# MINIREVIEW

# Membrane Cholesterol Dynamics: Cholesterol Domains and Kinetic Pools (43185)

FRIEDHELM SCHROEDER,*[,1] JOHN R. JEFFERSON,* ANN B. KIER,[†] JAMES KNITTEL,*
TERRENCE J. SCALLEN,[‡] W. GIBSON WOOD,[§] AND IVAN HAPALA*

*Division of Pharmacology and Medicinal Chemistry,* Department of Pharmacology and Cell Biophysics,[1] University of Cincinnati Medical Center, Cincinnati, Ohio 45267-0004; Department of Pathology and Laboratory Medicine,[†] and Department of Laboratory Animal Medicine,[†] University of Cincinnati Medical Center, Cincinnati, Ohio 45267-0529; Department of Biochemistry,[‡] University of New Mexico School of Medicine, Albuquerque, New Mexico 87131; and Geriatric Research and Education Center, Veterans Administration Medical Center and Department of Pharmacology[§], University of Minnesota School of Medicine, Minneapolis, Minnesota 55417*

*Abstract.* **Nonreceptor mediated cholesterol uptake and reverse cholesterol transport in cells occur through cellular membranes. Thus, elucidation of cholesterol dynamics in membranes is essential to understanding cellular cholesterol accumulation and loss. To this end, it has become increasingly evident that cholesterol is not randomly distributed in either model or biologic membranes. Instead, membrane cholesterol appears to be organized into structural and kinetic domains or pools. Cholesterol-rich and poor domains can even be observed histochemically and physically isolated from epithelial cell surface membranes. The physiologic importance of these domains is 2-fold: (i) Select membrane proteins (receptors, transporters, etc.) are localized in either cholesterol-rich or cholesterol-poor domains. Consequently, the structure and properties of the domains rather than of the bulk lipid may selectively affect the function of proteins residing therein. (ii) Kinetic evidence suggests that cholesterol transport through and between membranes may occur through specific domains or pools. Regulation of the size and properties of such domains may be controlling factors of cholesterol transport or accumulation in cells. Recent technologic advances in the use of fluorescent sterols have allowed examination of cholesterol domain structure in model and biologic membranes. These techniques have been applied to examine the role of high-density lipoprotein, cholesterol lowering drugs, and intracellular lipid transfer proteins in membrane sterol domain structure and sterol movement between membranes.**

[P.S.E.B.M. 1991, Vol 196]

The great success of the Singer and Nicolson (1) model of membrane structure is the acceptance of the idea that membrane proteins are embedded in or attached to a lipid bilayer. The major limitation of this concept is that the lipids, unlike the proteins, are thought of in the bulk sense: namely, lipids are randomly distributed throughout the membrane bilayer without discrete domain organization of their own. In contrast, increasing evidence that discrete lipid domains co-exist in biologic membranes (2–12) at physiologic temperatures requires further elaboration of the fluid mosaic hypothesis. This review focuses on the domain structure of cholesterol in membranes and factors that regulate cholesterol domains. At least three classes of cholesterol domains in the cell are recognized (Table I).

First, the cholesterol content of subcellular membranes differs by an order of magnitude with plasma membranes > endoplasmic reticulum > mitochondria (reviewed in Ref. 11). Vesicular transport (13–16), intracellular lipid transfer proteins (17–26), or membrane-bound sterol transfer protein (27) may account

[1] To whom requests for reprints should be addressed at Division of Pharmacology and Medicinal Chemistry, Department of Pharmacology and Cell Biophysics, University of Cincinnati Medical Center, Cincinnati, OH 45267-0004.

0037-9727/91/1963-0235/$3.00/0

for this nonequilibrium intracellular cholesterol distribution. Several lines of evidence are consistent with a physiologic role of sterol carrier proteins in regulating the intermembrane sterol distribution. For example, extensive studies with cultured tumor cells indicate that in highly metastatic cells the level of a cytosolic sterol transfer protein is lower than in less malignant cultured primary tumor cells (26, 28–35). It is important to note that this tumor cell sterol carrier protein is different from that of nontransformed tissues such as liver (36) and does not cross-react with antibody against recombinant sterol carrier protein from liver (L-FABP) or intestine (I-FABP) (37, 38). Plasma membranes isolated from the cultured highly metastatic cells have a lower sterol to phospholipid ratio than those of the cultured primary tumor cells, whereas microsomes and mitochondria do not differ significantly in sterol to phospholipid ratio (28–31, 33–35). Thus, a high level of tumor cell sterol carrier protein correlated with the high plasma membrane sterol to phospholipid ratio and low metastatic ability of the tumor cell. In contrast, recently a cultured tumor cell line, L cells, was transfected with the cDNA encoding for the liver sterol carrier protein (L-FABP) and both high- and low-expression clones were isolated (37). The plasma membrane of the high-expression clones had a lower sterol to phospholipid ratio and an enhanced rate of sterol transfer from plasma membrane to endoplasmic reticulum (38). In short, the different sterol carrier proteins may function in regulating intracellular sterol distribution depending on whether the tissue source of the protein is normal or transformed. The potential role of intracellular sterol carrier proteins is particularly intriguing since these proteins may also be involved in targeting of sterol to specific membranes. An intestinal sterol carrier protein has been reported to be bound to membranes of the intestinal microvillus and is thought to accelerate sterol transfer into the intestinal enterocyte from the intestinal lumen (27).

Second, the transbilayer cholesterol distribution within the cell surface membrane is asymmetric. Cholesterol appears to be enriched in the inner leaflet of most mammalian cell surface membranes examined (reviewed in Refs. 4, 5, 29, and 39–44). The asymmetric transbilayer distribution of cholesterol in membranes is highly sensitive to incorporation of polyunsaturated fatty acids (41–43), to the presence of oxidized sterols (29, 41–43), to exposure to chronic ethanol (44), and to the expression of cytosolic sterol carrier proteins (L-FABP) (38). Although the transbilayer distribution of sterol in membranes and the regulation thereof have been reviewed in detail earlier (4), one aspect of transbilayer sterol domains, namely, the transbilayer migration rate of sterol, is also important to understanding the lateral distribution of sterols in membranes. Kinetic exchange assays of cholesterol (or fluorescent dehydroergosterol) indicating the existence of multiple sterol domains in biologic or model membranes may not discriminate the membrane leaflet origin of the sterol. For example, sterol exists in both lateral and transbilayer domains. Thus, interpretation of the physical location of sterol domains in membranes is highly dependent on the rate of exchange as compared with the rate of sterol transbilayer migration. If the latter rate is fast, then exchange assays reflect primarily lateral sterol domains. Although some reported values for sterol transbilayer migration time in biologic membranes range up to 18 days, depending on the assay used and membrane examined (reviewed in Ref. 4), more recent assays using fluorescence (4, 40, 44, 45) and cholesterol oxidase (4, 46) are consistent with a transbilayer migration time of a few minutes ($t_{1/2}$ between 1 and 6 min). Since exchange assays of cholesterol between biologic membranes indicated exchange times between membranes on the order of hours (47, 48), it is therefore unlikely that the transbilayer migration rate of sterol in biologic membranes will be a limiting factor confounding examination of sterol lateral domains. Whether transbilayer migration of sterol is fast or slow in model membranes is far from clear (see Ref. 4 for a review). Exchange methods show half-times of transbilayer migration of days (49, 50), whereas sterophenol, chemical labeling, and cholesterol oxidase methods show half-times of transbilayer migration of minutes (51–54). Highly significant is the observation that under nonequilibrium conditions the rate of transbilayer sterol migration measured by the exchange method was much faster than under equilibrium conditions (50). Thus, the transbilayer migration of sterol in model membranes is also likely to be fast. The method and conditions of measurement can dramatically alter determination of the transbilayer migration rate. Under conditions of net cholesterol flux into or out of the cell, the exchange conditions would be expected to be nonequilibrium and the transbilayer migration rate of sterol would be expected to be fast. Last, whether kinetic exchange data reflect lateral versus transbilayer sterol domains can be resolved by examination of transbilayer structural as well as kinetic domain data as follows: At sterol to phospholipid molar ratios in excess of 0.45, approximately 50–70% of total

**Table I.** Asymmetric Distribution of Cholesterol in Cells

| Type |
|---|
| Intracellular |
| Plasma membrane > microsomes > mitochondria |
| Intramembrane-transbilayer |
| Intramembrane-lateral |
| Macroscopic domains |
| Microscopic domains |

sterol appears to be localized in the outer leaflet of model membranes. If transbilayer migration is slow, then the inner leaflet sterol (about 30–50% of total) should be essentially nonexchangeable. Indeed, some data on nonexchangeable pools using [$^3$H]cholesterol (55–57) or dehydroergosterol (55, 58, 59) indicate the presence of a nonexchangeable pool of this size. Although this correlative explanation is appealing, it is not correct. Reversal of the transbilayer sterol asymmetry in model membranes did not alter the size of the nonexchangeable domain (4). In addition, lifetime and acrylamide quenching data as well as [$^3$H]cholesterol exchange data indicate that the nonexchangeable pool is a laterally segregated sterol domain rather than a transbilayer sterol domain (55–57, 59, 60).

Third, cholesterol is segregated in cholesterol-rich and poor domains in the lateral plane of the membrane (10, 41). This review is primarily focused on presentation of evidence consistent with the existence of cholesterol domains in the lateral plane of model and biologic membranes. Although the exact relationship between kinetic and structural sterol domains is not known, some correlations between the structural and kinetic sterol domains have been proposed (4, 55, 58). Sterol in the rapidly exchanging domain may be the same as a small sterol pool that, in the case of fluorescent sterol, has a longer lifetime and is more accessible to acrylamide quenching agents as compared with sterol in the slowly exchangeable or sterol-rich domain. It is important to note that certain proteins are localized in sterol-rich membrane domains (acetylcholine receptor) (61, 62), in sterol-poor domains (63–70), and/or require sterol for activity (71–73). The possibility that the cholesterol domain distribution and the characteristics of these domains may affect cellular functions must be considered. In addition, the role of representative factors (cholesterol-lowering agents, serum lipoproteins, and intracellular lipid transfer proteins) in regulation of structural sterol domains and intermembrane sterol transport remains to be explored.

### Macroscopic Cholesterol Domains in Cell Membranes

The best characterized large-scale membrane lipid domains have been reported for epithelial cells of liver (74–77), intestine (78–80), and kidney (81). These cells have sinusoidal, basolateral, and brush border or canalicular domains that can be separated by density gradient centrifugation. Each membrane domain is characterized not only by distinct enzymatic and functional activities but also by substantial differences in lipid composition. In these cell types, the microvillar or canalicular membranes represent cholesterol-rich domains, whereas the basolateral or serosal membranes are cholesterol-poor domains. Although these membrane domains are contiguous in each type of epithelial cell, the mechanism preventing lateral diffusion and intermixing of components is not understood. It has been postulated that tight junctions may prevent the lateral diffusion of lipids between epithelial cell domains (82, 83). Significantly, when determined by filipin freeze-fracture histochemical determination, cholesterol appears less abundant in or absent from hepatocyte intercellular junctions (gap, tight, and desmosome) (84, 85), kidney podocyte foot processes (86), certain areas of smooth muscle cells (87), and intestinal villus basolateral membranes (88). It should be noted that although the filipin histochemistry results are in general agreement with lipid compositional data, negative findings with filipin (89) must be viewed with caution (90). For example, the protein coat of coated endocytic vesicles appears to prevent interaction with filipin. Likewise, the charge and other properties of the membrane surface also can restrict filipin interaction with cholesterol (5, 7, 90). Membranes of shed vesicles from cells (91, 92) represent macroscopic cholesterol-rich domains, whereas other shed vesicles from cells (31) or retinal rod outer segment (93–95) represent cholesterol-poor domains.

### Microscopic Lateral Cholesterol Domains in Cell Membranes

Initially, investigation of cholesterol domains has encountered difficulty due to the lack of suitable reporter groups in the cholesterol molecule. Recently, these studies have been significantly enhanced through use of fluorescent sterols such as dehydroergosterol. Justification for the use of dehydroergosterol and the evidence for microscopic cholesterol domains in biomembranes are summarized in the following sections.

**Suitability of Dehydroergosterol as a Fluorescent Sterol Analog.** Since the major point of this review is that all cholesterol molecules are not alike, i.e., they exist in discrete domains in membrane, it is absolutely essential that probe molecules used to determine sterol domains accurately represent the properties of cholesterol. The disadvantages of nuclear magnetic resonance or spin-labeled probes and cholesterol accessibility to cholesterol oxidase have been reviewed elsewhere (4, 5, 96). Although the fluorescent sterols dehydroergosterol and cholestatrienol serve as excellent analogs of cholesterol, it must be pointed out that not all fluorescent sterol probes are useful cholesterol analogs. For example, pyrene-labeled sterols exchange 4-fold faster than radiolabeled cholesterol under identical conditions (97). In contrast, the fluorescent sterols dehydroergosterol and cholestatrienol do not contain bulky side groups as reporter molecules (Fig. 1). In model membranes, dehydroergosterol and cholestatrienol behave nearly identically as cholesterol as determined by differential scanning calorimetry (40, 98, 99), surface pressure (100), and fluorescence (98, 100–102) assays. Most important, dehydroergosterol and [$^3$H]cholesterol demonstrate

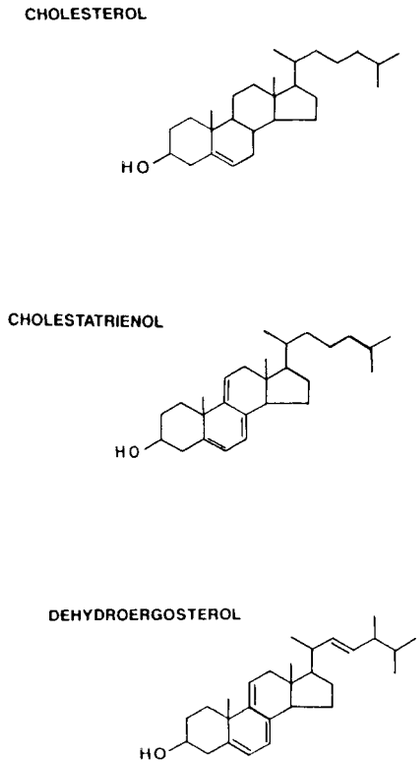


**Figure 1.** Structures of cholesterol and fluorescent sterols.

nearly identical kinetics (half-times, number of domains, and size of domains) in two different sterol exchange assays (55, 58). In addition, dehydroergosterol is a natural product (103, 104) comprising up to 20% of some yeast membrane sterols (105). This is very important to biologic membranes. Unlike many other spin-labeled or fluorescent sterol probe molecules, dehydroergosterol is nonperturbing in biologic membranes (4, 5, 7, 26, 41). Dehydroergosterol is nontoxic to cells and has been incorporated into cultured fibroblast plasma membranes, microsomes, and mitochondria (24, 29, 39–43), brain synaptosomal plasma membranes (44, 106), red blood cell membranes (2, 39, 45, 107), and microorganism plasma membranes (108, 109). This fluorescent sterol did not alter the growth properties of cultured fibroblasts [even when 70–85% of endogenous sterol was replaced by dehydroergosterol (39, 40)], *Tetrahymena pyriformis* (108), and *Mycoplasma mycoides* (109). Dehydroergosterol replaced more than 85% of the native sterol of cultured LM fibroblasts without effect on activities of membrane enzymes, phospholipid composition, phospholipid fatty acid composition, or sterol to phospholipid ratio in isolated plasma membrane, microsomal, and mitochondrial membrane fractions (40). Dehydroergosterol and cholesterol incorporated similarly into synaptosomal plasma membranes and erythrocytes (44, 45), serum lipoproteins [very low-density lipoprotein (110–112), low-density lipoprotein (113), high-density lipoprotein (107, 113)], and in binding to intracellular lipid transfer proteins [L-FABP (24–26), sterol carrier protein-2 (SCP-2) (17, 18)]. In summary, dehydroergosterol appears to be an excellent nonperturbing probe of cholesterol behavior in model membranes, biologic membranes, and lipoproteins.

**Structural Studies of Static Lateral Cholesterol Domains.** Structural analysis by electron microscopic, cholesterol oxidase, and electron spin resonance techniques reveal the existence of cholesterol-rich and cholesterol-poor domains in red blood cell membranes (63, 64, 90, 114), platelet membranes (65), and human immunodeficiency virus (66, 67). These studies have been substantially enhanced through use of the fluorescent sterol dehydroergosterol to examine structural and kinetic sterol domains in model membranes and in cultured fibroblast plasma membranes. Lifetime analysis (nonlinear least squares or Lorentzian distributional) of dehydroergosterol in ethanol (below the critical micelle concentration) revealed the presence of only a single lifetime near 0.4 nsec with distributional width of 0.05 nsec (55). In contrast, lifetime analysis of dehydroergosterol in LM fibroblast plasma membranes revealed the presence of at least two dehydroergosterol structural domains (26) with the following properties. First, in the plasma membranes dehydroergosterol displays two fluorescence lifetime components of nearly equal intensity near 0.8 and 3.3 nsec (Fig. 2). These two components are revealed by both nonlinear least squares and Lorentzian distributional lifetime analyses (Table II). Second, the shorter lifetime component near 0.8 nsec has a wider distributional width than the longer lifetime component, indicating a more heterogeneous environment. Third, the fractional intensities of the two lifetime components, indicative of the relative size of the two domains, are dependent on the cell type (Table II). The fractional intensity of the short lifetime component was significantly larger in the L-929 cell line, the parent cell line of the LM cells, perhaps indicating a difference in size of the sterol-rich and poor

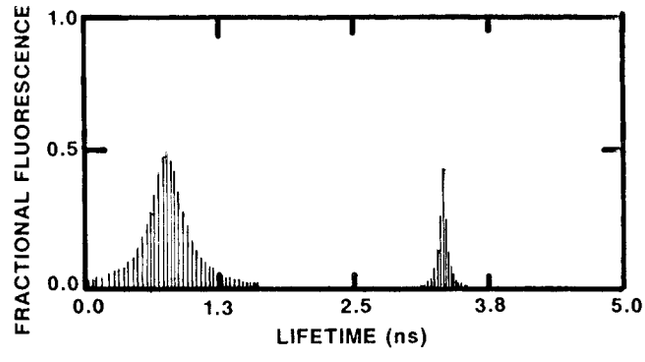


**Figure 2.** Lorentzian distributional lifetime analysis of dehydroergosterol in cultured fibroblast plasma membranes. All conditions were as described in footnote *a* to Table II.

**Table II.** Fluorescence Lifetime Analysis of Dehydroergosterol in Fibroblast Plasma Membranes[a]

| Cell line | Nonlinear least squares analysis[b] | | | | Lorentzian distributional analysis[b] | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | $\tau_1$ (nsec) | $f_1$ | $\tau_2$ (nsec) | $f_2$ | $C_1$ (nsec) | $W_1$ (nsec) | $f_1$ | $C_2$ (nsec) | $W_2$ (nsec) | $f_2$ |
| LM | 1.00 ± 0.18 | 0.53 ± 0.08 | 3.78 ± 0.61 | 0.47 ± 0.08 | 0.76 ± 0.02 | 0.38 ± 0.04 | 0.51 ± 0.02 | 3.34 ± 0.01 | 0.05 ± 0.01 | 0.49 ± 0.02 |
| L-929 | 1.29 ± 0.09 | 0.83 ± 0.01 | 10.20 ± 2.40 | 0.17 ± 0.01 | 0.86 | 0.36 | 0.78 | 7.45 | 0.05 | 0.22 |

[a] Cells were cultured with dehydroergosterol and plasma membranes were isolated as described earlier (29, 39–43). Dehydroergosterol comprised 21% of total plasma membrane sterol. Lifetime data were acquired at 24°C by multifrequency phase and modulation techniques (10–14 frequencies) and analyzed by nonlinear least squares (exponential) and Lorentzian (continuous) distributional analysis. Fits to two components had significantly better $\chi^2$ values (2–5 vs 30–50).

[b] In the nonlinear least squares analyses $\tau$, and $f_2$ refer to lifetime and fractional contribution of component one. Similar notations are used for component two. In the Lorentzian distributional analysis $C_1$, $W_1$, and $f_1$ refer to the center of lifetime distribution, width of lifetime distribution at peak half-height, and fractional contribution of component one, respectively. Similar notations are used for component two.

domains. In addition, the second lifetime component $\tau_2$ was more than 2-fold longer in L-929 cells (Table II). Elsewhere using model membrane systems, our laboratory also demonstrated the existence of two lifetime components for dehydroergosterol, the longer of which appeared to be more readily quenched by acrylamide (55). This observation was consistent with a dehydroergosterol population (corresponding to $\tau_2$) as being more exposed to the aqueous environment than the rest of the sterol with $\tau_1$. Extension of these observations with model membranes to plasma membranes implies that in the L-929 plasma membrane, a pool of sterol represented by $\tau_2$ also was more exposed to the aqueous environment but comprised a smaller percentage of the total plasma membrane sterol than in LM cells. Interestingly, fluorescence polarization and limiting anisotropy of diphenylhexatriene in L cell plasma membranes are much lower than in LM cell plasma membranes (35, 43). The higher fluidity of the L cell plasma membranes may allow greater penetration of aqueous solvent into the plasma membrane bilayer region in which the fluorescent sterols represented by $\tau_2$ are localized. In summary, the analysis of fluorescent sterol lifetime(s) in fibroblast plasma membranes has detected multiple sterol domains, and the properties of these domains are highly dependent on cell membrane source.

**Lateral Distribution of Proteins in Lateral Cholesterol Domains.** The presence of cholesterol domains in biologic membranes is indicated indirectly by the analysis of protein-lipid associations. Membrane proteins are localized in cholesterol-rich domains (64, 65), cholesterol-poor domains (66, 67), or even specifically associated with cholesterol (reviewed in Ref. 67). Cholesterol is specifically required for sodium and chloride coupled γ-aminobutyric acid transport (71), L-glutamate transport (71), $Na^+$,$K^+$-ATPase (72), $Na^+$,$Ca^{2+}$ exchanger (72), and ion channels (73). These effects appear to be due not only to changes in bilayer permeability to ions associated with cholesterol but also to direct effects of cholesterol. Some data suggest that cholesterol modulates the cooperativity of allosteric enzymes such as $Na^+$,$K^+$-ATPase (115) and that there may be an optimum level of membrane cholesterol for the function of the $Na^+$,$K^+$-ATPase (116). Cholesterol influences other membrane proteins including acetylcholine receptor (64, 65), insulin-coupled glucose and amino acid transport (117), band 4.5 glucose transport protein (118), band 3 protein (119), and erythrocyte surface antigen exposure (120, 121). It has been postulated that the effects of cholesterol are not direct but rather are because phase separation of cholesterol promotes aggregation of transporter (71) or membrane protein (118), where the active species is the aggregated transporter or protein.

**Kinetic Investigations of Dynamic Lateral Cho-**

**lesterol Domains.** Other evidence for the existence of sterol domains comes from kinetics of sterol exchange between membranes. Until recently only a limited amount of information was known regarding the existence of multiple exchangeable pools of cholesterol in membranes. As pointed out earlier in the text, such knowledge may be crucial to understanding the nonreceptor-mediated internalization of cholesterol and the reverse transport of cholesterol in cells. Even the limited amount of kinetic evidence of cholesterol exchange is consistent with the presence of multiple pools of cholesterol in erythrocyte membranes (47, 48) and model membranes (122–124). The difficulty in obtaining more information on sterol domains in biologic membranes arises from three principal limitations: lack of suitable nonperturbing probe molecules for cholesterol, uncertainty of the transbilayer migration rate of cholesterol, and limitations of existing sterol exchange assays. As pointed out above, dehydroergosterol is an excellent nonperturbing probe molecule for sterols in membranes. In addition, the transbilayer migration rate of sterol appears to be fast. The last issue is less clear. A limitation of previous assays used to measure kinetics of cholesterol exchange is that they require separation of donor and acceptor membranes. Recently, our laboratory developed an assay for sterol exchange based on fluorescence polarization of a fluorescent sterol analog, dehydroergosterol (17, 18, 55, 58). The assay has the following advantagess: (i) The large number of data points taken continuously allows for better resolution of rapidly exchanging sterol pools. Over 540 data points are continuously taken by computer over a selected time frame. These data are then computer fitted to one or more exponential terms. As shown below, this allows for accurate resolution of half-times of exchange in the range of 1–30 min. This is a crucial point because there is some controversy regarding the existence of a rapidly exchangeable sterol pool, with some investigators reporting such a pool (55, 58, 122–125) and others not observing it (56, 57, 97, 126–128). Although some earlier investigators reported the existence of a rapidly exchangeable sterol pool (distinct from oxidized sterol), they were unable to reproducibly resolve it due to the paucity of data points in the first 30 min of exchange (122–124). Nevertheless, some of these investigators estimated that a rapidly exchangeable cholesterol domain, accounting for 9–15% of cholesterol, was present in model membranes (122, 123) and erythrocytes (124). In contrast, due to the large number of data points taken, the dehydroergosterol polarization exchange assay allows accurate resolution of this domain. (ii) In previous investigations of sterol exchange assays, the donor and acceptor differed in charge, glycolipid content, size, or density. Not only can these factors all affect sterol exchange kinetics but also, as shown in the following sections, acidic phospholipids can modify the number and/or size of sterol domains. The latter point is especially relevant to observation of a nonexchangeable sterol pool.

**Kinetic Investigation of Sterol Domains Using Dehydroergosterol in LM Fibroblast Plasma Membranes.** As described above, the fluorescent sterol dehydroergosterol closely resembles cholesterol in its properties and is easily incorporated into membranes without perturbation.

*Fluorescent sterol exchange assay between biomembranes.* The fluorescent sterol exchange assay for biomembranes is essentially the same as that first developed for small unilamellar vesicles and detailed in the preceding section (55, 58). The only modification is that the fluorescent sterol is bioincorporated into cell membranes whereas in artificial membrane vesicles it is simply added during vesicle preparation. The exchange of sterol is determined using donor plasma membranes containing dehydroergosterol and acceptor membranes without dehydroergosterol. Briefly, L cell fibroblasts were cultured in the absence or presence of dehydroergosterol and plasma membrane fractions were isolated (29, 39, 40, 42, 43). This provided acceptor and donor plasma membrane vesicles. In the donor membrane vesicles the fluorescent sterol replaced the native sterol without altering the sterol to phospholipid ratio. However, at the high concentration of dehydroergosterol in the donor plasma membrane vesicles, the fluorescence polarization has a low value due to frequent dehydroergosterol-dehydroergosterol interactions (nonradiative energy transfer) resulting in fluorescence self-quenching (Fig. 3, time 0). Fluorescence polarization of dehydroergosterol in the donor membrane vesicles is stable over the time period examined (Fig. 3, Curve 1). Upon addition of acceptor plasma membrane vesicles, dehydroergosterol is exchanged for cholesterol. As dehydroergosterol appears in the acceptor mem-

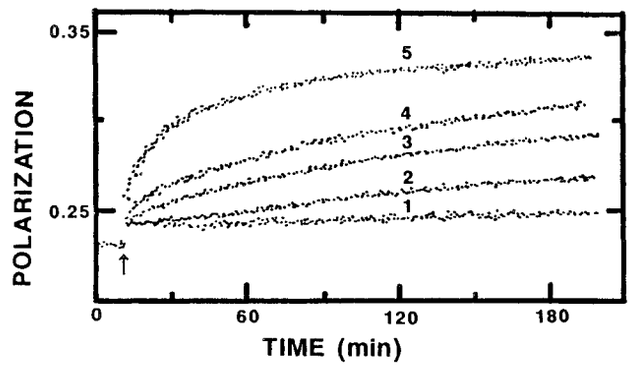


**Figure 3.** Dehydroergosterol exchange between cultured fibroblast plasma membranes. All conditions were as described in footnote *a* to Table III. Curve 1, donor only at 24°C and at 37°C (addition of 1.5 $\mu M$ SCP-2 to donor only yielded the same curve); Curve 2, donor plus acceptor at 24°C; Curve 3, donor plus acceptor at 37°C; Curve 4, donor plus 1.5 $\mu M$ SCP-2 plus acceptor at 24°C; Curve 5, donor plus 1.5 $\mu M$ SCP-2 plus acceptor at 37°C.

brane, dehydroergosterol-dehydroergosterol interactions are very infrequent and fluorescence increases due to release from self-quenching. Concomitantly, the polarization of the donor acceptor vesicle population increases with time (Fig. 3, Curve 2). The polarization of dehydroergosterol is continuously monitored and 540 data points are collected over 3 hr. This allows computer curve fitting to multiple exponential components. The degree of polarization change is proportional to the amount of sterol transferred (125). The results for dehydroergosterol exchange between L cell plasma membranes at 24°C best fit two components: a slowly exchanging pool with $t_{1/2}$ = 227 min comprising 70% of the total and a very slowly exchanging or nonexchangeable pool comprising 30% of the total, but with $t_{1/2}$ so slow that it was not measurable (Table III). The following sections summarize factors that can alter the domain structure of sterol in biomembranes as determined by kinetics of sterol exchange between vesicles.

*Effect of temperature on sterol exchange between biomembranes.* At room temperature (24°C), several kinetic sterol domains are resolved in L cell plasma membranes: $f_1$, a rapidly exchanging domain is either not observed, or if observed accounts for less than 1% of the fractional intensity; $f_2$, a slowly exchanging domain; and $f_3$, a nonexchangeable domain (Table III). However, at physiologic temperature (37°C), a rapidly exchanging domain, $f_1$, with a half-time for exchange of the rapidly exchanging domain ($^1t_{1/2}$) equal to 20 min is clearly resolved, whereas the half-time of exchange of the slowly exchangeable sterol domain ($^2t_{1/2}$) is decreased from 227 to 117 min. The fraction $f_1$ of the exchange represented by the rapidly exchanging domain, 14%, appears concomitant with a decrease in the fraction $f_3$ of nonexchangeable domain from 30 to 10%. Significant change in the size of the slowly exchanging domain $f_2$ was not observed. Thus, changing the temperature from 24 to 37°C not only accelerated sterol transfer, but in addition elicited the appearance of a rapidly exchangeable sterol domain concomitant with a reduction in the size of the nonexchangeable domain. It is significant to note that temperature dependencies of enzyme activities and of lipid structure have been observed near 24°C of plasma membranes from a variety of cells and tissues (129–133) including L cells (30, 33, 134). Essentially what may be observed is a temperature-dependent phase separation of sterol that results in aggregation of the enzyme in a more active form in a sterol-rich domain. Such a mechanism has been proposed for activation of some ion transport proteins (71).

*Effect of lipid transfer proteins on biomembrane sterol domains.* A critical issue is the role of lipid transfer proteins in regulating biomembrane sterol domains. Three intracellular lipid transfer proteins were examined: SCP-2 (also called nonspecific lipid transfer protein), L-FABP (also called sterol carrier protein), and I-FABP. SCP-2 (17) and L-FABP (24–26) both bind cholesterol and dehydroergosterol in 1:1 *M* ratio, whereas I-FABP does not (135). SCP-2 and, to some extent, L-FABP both increase sterol exchange but by different mechanisms. SCP-2 increases the size of the rapidly exchangeable pool $f_1$ 4-fold at the expense of $f_3$, the nonexchangeable pool (which decreases from 13% to 0% of total sterol), whereas L-FABP does not significantly alter pool size (Table IV). SCP-2 markedly decreases both $^1t_{1/2}$ and $^2t_{1/2}$ by a factor of 2, whereas L-FABP decreases $^1t_{1/2}$ slightly less than does SCP-2 and decreases $^2t_{1/2}$ much less so than does SCP-2 (Table IV). I-FABP was essentially without effect on kinetically resolved sterol domains in plasma membranes.

The concentration of these proteins used in the assays, 1.5 $\mu M$, is in the range observed in cytosol of many tissues. FABP concentrations between 5 and 300 $\mu M$ (1–57 $\mu$g/mg cytosolic protein) have been reported in a variety of tissue cytosols (136, 137). Since SCP-2 concentrations are approximately 100-fold lower than FABP concentrations in cell cytosols (137), the SCP-2 physiologic concentration is less than 3 $\mu M$ (0.5 $\mu$g/mg cytosolic protein; Ref. 137). Moreover, the intracellular distribution of these proteins is also not uniform (20, 138, 139) and local concentrations in the cytosol can differ severalfold.

It was previously reported that in model mem-

**Table III.** Effect of Temperature on Sterol Domains in Membranes

| Membrane | °C | $f_1$ | $f_2$ | $f_3$ | $^1t_{1/2}$ (min) | $^2t_{1/2}$ (min) |
|---|---|---|---|---|---|---|
| Plasma membrane[a] | 24 | — | 0.70 ± 0.02 | 0.30 ± 0.02 | — | 227 ± 2 |
| | 37 | 0.14 ± 0.02[b] | 0.76 ± 0.02 | 0.10 ± 0.02[b] | 20 ± 3[b] | 117 ± 7[b] |
| SUV[c] | 24 | 0.10 ± 0.04 | 0.81 ± 0.06 | 0.10 ± 0.06 | 20 ± 8 | 125 ± 14 |
| | 37 | 0.22 | 0.78 | 0 | 10 | 55 |

[a] L cell fibroblasts were cultured with or without dehydroergosterol and plasma membranes were isolated as described earlier (29, 39–43). Donor plasma membranes (with dehydroergosterol) and acceptor plasma membranes (without dehydroergosterol) were mixed 1:10 in 10 m*M*;1,4-piperazine(ethanesulfonic acid), 0.02% $NaN_3$ (pH 7.3) to a final protein concentration of 35 $\mu$g/ml. Exchange was determined at the indicated temperature.

[b] $P < 0.05$ by Student's *t* test as compared with 24°C.

[c] Small unilamellar vesicles [PC:PS:sterol (55:10:35), where sterol is dehydroergosterol (donor) or cholesterol (acceptor)] were prepared, and exchange was determined at the indicated temperature as described earlier (8, 25, 125).

**Table IV.** Effect of Lipid Transfer Proteins and Lipid-Lowering Agents on Sterol Domains in L Cell Fibroblast Plasma Membranes[a]

| Addition | $f_1$ | $f_2$ | $f_3$ | $^1t_{1/2}$ (min) | $^2t_{1/2}$ (min) |
|---|---|---|---|---|---|
| Control | 0.09 ± 0.01 | 0.76 ± 0.02 | 0.13 ± 0.02 | 17 ± 2 | 111 ± 14 |
| SCP-2 | 0.34 ± 0.02[b] | 0.66 ± 0.03[b] | 0[b] | 8 ± 1[b] | 70 ± 6[b] |
| L-FABP | 0.10 ± 0.04 | 0.70 ± 0.04 | 0.20 ± 0.04 | 11 ± 2[b] | 86 ± 2[b] |
| I-FABP | 0.16 | 0.84 | 0.12 | 19 | 144 |
| Control | 0.12 ± 0.02 | 0.69 ± 0.03 | 0.19 ± 0.04 | 29 ± 4 | 131 ± 10 |
| Probucol | 0.13 | 0.81 | 0.06 | 27 | 144 |
| Neomycin | 0.16 ± 0.01[b] | 0.75 ± 0.01 | 0.09 ± 0.06 | 10 ± 1[b] | 103 ± 11[b] |
| JK-1 | 0.13 | 0.75 | 0.12 | 29 | 161 |
| MDP[c] | 0.25 | 0.76 | 0 | 33 | 239 |

[a] All measurements were made at 37°C as described in footnote *a* to Table III. Proteins were 1.5 $\mu M$; probucol was 75 $\mu M$; neomycin was 5 $\mu M$; JK-1 peptide was 100 $\mu M$; MDP peptide was 15 $\mu M$.
[b] $P < 0.05$ by Student's *t* test as compared with control.
[c] MDP, MDL28,209-01.

branes the rate-limiting step in sterol transfer is the rate of sterol desorption from the membrane (97, 126). The rate of exchange will be influenced by domains only when the rate of exchange between domains is slower than the rate of exchange between the membranes, regardless of the distribution of cholesterol between domains. The assumption of rapid exchange between domains on the time scale of the experiments leads to one kinetic pool, but the possibility of multiple pools on different time scales was not ruled out in early experiments on cholesterol exchange. To date, nothing is known about the rate of sterol exchange between domains. Likewise, it is not known whether cholesterol desorbs from separate domains or transfers to the rapidly exchanging domain from which it desorbs. On the basis of model membrane data, the latter possibility has been suggested (55, 58).

In conclusion, if multiple sterol domains exist, then the rate of sterol exchange will also be governed by the distribution of sterol between the domains. In other words, sterol flux will be determined both by rate of desorption and by size of sterol domains. Interestingly, the intracellular cholesterol carrier proteins may enhance intermembrane cholesterol transfer by increasing the rate of desorption (decreasing $t_{1/2}$) or by decreasing the nonexchangeable pool. SCP-2 may act by doing both, whereas L-FABP may act only by enhancing the rate of sterol desorption. As pointed out in the following section, these proteins could affect reverse cholesterol transport from intracellular sites by modulating the flux of cholesterol through plasma membrane cholesterol domains.

*Effect of lipid-lowering drugs and peptides on biomembrane sterol domains.* Reverse cholesterol transport represents the movement of cholesterol out of the cell (140). Reverse cholesterol transport has come to include transfer to the liver, so it requires an acceptor and flux to the liver. Thus, not only is the rate important, but so is the relative chemical potential of cholesterol in cellular membrane (peripheral and liver) and plasma lipoprotein pools. To consider reverse cholesterol transfer, one must also treat these membrane and lipoprotein pools as "domains" among compartments in the whole organism. A great many of the earliest experiments on cholesterol exchange dealt with this problem of what determines the preference of a particular compartment for cholesterol. The focus of this review, however, is to examine cholesterol domains within membranes, since reverse cholesterol movement must occur through the cell surface membrane. Thus, there is a potential role for cholesterol lowering agents in modulating membrane sterol domains and consequently regulating the flux of sterol movement out of the cell. Therefore, the effects of several cholesterol-lowering drugs and several amphipathic peptides were examined. Probucol and neomycin are cholesterol-lowering drugs (141). Although probucol is now believed to act primarily as an antioxidant, some studies indicate that it may be involved in reverse cholesterol transport (142) whereas neomycin is thought to lower plasma cholesterol by complexing bile acids in the gut, in a manner similar to that of cholestyramine (141). However, some of the cholesterol-lowering agents may have other actions heretofore not reported (i.e., a role in modulating sterol exchange kinetics).

Probucol had essentially no effect on sterol transfer between L-cell fibroblast plasma membranes (Table IV). In contrast, the aminoglycoside neomycin significantly reduces both $^1t_{1/2}$ and $^2t_{1/2}$ by 50 and 22%, respectively, and increases the fraction $f_1$ of rapidly exchanging pool, apparently at the expense of $f_3$ the nonexchangeable pool. In addition, the effects of two synthetic amphipathic helical peptides expected to interact with membrane lipids were examined. First, the amino acid sequence of SCP-2 indicates that a region of amphipathic $\alpha$-helical structure might reside near the amino terminus [amino acid residues 13–24 (143)]. Based on this sequence, the peptide $CH_3CO$-Phe-Lys-Glu-Ile-

Glu-Lys-Lys-Leu-Glu-Glu-Glu-Gly-$NH_2$ (mol wt 1861.64), designated as JK-I, was synthesized. This peptide did not enhance sterol transfer between plasma membranes. The second amphipathic peptide was MDL28,209-01 (generously provided by Merrell Dow Research Laboratories, Cincinnati, OH). The amino acid sequence of this peptide is *N*-$\alpha$-succinyl-Leu-Leu-Glu-Lys-Leu-Leu-Glu-Lys-Leu-Lys-$NH_2$ (mol wt 1324.83). Although this peptide abolished the nonexchangeable sterol pool $f_3$ and increased the rapidly exchangeable sterol pool $f_1$ 2-fold, it decreased the rates of sterol transfer between plasma membranes (Table IV). These drugs and peptides illustrate the need to consider modulation of cholesterol exchange kinetics as a potential mechanism of action. More important, they illustrate an important concept: could serum cholesterol be lowered by agents that enhance cholesterol transfer to the reverse cholesterol transfer pathway?

### Microscopic Lateral Cholesterol Domains in Model Membranes

A large variety of physical techniques including nuclear magnetic resonance, ESR, fluorescence, x-ray diffraction, and electron microscopy have been applied to the examination of cholesterol domains in membranes (4). Considerably more extensive structural and kinetic evidence has accumulated with regard to the presence of multiple cholesterol domains in model membrane studies than with biomembranes.

**Structural Studies of Static Lateral Cholesterol Domains.** On the basis of physical measurements, investigators have constructed phase diagrams of cholesterol/dimyristoylphosphatidylcholine membranes (144, 145) and cholesterol/dipalmitoylphosphatidylcholine membranes (146, 147). Phase boundaries near 6–8% cholesterol and 20% cholesterol were obtained in the cholesterol/dipalmitoylphosphatidyl choline and cholesterol/dimyristoylphosphatidylcholine membranes, respectively. Close examination of such phase diagrams shows the existence of at least three regions of two-phase coexistence (0–6% cholesterol, $L_a$/gel phase; 7.5–22%, gel/$L_b$ phase; 7.5–20%, $L_a/L_b$) as well as three regions comprised of single phases ($L_a$ liquid crystalline phase; gel phase; $L_b$ high cholesterol phase). An additional critical point appears at 33 mol% cholesterol. An examination of fluorescence properties of three different fluorescent sterols [dehydroergosterol (96, 98), cholestatrienol (101, 102), and naphthylcholesterol (148)] in phosphatidylcholine membranes also revealed critical points near 6 and 33 mol% sterol. In addition, lifetime analysis of the fluorescent sterols in phosphatidylcholine vesicles revealed the presence of two sterol domains (55, 98–102). From these (55, 98–102) and other data (67, 100, 148–150, and reviewed in Ref. 69) the following points may be made: First, below 6 mol% sterol, the sterol behaves as if it were a monomeric dispersion in phospholipid (i.e., essentially a cholesterol-poor domain). Second, above 6 mol% sterol, the sterols phase separately into sterol-poor and sterol-rich domains. Between 6 and 20 mol% sterol, it has been postulated that cholesterol and phospholipid form mixed domains, and at 20 mol% these domains achieve connectivity, possibly linear arrays. This results in decreasing the total interphase area and altering lateral diffusion of lipids. Third, at 33 mol% sterol, the phospholipid phase transition completely disappears, implying loss of pure phospholipid domains and complete connectivity between sterol/phospholipid arrays. The latter transition is also detected with the fluorescent sterols. With increasing concentration above 6 mol%, the fluorescent sterols interact to form dark (nonfluorescent) complexes. The formation of such complexes is maximal at 33 mol% dehydroergosterol. The fluorescent sterols codistribute with cholesterol and do not form separate fluorescent sterol-rich domains apart from cholesterol-rich domains (67, 96, 98, 101, 102). Pure cholesterol phases do not form in phospholipid membranes below 50 mol% sterol (69). Above 33 mol% fluorescent sterol, the sterol forms a sterol phospholipid (1:1 *M* ratio)-rich phase termed an ordered bimolecular mesomorphic lattice (100). Energy transfer data indicate that in this lattice the sterols are located approximately 10.6 Å from each other, each sterol is surrounded by four phospholipid molecules, and each phospholipid molecule is surrounded by four sterol molecules. Since the Forster energy transfer distance for 50% transfer efficiency, $R_0$, equals 13.2 Å for energy transfer between the fluorescent sterols, this arrangement allows for efficient radiationless energy transfer within a membrane leaflet (but not across the membrane bilayer). Other investigators have proposed an alternative mechanism of fluorescent sterol self-quenching in membranes, based on collisional quenching (151). Fourth, in fluid phase phospholipids, microimmiscibility can result in the co-existence of cholesterol-rich and cholesterol-poor domains (100, 152). Such co-existing fluid phase domains are also predicted by phase diagrams (144–147). The latter point is especially important to biologic membranes since most biomembrane lipids are in the fluid phase.

Under the conditions of high mol% cholesterol and temperatures above the phospholipid phase transitions, the co-existence of immiscible fluid-phase sterol-rich and sterol-poor domains in small unilamellar vesicles (SUV) is a realistic possibility. These conditions are also observed in biomembranes. The motional dynamics of sterols in such domains may differ dramatically. Fluorescent sterols (96, 98, 101, 102, 148) as well as spin-labeled sterols (100) indicate that in the sterol-poor domain the sterol is much less restricted in motion (lower limiting anisotropy or order parameter) and more mobile (faster rotational relaxation rate) than in

sterol-rich domain and is more accessible to the aqueous solvent as indicated by acrylamide quenching data (4, 55). The higher mobility of the sterols in the sterol-poor domain may facilitate their ability to desorb from the membrane, thereby accounting for the rapidly exchangeable sterol pool.

**Kinetic Investigations of Dynamic Lateral Cholesterol Domains in Model Membranes.** The kinetics of cholesterol exchange have been extremely useful in defining the presence of domains in model membranes. However, kinetic data based on [$^3$H]cholesterol exchange between donor and acceptor membrane vesicles have not clearly resolved whether one or multiple exchangeable sterol domains co-exist in membranes. There is disagreement in the literature with some investigators reporting multiple cholesterol domains [rapidly, slowly, and nonexchangeable (17, 55, 58); slowly and nonexchangeable (56, 57, 59); rapidly and slowly exchangeable (122–124)], and others finding only a single exchangeable and no nonexchangeable cholesterol domain (97, 126–128). As pointed out in the preceding sections, the reasons for the discrepancy appear to be based on the assays necessarily requiring separation of dissimilar donor and acceptor membrane vesicles. The properties of the acceptor membrane as well as those of the donor membrane influence sterol exchange. In addition, with assays requiring separation of donor and acceptor membrane vesicles, it is difficult to acquire sufficient data during the initial phase of exchange to allow resolution of rapidly exchanging sterol domains. The new dehydroergosterol exchange outlined above does not require separation of donor and acceptor membrane vesicles and does not suffer from these disadvantages (17, 55, 58). A detailed description of dehydroergosterol exchange assays based on fluorescence polarization (58), fluorescence intensity (55, 58, 59), and lifetime (55) is provided in the cited references and will not be reiterated here. The polarization assay has been modified for continuous polarization measurement with data acquired by computer (up to 540 data points in 3 hr) and computer fitted to single or multiple components (17). Initial rate of fluorescence polarization change is directly proportional to sterol transfer (18, 125, 153), whereas the polarization change of the entire exchange process is best simulated by a polynomial equation (117, 153, 154). As pointed out earlier in the text, the transbilayer migration rate of sterol must not be rate limiting in order to elucidate lateral sterol domains by exchange kinetics.

**Kinetic Investigation of Sterol Domains Using Dehydroergosterol in SUV.** The above sterol exchange assay was used to examine spontaneous sterol exchange between 1-palmitoyl-2-oleoylphosphatidylcholine (POPC):sterol SUV (Fig. 4). Fluorescence polarization of dehydroergosterol in the donor POPC:dehydroergosterol SUV was stable over the 3-hr time period

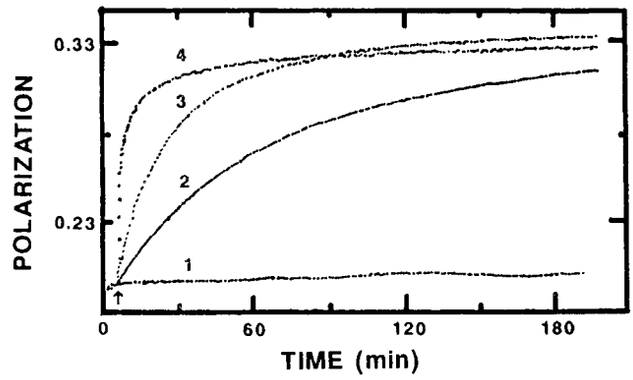


**Figure 4.** Dehydroergosterol exchange between model membranes. All conditions were as described in footnotes to Tables IV and V. Curve 1, donor only at 24°C and at 37°C (addition of 1.5 $\mu M$ SCP-2 to donor only yielded the same curve; Curve 2, donor plus acceptor at 24°C; Curve 3, donor plus acceptor at 37°C; Curve 4, donor plus 1.5 $\mu M$ SCP-2 plus acceptor at 37°C.

investigated (Fig. 4, Curve 1). Addition of 10-fold excess acceptor vesicles comprised of POPC:cholesterol resulted in increased polarization with time (Fig. 4, Curve 2). These data were best fitted to two exchangeable and one nonexchangeable components (Table V). A rapidly ($^1t_{1/2}$ = 20 min), slowly ($^2t_{1/2}$ = 125 min), and a nonexchangeable ($t_{1/2}$ greater than 12 hr or too slow to be measured) sterol domain were resolved. The fractional contributions of the three domains were 8–10%, 76–81%, and 10–16%, respectively. The effect of a variety of parameters on these sterol domains in model membranes was examined.

*Effect of temperature on sterol exchange between SUV.* The half-time for cholesterol and dehydroergosterol (Fig. 4 and Table III) exchange between model membranes decreases with increasing temperature (56, 57, 60, 97, 118). This is shown by increased rate of polarization change (Fig. 4, Curve 3). Increasing the temperature from 24 to 37°C decreases the half-times of exchange from the fast and slowly exchangeable sterol pool nearly equally about 2-fold. Similar results with dehydroergosterol were obtained with a fluorescence intensity assay (59). Increasing temperature also reduced the size of the nonexchangeable pool $f_3$ (Table III), a result consistent with that reported by others (56, 57). Thus, the effect of temperature on sterol domains in model membranes identified by kinetic parameters is similar to that observed in biomembranes (Table III). If the temperature effect is mediated through lipid fluidity, then one might postulate that drugs, hormones, or ions that modulate lipid fluidity of biomembranes might alter sterol domains.

*Effect of phospholipid composition on model membrane sterol domains.* Several kinetic exchange studies with model membrane sterols identified an exchangeable and a nonexchangeable pool (56, 57, 59). However, the exchangeable pool was not further resolved into a

**Table V.** Effect of Lipid Transfer Proteins and Lipid-Lowering Agents on Sterol Domains in Small Unilamellar Vesicle Membranes[a]

| Addition | $f_1$ | $f_2$ | $f_3$ | $^1t_{1/2}$ (min) | $^2t_{1/2}$ (min) |
|---|---|---|---|---|---|
| POPC:sterol SUV | | | | | |
| Control | 0.08 ± 0.02 | 0.76 ± 0.02 | 0.16 ± 0.04 | 20 ± 4 | 128 ± 12 |
| SCP-2 | 0.14 ± 0.01[b] | 0.46 ± 0.01[b] | 0.40 ± 0.01[b] | 11 ± 2[b] | 93 ± 3[b] |
| L-FABP | 0.10 ± 0.03 | 0.72 ± 0.03 | 0.18 ± 0.05 | 24 ± 6 | 123 ± 27 |
| I-FABP | 0.10 ± 0.09 | 0.70 ± 0.09 | 0.20 ± 0.20 | 21 ± 13 | 158 ± 73 |
| $HDL_3$ | 0.34 | 0.66 | — | 30 | 110 |
| Probucol | 0.06 | 0.53 | 0.41 | 32 | 100 |
| JK-1 | 0.01 | 0.54 | 0.45 | 10 | 98 |
| MDP[c] | 0.39 | 0.61 | 0 | 76 | 693 |
| POPC:PS:sterol SUV | | | | | |
| Control | 0.10 ± 0.04 | 0.81 ± 0.04 | 0.10 ± 0.04 | 20 ± 8 | 125 ± 14 |
| SCP-2 | 0.48 ± 0.03[b] | 0.27 ± 0.01[b] | 0.25 ± 0.03[b] | 1 ± 0.1[b] | 24 ± 3[b] |
| L-FABP | 0.15 | 0.61 | 0.24 | 33 | 151 |
| I-FABP | 0.17 | 0.61 | 0.24 | 44 | 193 |
| Neomycin | 0.05 | 0.95 | 0 | 31 | 257 |
| JK-1 | 0.19 | 0.76 | 0.05 | 29 | 187 |
| MDP | 0.17 | 0.83 | 0 | 48 | 178 |

[a] All measurements were made at 24°C as described earlier (18, 25, 98). SUV were POPC:sterol (65:35) or POPC:PS:sterol (55:10:35) with dehydroergosterol in the donor and cholesterol in the acceptor SUV. Donor:acceptor ratios were 1:10. Human $HDL_3$ was generously provided by Dr. D. Hui (Department of Pathology and Laboratory Medicine, University of Cincinnati Medical Center, Cincinnati, OH). $HDL_3$ was 2.6 μg/ml, whereas other proteins were 1.5 μM; probucol was 75 μM; neomycin was 5 μM; JK-1 peptide was 100 μM; MDP peptide was 15 μM.
[b] $P < 0.05$ by Student's $t$ test as compared with control.
[c] MDP, MDL28,209-01.

rapid and a slowly exchangeable pool. Nevertheless, these investigators observed that not only the rate of cholesterol or dehydroergosterol exchange but also the size of the nonexchangeable pool were dependent on the type of phospholipid used (56, 57, 59). In binary phospholipid donor systems, above the phase transition temperatures of the lipids, the exchange rate was fastest when the matrix phospholipid contained unsaturated or short-chain fatty acids. However, phospholipid fatty acid composition did not alter the size of the nonexchangeable pool (56). In contrast, phospholipid polar head group composition dramatically altered both the rate of sterol transfer and the nonexchangeable pool size. The presence of 10 mol% acidic phospholipid (phosphatidylserine, phosphatidylinositol, or cardiolipin) increased the initial rate of sterol transfer by up to 2-fold (18), whereas the presence of large concentrations of sphingomyelin decreased the rate of sterol transfer and converted much of the sterol from exchangeable domain to nonexchangeable domain (56, 59, 155–158).

*Effect of lipid transfer proteins on model membrane sterol domains.* In Table IV, the effect of lipid transfer proteins on kinetically resolved sterol domains in biomembranes was detailed. However, it is not known if the observed effects are due to biomembrane lipids or to some interaction of the transfer proteins with membrane proteins. Therefore, the effect of three intracellular lipid transfer proteins on model membrane SUV sterol exchange was examined: SCP-2 (also called nonspecific lipid transfer protein); L-FABP (also called sterol carrier protein); and I-FABP. In contrast to data obtained with biomembranes, SCP-2 but not L-FABP increases sterol exchange and increases the size of the rapidly exchangeable pool $f_1$. In phosphatidylcholine (PC) containing SUV, SCP-2 markedly decreases the half-times for exchange, $^1t_{1/2}$ and less so $^2t_{1/2}$ (Table V). More importantly, in SUV-containing anionic phospholipids such as phosphatidylserine, the effects of SCP-2 on the initial rate of sterol exchange (18) and half-times, $^1t_{1/2}$, and $^2t_{1/2}$ of sterol exchange (Table V), are markedly potentiated 35-, 20-, and 5-fold, respectively, as compared with 2-, 2-, and 0.3-fold, respectively, in SUV containing only phosphatidylcholine as the matrix phospholipid. In addition, as shown in Table V, SCP-2 effects on sterol domains were also highly dependent on the presence of phosphatidylserine. SCP-2 increased $f_1$ and decreased $f_2$ in phosphatidylserine containing SUV in a similar but exaggerated manner as compared with phosphatidylcholine SUV (Table V). In contrast to observations with biomembranes (Table IV), SCP-2 increased the size of the nonexchangeable sterol domain, $f_3$, both in phosphatidylcholine and in phosphatidylserine containing SUV (Table V). This observation indicates that the complexity of biomembranes may not always be accurately modeled by SUV.

Although L-FABP effectively altered sterol domains and half-times of exchange between biomembranes (Table IV), it was without effect in phosphatidylcholine SUV whether or not phosphatidylserine was present (Table V). As for biomembrane sterol exchange

(Table IV), I-FABP was essentially without effect on kinetically resolved sterol domains in SUV membranes (Table V). I-FABP does not bind cholesterol (but it does bind fatty acid as does L-FABP) and therefore makes an excellent negative control protein. Thus, it appears that SCP-2-mediated effects on sterol domains and sterol exchange between membranes do not require the presence of membrane proteins. As compared with SCP-2, L-FABP effects on sterol exchange kinetics are much smaller in biomembranes and insignificant in SUV. Also, it appears that L-FABP-enhanced sterol exchange between biomembranes is mediated through membrane proteins or perhaps membrane glycolipids (which were not tested). Indeed, another group of investigators showed that treatment of membranes with protease abolished the sterol transfer ability of L-FABP, also called sterol carrier protein (159).

Low concentrations of high density lipoprotein ($HDL_3$), a plasma lipoprotein that may carry cholesterol in reverse cholesterol transport, dramatically altered the proportion of sterol domains ($f_1$, the rapidly exchanging pool, increased from 8 to 34%) without significantly altering the half-times of sterol exchange (Table V). (It should be noted that nonexchangeable sterol pools were not taken into account in this calculation.) At these concentrations, fusion of liposomes with $HDL_3$, as indicated by light scattering, was not observed. In contrast, high concentrations of $HDL_3$ actually resulted in disruption of the SUV and solubilization of the membranes as indicated by light scatter changes (data not shown). It is of interest to note that receptor binding of $HDL_3$ has been reported to facilitate removal of cholesterol from specific intracellular pools by initiating translocation of intracellular cholesterol to the plasma membrane of human fibroblasts (160). The identity of such a receptor has tacitly been assumed to be a protein. As shown herein, $HDL_3$ can interact with membrane lipids alone to elicit an effect on sterol domains.

*Effect of lipid-lowering drugs and peptides on model membrane sterol domains.* Although the effects of several lipid-lowering drugs and amphipathic peptides on kinetically resolved sterol domains between biomembranes were tested (Table IV), it is not known whether the observed effects might be due to interactions with membrane lipids alone or whether membrane protein(s) might be necessary. Therefore, the effects of these agents on sterol domains in SUV model membranes were examined. Probucol and neomycin are cholesterol-lowering drugs (141), and the former may be involved in reverse cholesterol transport (142). Probucol had no effect in either type of SUV on any of the sterol exchange parameters tested (Table V). Neomycin abolished the nonexchangeable pool and increased the half-times of exchange for phosphatidylserine (PS) containing SUV. Since neomycin essentially inhibited sterol transfer between model membranes while enhancing sterol transfer between biomembranes, the stimulatory effect noted between biomembranes would appear to be mediated through membrane protein(s) and might actually be much larger if corrected for the inhibitory effect on the lipids alone. The peptide derived from the SCP-2 structure, JK-1, decreased $^1t_{1/2}$ and the size of $f_1$ while increasing the size of the nonexchangeable pool $f_3$ in phosphatidylcholine SUV. Peptide JK-1 also increased pool $f_3$ in biomembranes. However, biomembranes do contain acidic phospholipids. Interestingly, in phosphatidylserine-containing SUV, JK-1 was essentially without effect. In contrast, MDL28,209-01 slowed the sterol exchange rate between PS-containing SUV, an effect similar to that noted with biomembrane sterol exchange. In addition, in both types of SUV and in biomembranes the MDL28,209-01 peptide abolished the nonexchangeable sterol pool. It is interesting that the effect of $HDL_3$ above is much like that of the peptide MDL28,209. Possibly apoA-I from $HDL_3$ transfers to the lipid phase and has similar amphipathic $\alpha$-helical effects as MDL28,209 in terms of kinetic pools of cholesterol. It is not known how the peptide slows sterol exchange. Perhaps it may interact directly with sterols within the membrane in a manner similar to that described for many of the erythrocyte membrane proteins (119). In summary, the effects of the lipid lowering drugs observed with SUV were highly dependent on the particular agent. Nevertheless, modification of membrane sterol domains and exchange must now be recognized as a possible mechanism whereby drugs may modify sterol transfer through membranes.

*Oxidized cholesterol and lateral cholesterol domains in model membranes.* Oxidized sterols differ markedly from cholesterol in their properties. For example, the molecular area of cholestenone is much larger than that of cholesterol (52 vs 32 $Å^2$) (161). Likewise, the membrane phospholipid-condensing and structural ordering effects of cholestenone are much less effective than with cholesterol (162). More importantly, cholestenone does not form cholestenone-rich domains in membranes (163). Cholestenone is randomly dispersed in the bilayer plane. Thus, it is likely that cholestenone could also alter cholesterol domains in the membrane. Several investigators have suggested that cholestenone may enhance transbilayer migration of cholesterol (153, 164, 165). Moreover, cholestenone and other oxidized sterol altered the equilibrium transbilayer distribution of sterol in LM fibroblast plasma membranes (41).

## Cholesterol Domains in Membranes: The Role of Sphingomyelin

Although it can be modified by a variety of external factors, the origin of the sterol domains detailed above appears to be intrinsically a property of the specific

lipids involved. These domains in part determine the relative chemical potentials of cholesterol in pools distributed within the organism. It is therefore interesting to speculate that some lipids might play a greater role in regulating sterol domain structure than others. In this regard, sphingomyelin and cholesterol are both highly enriched in brain lipids, red blood cell membranes, plasma lipoproteins, and lipid deposits in the vasculature. A considerable amount of literature has focused on a postulated preferential interaction of cholesterol with sphingomyelin in the above tissues and especially the plasma membrane (reviewed in Refs. 156 and 158). The idea of 1:1 sphingomyelin:cholesterol complexes initially seems attractive because the levels of sphingomyelin and cholesterol in serum and in cell plasma membranes were directly correlated. In high concentration, sphingomyelin dramatically slows down sterol transfer between membranes (56, 57, 157, 158, 166, 167). The results with SUV containing 33 mol% sphingomyelin demonstrate that this high level of sphingomyelin abolishes the rapidly exchanging sterol domain and increases $^2t_{1/2}$ nearly 3-fold (Table VI). However, with lower mol% sphingomyelin (e.g., 10 mol%), no significant effect is observed (Table VI). With the exception of some red blood cell membranes and brain membranes, most plasma membranes from mammalian cells have sphingomyelin levels less than 10 mol% of total lipids (less than 29 mol% of total phospholipids). If sphingomyelin formed high-affinity 1:1 complexes with cholesterol but phosphatidylcholine did not, then the rate of transfer or the domain structure of sterols should have been altered even at the lower sphingomyelin content (Table VI). Other data from biomembranes and model membranes are also not consistent with a 1:1 sphingomyelin:cholesterol complex formation or preferential interaction of sphingomyelin with cholesterol in membranes.

First, in the cell plasma membrane sphingomyelin is almost exclusively an exofacial leaflet phospholipid (168). Thus, one might expect that cholesterol should be enriched in the exofacial leaflet of cells. In contrast, cholesterol has been reported to be equally distributed (159) or enriched (4, 45, 149) in the plasma membrane cytofacial leaflet (devoid of sphingomyelin) of the erythrocyte. Likewise, cholesterol is enriched in the plasma membrane cytofacial leaflet of synaptic plasma membranes (4, 106) and cultured fibroblasts (4, 29, 39–43).

Second, cholesterol rotates around its long axis with a correlation time near 0.1 to 0.4 nsec (67, 96, 98, 101, 102, 148, 169–171). In contrast, phospholipids rotate with a correlation time near 10 nsec (169). Long-lived phospholipid:cholesterol complexes, which would increase the rotational correlation time of cholesterol, were not observed.

Third, several investigations indicate that cholesterol and fluorescent sterols preferentially partition into fluid phases rather than interact with specific phospholipids as chemical entities (reviewed in Refs. 4, 60, and 152).

Therefore, it is clear that at high concentrations, sphingomyelin can affect cholesterol domains in membranes (56, 59, 143, 157, 167). But, even at high concentrations, sphingomyelin does not preferentially bind to cholesterol. Instead, sphingomyelin appears to alter the distribution sterol between rapidly and slowly exchanging sterol domains. At lower, physiologic levels of sphingomyelin, kinetic sterol domains are not affected.

## Conclusions

The investigations summarized in this review allow the following conclusions and some speculations on the significance of sterol domains in membranes:

The preponderance of evidence with biomembranes and model membranes indicates that at least three kinetically distinct cholesterol domains may be resolved. Differences in sterol domains and responsiveness to transfer proteins observed between biomembranes and model membranes point to potential for specific proteins (receptors) to directionally affect intermembrane sterol transfer by targeting specific intracellular membranes. A corollary is that sterol domains should be studied in both biomembranes and model membranes to more clearly understand regulation of these domains.

Although the physiologic function of membrane sterol domain structure is not clear, the fact that some proteins are localized in sterol-rich while others are localized in sterol-poor domains is indicative that the

**Table VI.** Effect of Sphingomyelin (SP) on Sterol Domains in SUV Membranes[a]

| SUV phospholipid | $f_1$ | $f_2$ | $f_3$ | $^1t_{1/2}$ (min) | $^2t_{1/2}$ (min) |
|---|---|---|---|---|---|
| POPC:cholesterol (65:35) | 0.08 ± 0.02 | 0.76 ± 0.02 | 0.13 ± 0.02 | 20 ± 4 | 128 ± 12 |
| POPC:SP:cholesterol (55:10:35) | 0.06 ± 0.02 | 0.81 ± 0.02 | 0.15 ± 0.07 | 10 ± 2 | 114 ± 11 |
| POPC:SP:cholesterol (33:33:34) | 0[b] | 1.00[b] | 0[b] | — | 352 ± 39[b] |

[a] All measurements were made at 24°C as described in footnote *a* to Table V except that bovine brain sphingomyelin replaced a portion of POPC as indicated.
[b] $P < 0.05$ by Student's *t* test as compared with POPC:cholesterol (65:35).

properties of such domains would influence the function of the proteins. It is also clear that a variety of factors including lipid composition, temperature, lipid transfer proteins, and drugs may modulate the size and kinetics of these domains. Of special significance is the potential for regulation of sterol flux into or out of the cell by exchange mechanisms through the rapidly exchanging sterol pool. The size and properties of this pool and importantly its regulation could provide impetus for development of a new class of cholesterol-lowering agents that acts by modulating sterol domain structure and kinetics. Cholesterol efflux from membranes or deposits could be enhanced by increasing the size of the rapidly exchangeable pool, decreasing the size of the nonexchangeable pool, enhancing the rate of transfer, or any combination of these three factors. These promise to be exciting areas of future investigation into the function of sterol domains.

This work was generously supported in part by grants from the United States Public Health Service (DK41402, F. S.; AA07292, W. G. W.; RR00013, A. B. K.; AM32309 and AM10628, T. J. S.).

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