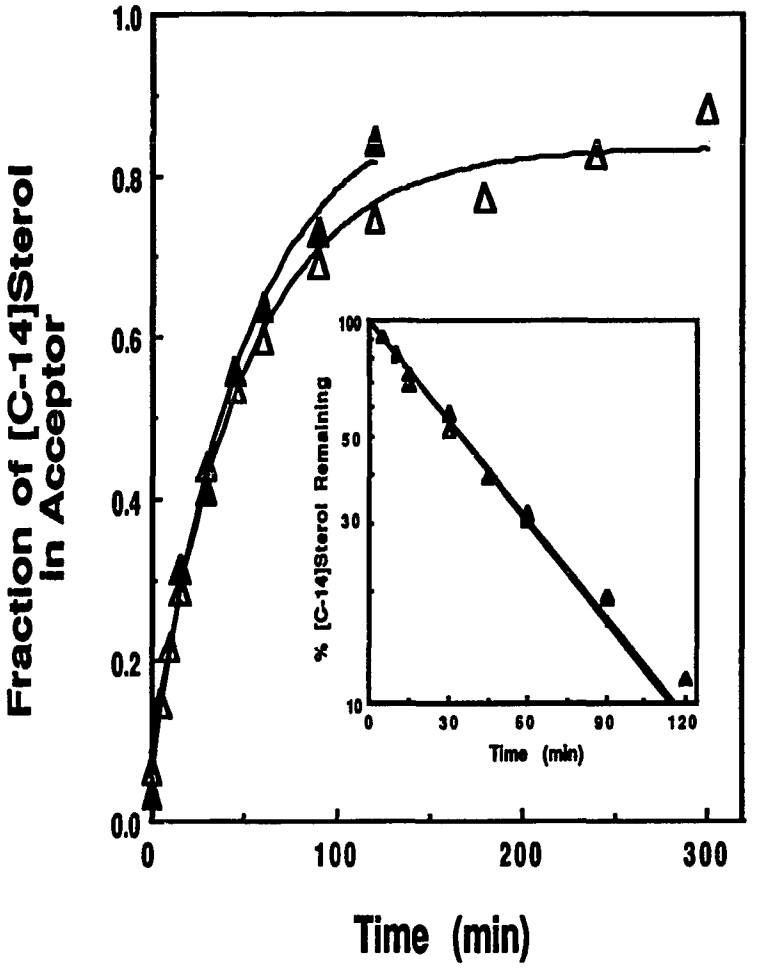


Figure 4-1B. Time course of [^{14}C]sterol exchange between DPPC/sterol unilamellar vesicles at 50 °C. The vesicles contained 24 mol % sterol. Inset, first-order plot of the exchange data. The sterols are epicholesterol (▲) and triethoxycholesterol (△). The incubation medium contained BSA (2 % w/v).

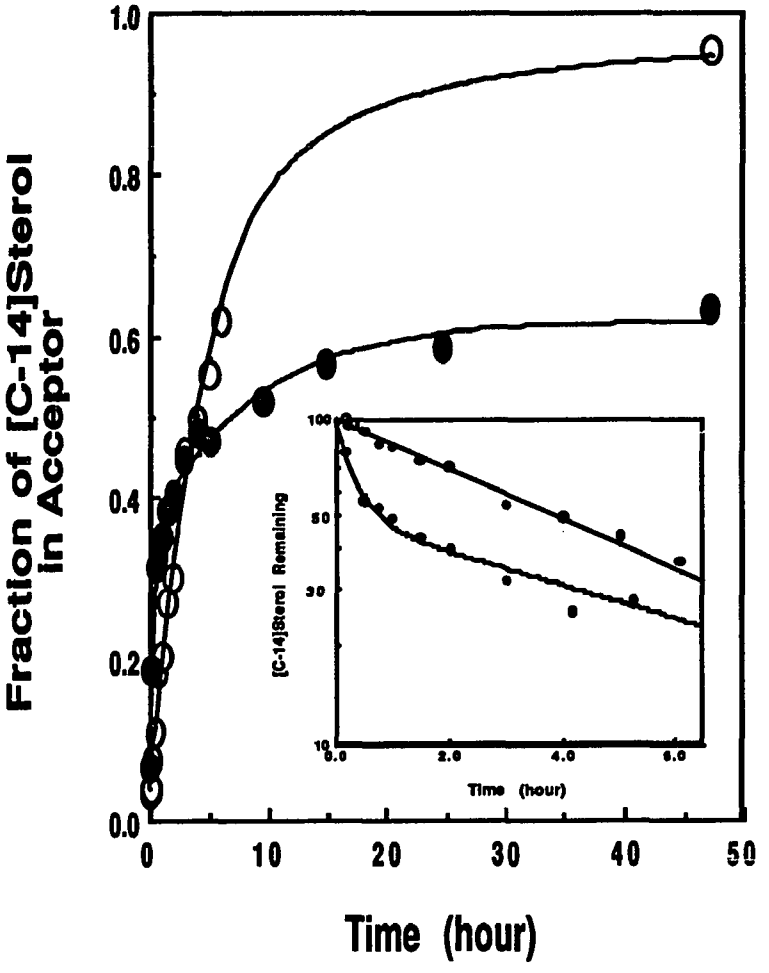


Figure 4-2A. Time course of [^{14}C]sterol transfer at 50° C DPPC/sterol donor vesicles to DPPC acceptor vesicles using the ion-exchange chromatography assay. The donor vesicles contained 6 mol % sterol. Inset, first-order plot of the transfer data. The sterols in the donor vesicles are cholesterol (o) and 3α-aminocholesterol (•).

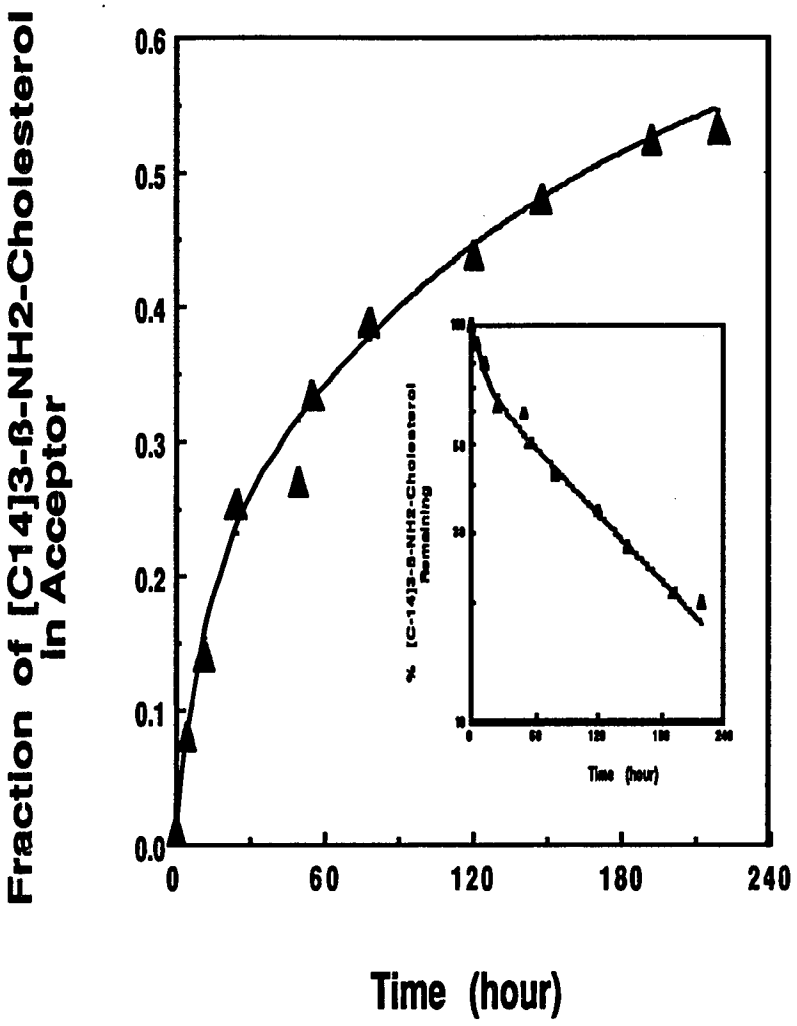


Figure 4-2B. Time course of [^{14}C]sterol transfer at 50° C DPPC/sterol donor vesicles to DPPC acceptor vesicles using the ion-exchange chromatography assay. The donor vesicles contained 6 mol % sterol. Inset, first-order plot of the transfer data. The sterol in the donor vesicles is 3β-aminocholesterol.

species and the acceptor contained no 3-aminocholesterol and cholesterol. The unidirectional transfer of cholesterol at 6 mol % displays a single kinetic phase ($t_{1/2}$, 256 min), which is different from that of 3α- and 3β-aminocholesterol (3α-aminocholesterol, $t_{1/2}$ values of 16 and 393 min; 3β-aminocholesterol, $t_{1/2}$ values of 558 and 7134 min for the fast and slow phases, respectively; see Figure 4-2). Since DCP present in donor vesicles may interact with 3-aminocholesterol and cause biphasic transfer kinetics, the lectin assay was used to study 3-aminocholesterol transfer.

The kinetics of [^{14}C]3β-aminocholesterol transfer from egg PC/sterol vesicles to egg PC acceptor vesicles was measured in an assay system that does not involve separation of donor and acceptor species based on charge. The assay system involves the incorporation of glycolipid into the donor vesicles; separation of donor from acceptor vesicles is achieved by agglutination after the addition of lectin. Fig. 4-3 shows that biphasic kinetics were still observed for 3β-aminocholesterol in this assay method, whereas cholesterol still underwent transfer as a single pool ($t_{1/2}$, 3β-aminocholesterol, 12 and 155 min for the fast and slow phases; cholesterol, 54 min; see Fig. 4-3A). The transfer of cholesteryl diazoacetate, the photoaffinity probe, also followed biphasic kinetics ($t_{1/2}$, 35.4 and 2468 min for the fast and slow phases; see Fig. 4-3B), indicating that the charge-bearing or the zwitterionic sterol molecules undergo movement with a rate-determining step different from that of cholesterol. Transbilayer migration of charged sterol molecules from the inner to the outer monolayer is expected to be much slower for 3-aminocholesterol and cholesteryl diazoacetate than for cholesterol. The flip-flop rate may become the rate-determing step in the desorption process of these sterol molecules from the lipid-water interface.

The last series of labeled sterols that were investigated differ from

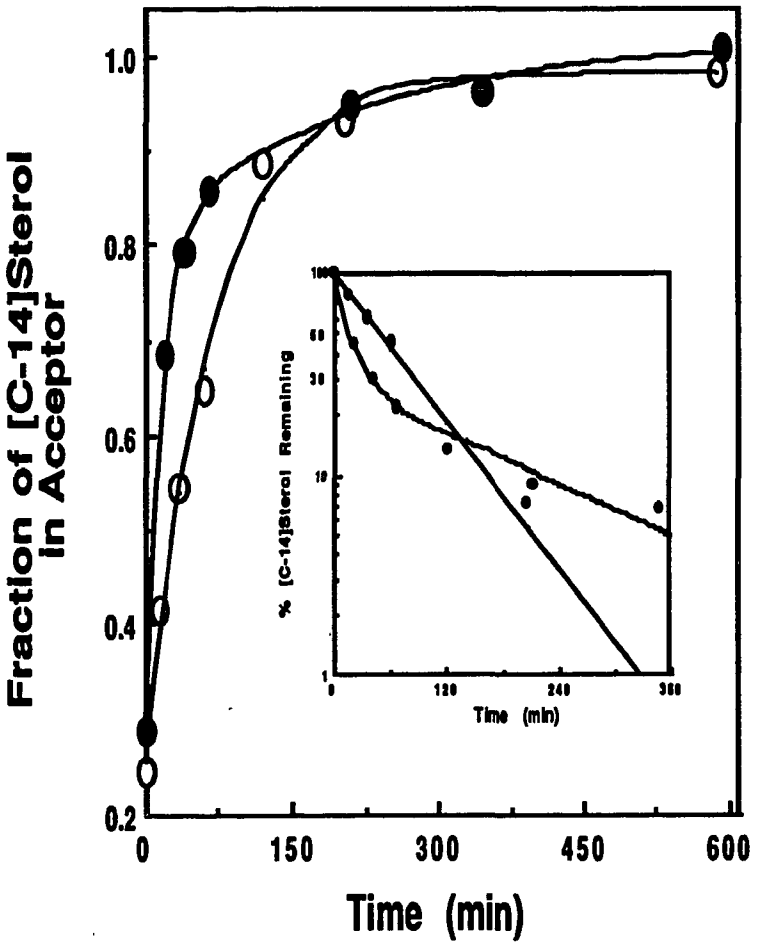


Figure 4-3A. Time course of [^{14}C]sterol transfer at 50° C from egg PC/sterol donor vesicles to egg PC acceptor vesicles using the lectin assay. The donor vesicles contained 24 mol % sterol, 56 mol % egg PC, and 20 mol % *N*-palmitoyldihydrolactocerebroside. Inset, first-order plot of the transfer data. The sterols in the donor vesicles are cholesterol (o) and 3β-aminocholesterol (•).

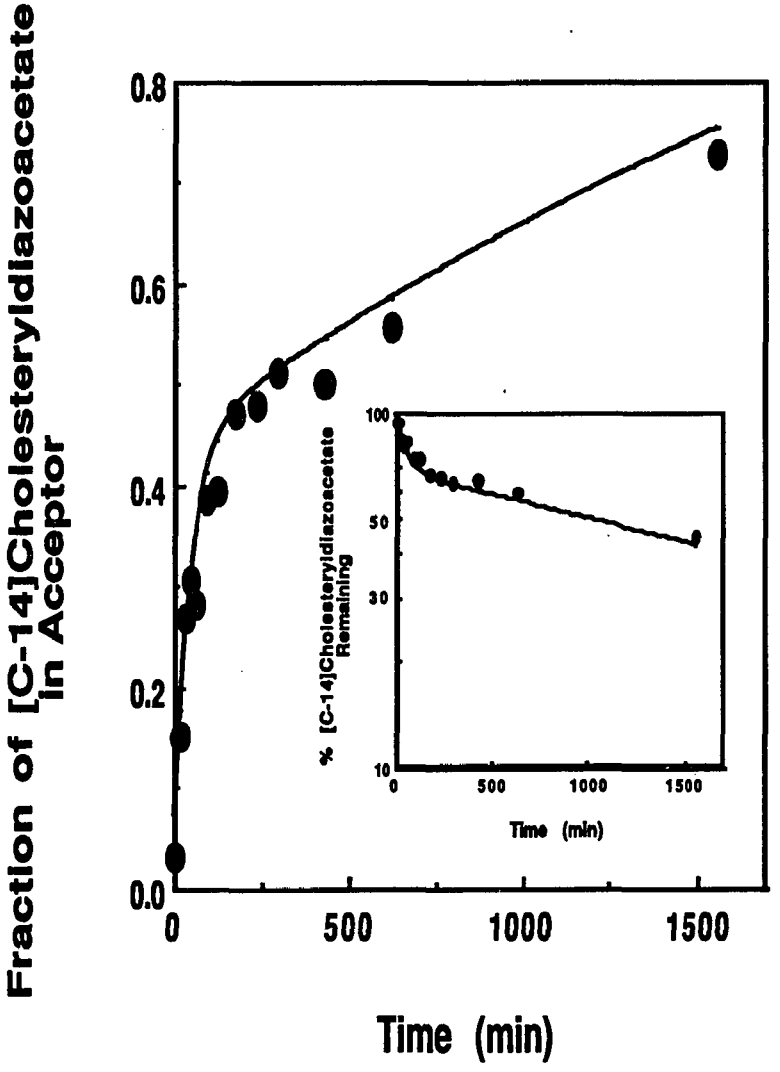


Figure 4-3B. Time course of [^{14}C]sterol transfer at 50° C from egg PC/sterol donor vesicles to egg PC acceptor vesicles using the lectin assay. The donor vesicles contained 24 mol % sterol, 56 mol % egg PC, and 20 mol % *N*-palmitoyldihydrolactocerebroside. Inset, first-order plot of the transfer data. The sterol in the donor vesicles is cholesteryl diazoacetate.

cholesterol 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 4-4 shows that the 7-oxysterols undergo exchange much more rapidly than cholesterol. The $t_{1/2}$ values of the data shown in Figure 4-4 are: 7-keto, 81 min; 7α-hydroxy, 16 min; 7β-hydroxy, 7 min; and cholesterol, 1825 min. The results are in agreement 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, the present finding of 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).

In previous studies of the kinetics of cholesterol transfer between membranes, the effects of alterations in phospholipid structure, 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; Bittman et al., 1985). Local domain structure, as influenced by membrane proteins and saturated phospholipid fatty acyl chains, affect the rate of cholesterol transfer from cell membranes (Clejan and Bittman, 1984c; Bellini et al., 1984). With regard to the influence of phospholipid structure on cholesterol uptake into bilayers, preferential interactions between cholesterol and phospholipids (as in the case of saturated glycerophospholipids and SPM) impede the rate of cholesterol release from the donor surface (Wattenberg and Silbert, 1983; Fugler et al., 1985; Yeagle and Young, 1986; Lund-Katz et al., 1988). In contrast to the

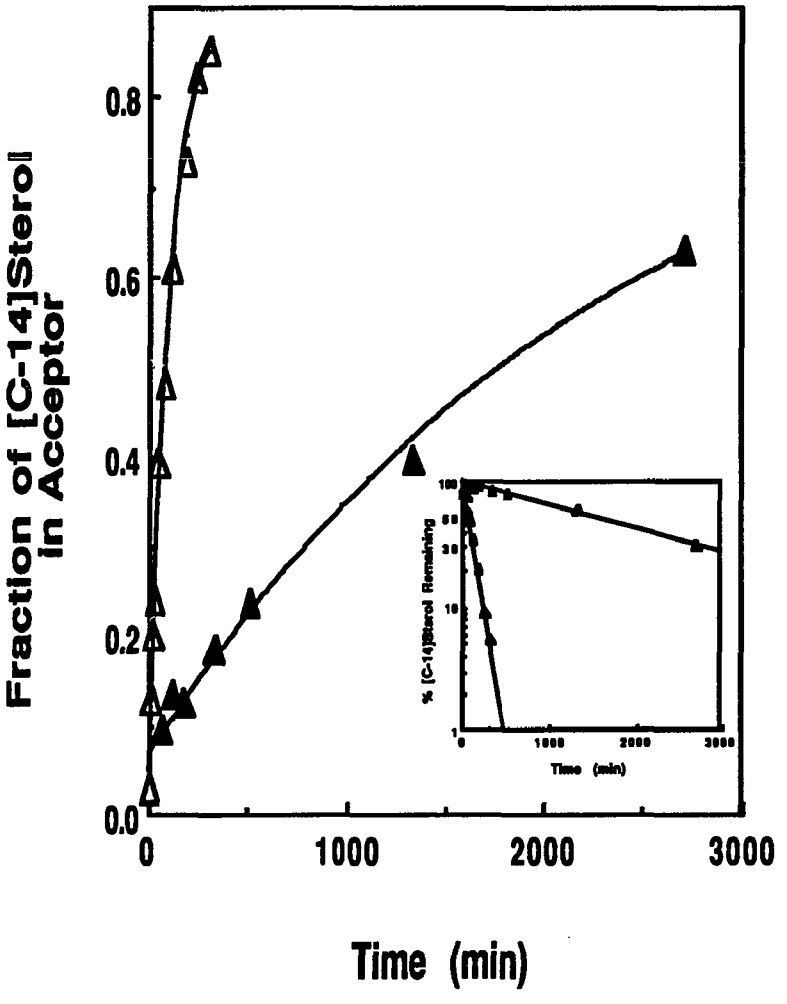


Fig. 4-4A. Time course of [^{14}C]cholesterol exchange at 37° C and [^{14}C]-7-ketocholesterol between DPPC/sterol unilamellar vesicles. The donor and acceptor vesicles contained 24 mol % cholesterol or 7-ketocholesterol. Inset, first-order plot of the exchange data. The sterols are cholesterol (△) and 7-ketocholesterol (▲).

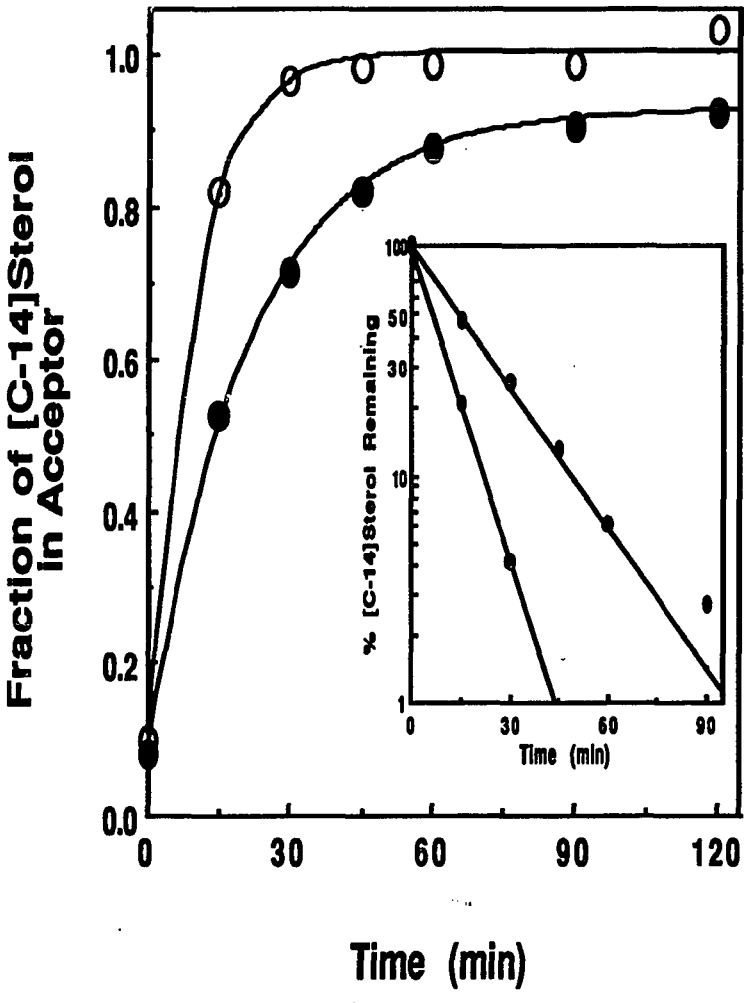


Figure 4-4B. Time course of [^{14}C]-7α- and 7β-hydroxycholesterol exchange at 37° C between DPPC/sterol unilamellar vesicles. The donor and acceptor vesicles contained 24 mol % 7α- or 7β-hydroxycholesterol. Inset, first-order plot of the exchange data. The sterols are: 7α-hydroxycholesterol (●) and 7β-hydroxycholesterol (o).

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. Previous studies 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 and Bittman, 1984a), and that [^{14}C]sitosterol undergoes slower exchange between phospholipid vesicles than does [^{14}C]cholesterol (Kan and Bittman, 1990). 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 and Sherry, 1984), and 7-ketocholesterol was taken up by erythrocytes faster than cholesterol (Wharton and Green, 1982). For further elucidation of the factors that are involved in the spontaneous distribution of cholesterol between membranes, a series of synthetic ^{14}C-labeled sterols that differ in either the nuclear or polar head group portion of cholesterol were used in kinetic studies of exchange or transfer between vesicles.

Aqueous monomeric sterol solubilities appear to play an important role in the rate of transfer between vesicles, since the cholesterol exchange rate is enhanced by chaotropic salts (Clejan and Bittman, 1984c), bile salts (Vlahcevic et al., 1990), and polar organic solvents (Bruckdorfer and Green, 1967; Quarfordt and Hilderman, 1970; Bruckdorfer and Sherry, 1984). However, the mechanism by which cholesterol undergoes intermembrane movement is not fully established. 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 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.

In this study it has been found 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 37 °C is ~ 25-fold lower than that of [^{14}C]cholesterol (Figs. 4-4A); [^{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 4-4B). The interaction of 7-ketocholesterol and 7α- and 7β-hydroxycholesterol with PC are weak relative to that of cholesterol, as estimated by the condensing effect in monolayers and permeability of liposomes (Bruckdorfer et al., 1968; Nakagawa et al., 1980; Demel et al., 1972; Theunissen et al., 1986). In addition, vibrational studies in bilayers indicated a looser packing of DPPC in the presence of 7-ketocholesterol and 7α-hydroxycholesterol (Rooney et al., 1986). The retention times of 7-keto- and 7α- and 7β-hydroxycholesterol on a reversed-phase HPLC column are similar, but the relative ease with which they desorb from DPPC/sterol vesicles differs significantly (Table 4-1). This observation suggests that the loose packing of these sterols with DPPC rather than their increased aqueous solubility is primarily responsible for the stimulation in the rate of exchange of [^{14}C]7-keto- and 7α- and 7β-hydroxycholesterol.

Table 4-1. Examination of a Relationship between the Retention Tmes of Sterols on a C18 Carbosphere HPLC Column and Their Half-times of Desorption from PC Vesicles

Sterol	k'^a	$t_{1/2}{}^b$	$t_{1/2}{}^c$	$t_{1/2}{}^d$	$t_{1/2}{}^e$
7-ketocholesterol[f]	2.27	81			
7α-hydroxycholesterol[f]	2.51	16			
7β-hydroxycholesterol[f]	2.46	7			
epicholesterol	4.51		35 ± 4		
triethoxycholesterol	7.68		33 ± 8		
3α-aminocholesterol	6.82			17 ± 1, 371 ± 32	
3β-aminocholesterol	7.42			474 ± 126, 6906 ± 324	8 ± 6, 138 ± 25
cholesteryl diazoacetate	8.12				35.4, 2467.9
cholesterol	9.24	1825	500 ± 78	253 ± 9	55 ± 1
sitosterol	10.80				
cholestanol	8.13[g]	600 ± 66			

[a] k' is a normalized HPLC retention value obtained by using the relationship $[(R_{t,sterol}/R_{t,solvent}) - 1]$. The retention times were measured by reverse-phase HPLC as described on p. 13.

[b] See captions in Figure 4-4.

[c] See captions in Figure 4-1.

[d] See captions in Figure 4-2.

[e] See captions in Figure 4-3.

[f] Donor vesicles were sonicated for 40 min.

[g] Value of k' was obtained by normalizing the k' value of Armstrong and Carey (1987) to the k' of cholesterol on k' column in this Table.

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-triethoxy-cholesterol between DPPC/sterol vesicles at 50 °C are about 15-fold lower than that of [^{14}C]cholesterol (Fig. 4-1B). It is well known that epicholesterol does not condense phospholipids to a similar extent to that of cholesterol (Bittman and Blau, 1972; Demel et al., 1972; Demel and 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 chain (Murari et al., 1986).

3-*O*-Alkylated sterols may alter the hydration capacity of lipids at lipid/water interface and may also impose steric constrains 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 DOPC bilayers to as large an extent as did cholesterol (Goodrich et al., 1988). The capacity of these sterols to inhibit the initial rate of [^{14}C]glucose efflux from DPPC vesicles at 50 °C was estimated. It was found that triethoxycholesterol and epicholesterol were much less effective in reducing the initial rate of glucose efflux compared with cholesterol (data not shown). The finding that retention times of epicholesterol and 3α-triethoxycholesterol differ significantly but that the half-times of exchange of these sterols are the same (Table 4-1) is another indication that the hydrophilicity of the sterol, as estimated by mobility on a C18-column, plays a less dominant role than does the interaction between the sterol and phospholipid in the bilayer of the host particle. In addition, the retention times of triethoxycholesterol and cholesterol are very similar, whereas their

half-times of exchange differ significantly.

Catalytic hydrogenation of the double bond in the sterol nucleus gave [^{14}C]cholestanol (dihydrocholesterol). Fig. 4-1A shows that the half-time of exchange is not affected significantly by removing the double bond from the sterol nucleus. In previous comparisons of the properties of these two sterols, cholestanol was found to be 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 and 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 and Silbert, 1986).

The data for exchange of [^{14}C]3α- and 3β-aminocholesterol are fitted to a two-exponential function, with a fast and slow phase (Figs. 4-2 and 4-3), whereas only one kinetic phase was found for exchange of all of the other ^{14}C-labeled sterols investigated. This is an interesting observation, since 3-aminocholesterol is the only sterol analog used here 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 DCP and consequently would be impeded with respect to its movement across the bilayer and desorption from the external surface, the assay system was changed to avoid the use of DCP. Fig. 4-3 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. The 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 (Figs. 4-2 and 4-3) suggests that transbilayer movement of these sterols 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 than does cholesterol, it was also found that cholesteryl diazoacetate (which is zwitterionic) also undergoes biphasic exchange kinetics (Fig. 4-3B).

In conclusion, It has been found that decreasing the relative hydrophobicity of the sterol molecule by insertion of polar groups enhances the rate of spontaneous sterol exchange. The rates of sterol movement are not correlated with their relative polarity, as estimated by reversed-phase HPLC. The results presented here suggest that decreasing the affinity of cholesterol for phospholipids in the donor membranes stimulates the rate of movement of cholesterol between membranes to a greater extent than increasing the sterol's aqueous solubility.

Chapter 5. Influence of Bilayer Cholesterol Content on Cholesterol Exchange Rate

Figure 5-1 shows the plot of mol % of cholesterol in DPPC and egg PC vesicles vs. half-time for [^{14}C]cholesterol exchange. In DPPC vesicles, the half-times increase from 1 to 24 mol % for cholesterol and then decrease with an increase of cholesterol content to 50 mol %; in egg PC vesicles, the half-times increase dramatically from 1 to 10 mol %, and increase less dramatically between 10 and 50 mol %. The relationship between half-times and cholesterol mol % in egg PC vesicles is in agreement with the data of McLean and Phillips (1982), in which exchange was measured at 37 °C. The difference in the relationship between half-time and cholesterol mol % in DPPC and egg PC vesicles was also observed in sitosterol exchange (using sitosterol purchased from Sigma, see Figure 5-2), although sitosterol exchanged at a higher rate at 24 mol % than at 1 and 6 mol % in egg PC vesicles. The kinetics of cholesterol exchange from cholesterol-rich domains or from "edges" between cholesterol-rich and pure phospholipid domains in vesicles may be sensitive to the size of the domains. It is possible that as the size of a sterol-rich domain increases in saturated PC (e. g., DPPC) vesicles, the rate of exchange decreases (see Figure 5-1 and 5-2 for cholesterol and sitosterol exchange from DPPC vesicles). The packing of lipid molecules in sterol-rich domain may also be sensitive to the size of the domain since the rate of sterol exchange is influenced by the lipid packing. It seems that when the size of the cholesterol-rich domain increases above a critical value , the rate of cholesterol exchange from this domain increases. Figure 5-3 shows that the rate of cholestanol exchange from DPPC vesicles decreases above 24 mol % cholestanol.

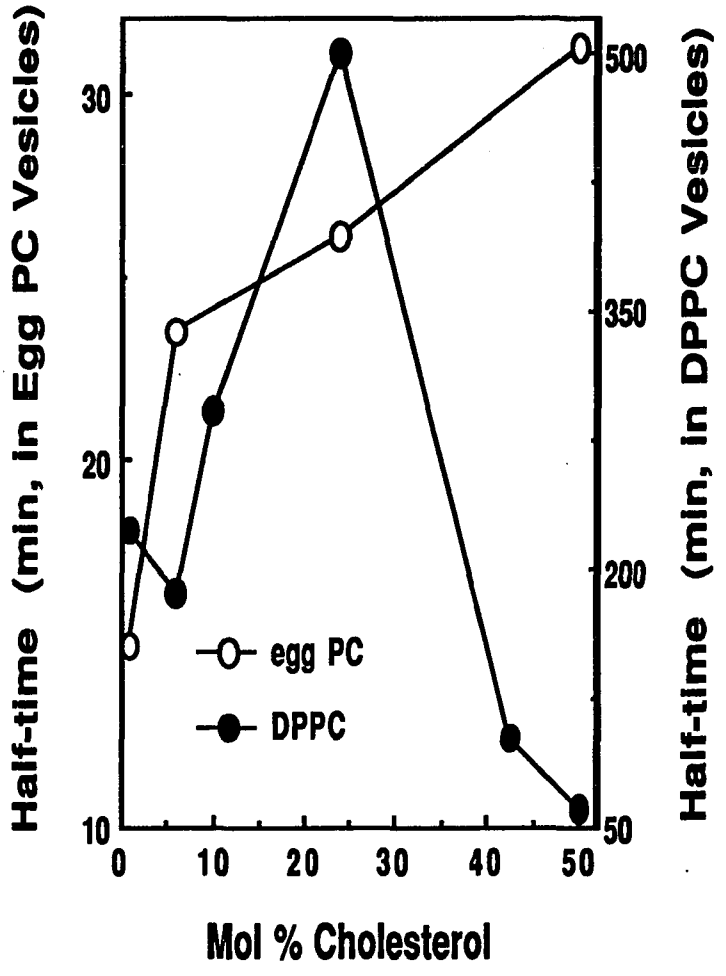


Figure 5-1. Plot of half-time for [^{14}C]cholesterol exchange vs. mol % of cholesterol in DPPC vesicles. Exchange underwent at 50 °C in the presence of albumin (2 % w/v). at least two preparations of vesicles were used for the measurement of kinetics.

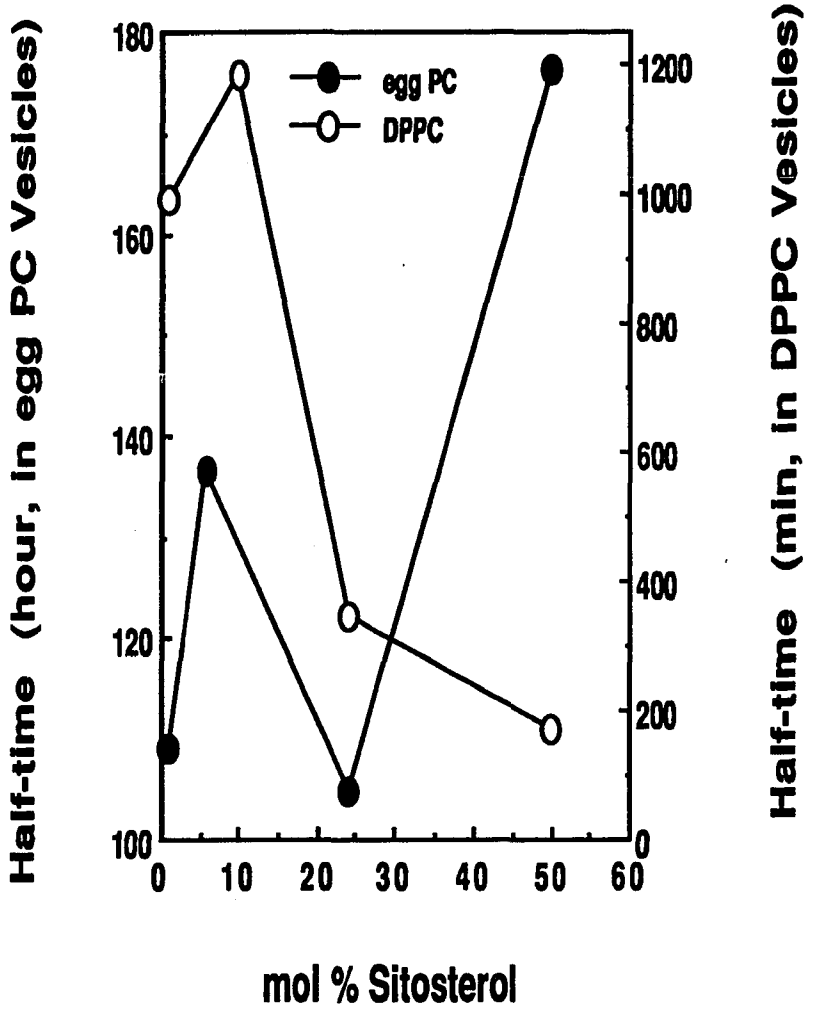


Figure 5-2. Plot of half-time for [^{14}C]sitosterol exchange vs. mol % of sitosterol in DPPC vesicles. Sitosterol was from Sigma and contained 45 % campesterol. Exchange underwent at 50 °C in the presence of albumin (2%, w/v). At least two different preparations of vesicles were used for the measurement.

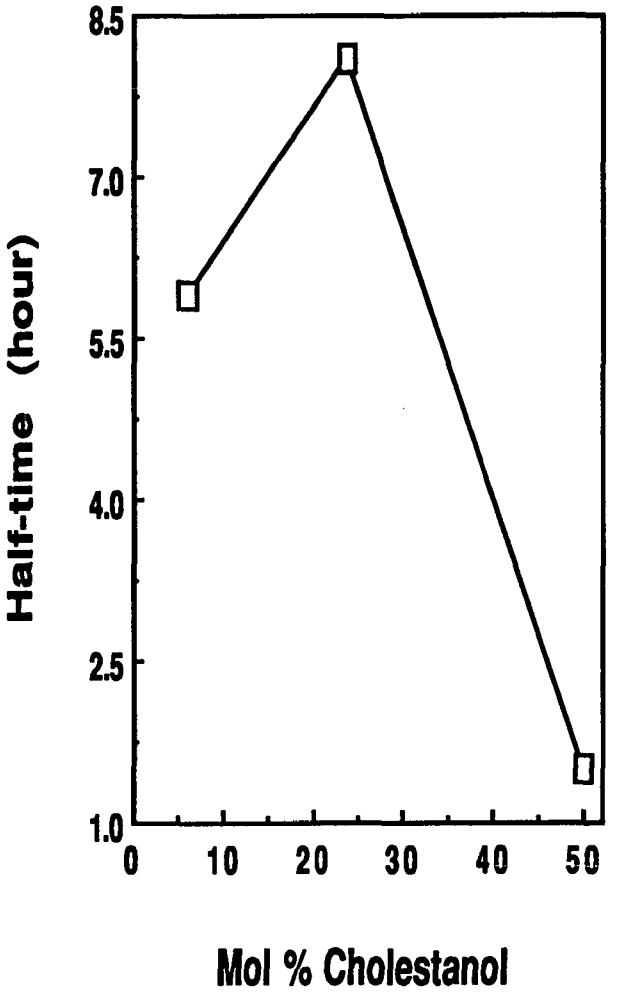


Figure 5-3. Plot of half-time for [^{14}C]cholestanol exchange vs. mol %. The kinetic measurements were taken at 50 °C in the presence of albumin (2 % w/v). At least two different preparations of vesicles were used.

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