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Fraction of Cholesterol Undergoing Spontaneous Exchange between Small Unilamellar Phosphatidylcholine Vesicles†

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ABSTRACT: The kinetics of the spontaneous exchange of [^{3}H]cholesterol between small unilamellar vesicles of phosphatidylcholine has been reexamined. Although first-order exchange kinetics were observed (k = 0.0117 min^{-1}), in good agreement with previous studies, about 20% of the total cholesterol was found to be nonexchangeable in the 8-h time frame of the experiments. The size of this nonexchangeable pool was found to depend on the type of phospholipid and the temperature. It seems probable that the two pools of cholesterol defined in these experiments reflect the complex phase structure of the cholesterol–phosphatidylcholine vesicles.

The spontaneous exchange of cholesterol between plasma and red blood cells was first reported by Hagerman and Gould (1951). Since then, exchange has been demonstrated to occur between a variety of biological membranes, lipoproteins, and liposomes (Bruckdorfer & Graham, 1976; Bell, 1978). The mechanism of cholesterol exchange has been studied in biological membranes (Phillips et al., 1981; Clejan & Bittman, 1984; Davis et al., 1984) as well as in model membranes, such as unilamellar vesicles (Backer & Dawidowicz, 1979; McLean & Phillips, 1981). It has been shown in these systems that the transfer of cholesterol proceeds by diffusion of cholesterol through the aqueous phase and follows single-exponential kinetics.

Although several investigators have found only a single pool of cholesterol (Backer & Dawidowicz, 1979; McLean & Phillips, 1981; Bloj & Zilversmit, 1977), others have observed a significant nonexchangeable portion, leading to the conclusion that membrane cholesterol may exist in two or more kinetically distinguishable pools (Bell & Schwartz, 1971; d'Hollander & Chevallier, 1972; Poznansky & Lange, 1978). Furthermore, a number of studies in a variety of systems have attempted to correlate this nonexchangeable pool with cholesterol on the inner vesicle surface by assuming a very slow transbilayer movement of cholesterol (Smith & Green, 1974; Poznansky & Lange, 1976; Lenard & Rothman, 1976).

In the investigation reported in this paper, we reexamine the magnitude of the fraction of exchangeable cholesterol in small vesicles formed from mixtures of cholesterol and several

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phosphatidylcholines. We find that in these systems cholesterol is present in two kinetically defined pools. Spontaneous exchange occurs from one pool while no exchange occurs from the other much smaller pool in the time frame of the experiments. The fraction of cholesterol in the two pools depends upon the temperature and the vesicle composition. The nonexchangeable pool cannot be identified with cholesterol on the inner vesicle surface.

Knowledge of the parameters governing the spontaneous transfer of cholesterol between bilayers is necessary in order to understand the role played by spontaneous exchange of cholesterol between membranes and lipoproteins in the distribution of cholesterol in biological systems (Lange & Matthies, 1984; DeGrella & Simoni, 1982).

Experimental Procedures

Materials. Cholesterol was obtained from NU-CHEK-PREP, Inc. (Elysian, MN). 1-Palmitoyl-2-oleoylphosphatidylcholine (POPC)[1] and 1-palmitoyl-2-oleoylphosphatidylglycerol (POPG) were supplied by Avanti Polar Lipids, Inc. (Birmingham, AL). [1,2-^{3}H]Cholesterol (specific activity 47.9 Ci/mmol) and [*oleate*-1-^{14}C]cholesteryl oleate (specific activity 56.6 mCi/mmol) were purchased from New England Nuclear (Boston, MA). Cholesterol (cholest-5-en-3β-ol) was freed from the α-cholesterol isomer by digitonin purification (Bladon, 1958).

The purity of the labeled and unlabeled lipids was assayed by TLC on silica gel in two solvent systems: (i) chloroform–methanol–water (65:25:4 v/v/v) for the phospholipids and (ii) hexane–ether–acetic acid (50:50:1 v/v/v) for cholesterol and cholesterol ester. Lipids were visualized either by spraying the plates with primuline or by exposure to iodine vapors. The purity of the phospholipids, the unlabeled cholesterol, and the [^{14}C]cholesteryl oleate (by radioactivity) was better than 99%. Up to 20% of the label in [1,2-^{3}H]cholesterol, depending upon the specific preparation, was due to the presence of oxidation products. These impurities, which transferred completely and much faster than cholesterol with a half-time of about 4 min, were removed by preparative TLC. After purification, all lipids were stored under nitrogen in chloroform stock solutions at −20 °C. We have observed that in phospholipid-tritiated cholesterol vesicles incubated for 2 days or more, substantial oxidation of cholesterol occurs. However, in the 8-h time frame of the transfer experiments at 37 °C, we can detect no cholesterol oxidation products.

Preparation of Small Unilamellar Vesicles. Small unilamellar vesicles (SUV) were prepared by the method of Barenholz et al. (1977) in a 10 mM Pipes buffer (pH 7.0) containing 50 mM KCl, 0.5 mM EDTA, and 0.02% NaN_3 to a final lipid concentration of 10 mg of lipid/mL. Aqueous dispersions were sonicated under nitrogen at 0 °C in a Heat System Ultrasonics Model W350 at setting 2 with a 50% duty cycle for 45 min in 9-min intervals separated by 2-min cooling periods. The clear supernatant obtained after differential centrifugation was equilibrated overnight at 37 ± 0.5 °C and then used for the kinetic studies.

Gel Filtration Chromatography. Estimation of the homogeneity of the vesicles and their stability to aggregation and fusion were studied by using gel filtration chromatography and photon correlation spectroscopy. The data were collected before and after the incubation at 37 °C for up to 24 h. Both Sepharose 2B-CL and Sephacryl S-500 were used with the same results for the gel filtration work.

Photon Correlation Spectroscopy (PCS). Photon correlation spectroscopy measurements were carried out by using a NiCOMP Model HN5-90 laser scattering spectrometer modified in our laboratory to measure light scattering at various fixed angles ≤90°. Detailed descriptions of the methods of data collection and analysis are given elsewhere (Goll & Stock, 1977).

Cholesterol Transfer. In all the experiments, two populations of POPC vesicles were used: neutral donors and negatively charged acceptors. After the incubation period, the charged acceptor vesicles were separated from the neutral donors on a DEAE-Sepharose CL-6B (Pharmacia Fine Chemicals) ion-exchange column by a procedure similar to that used by McLean and Phillips (1981). The donor vesicles contained [^{3}H]cholesterol and also [^{14}C]cholesteryl oleate as a nonexchangeable marker to monitor vesicle recovery. Acceptor vesicles in the equilibrium exchange experiments had the same cholesterol concentration as the donors plus 15 mol % 1-palmitoyl-2-oleoylphosphatidylglycerol (POPG) while in the net transfer experiments they contain no cholesterol. Acceptor vesicles were present in at least 10-fold excess over donors in all experiments in order to minimize back-exchange of [^{3}H]cholesterol. Disintegrations per minute were calculated from the counts by using an external standard. Typically, less than 1% of the charged vesicles and 70–85% of the neutral vesicles were recovered in the eluate from the ion-exchange column.

Kinetic Analysis. The fraction of label that remains in the uncharged donor vesicles at time t is given by

$$X(t) = \frac{(^3H/^{14}C)_t}{(^3H/^{14}C)_{mix}} \quad (1)$$

where $(^3H/^{14}C)_t$ and $(^3H/^{14}C)_{mix}$ represent the ratio of [^{3}H]cholesterol to [^{14}C]cholesteryl oleate concentrations in the eluate at time t and in the incubation mixture, respectively. If negatively charged donor vesicles are used instead, then the fraction of label remaining in the donors at time t is given by

$$X(t) = \frac{(^3H/^{14}C)_{mix} - (^3H/^{14}C)_t}{(^3H/^{14}C)_{mix}} \quad (2)$$

Kinetic data were fitted to one- or two-exponential functions and one equilibrium value by using an iterative nonlinear least-squares analysis (Johnson et al., 1985) on a Control Data Corp. Cyber 730 computer.

Results

Figure 1 shows a typical equilibrium exchange experiment at 37 °C using neutral donor and negatively charged acceptor vesicles both containing 10 mol % total cholesterol (●). When isotopic equilibrium is reached between donor and acceptor vesicles, the expected value for the percent of label remaining in the donors is 9.09% on the basis of the acceptor:donor vesicle ratio of 10. However, in Figure 1, the curve plateaus at about 20% at long times. These data and the data of many similar experiments are best described by a one-exponential function with a preexponential factor of 0.6667 ± 0.02, a rate constant of 0.0097 ± 0.001 min^{-1} ($t_{1/2}$ = 71 ± 9 min), and an equilibrium value of 23.0 ± 3.0% for the percent of [^{3}H]cholesterol remaining in the donor vesicles.

Stability of the Nonexchangeable Pool. After an exchange experiment was completed, the donors and the negatively charged acceptors were separated. Followed by a 15-h

[1] Abbreviations: POPC, 1-palmitoyl-2-oleoylphosphatidylcholine; POPG, 1-palmitoyl-2-oleoylphosphatidylglycerol; SUV, small unilamellar vesicle(s); TLC, thin-layer chromatography; Pipes, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); EDTA, ethylenediaminetetraacetic acid; PCS, photon correlation spectroscopy; DPPC, dipalmitoylphosphatidylcholine.

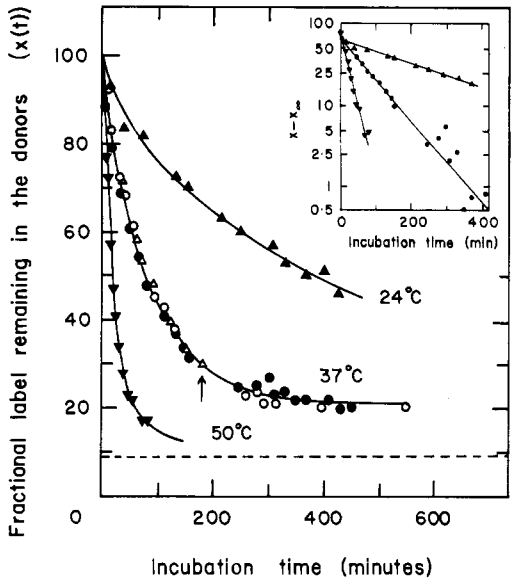

FIGURE 1: Typical curves for [^{3}H]cholesterol, at three temperatures, exchange from neutral donor vesicles containing 90 mol % POPC, 10 mol % cholesterol, and traces of [^{14}C]cholesteryl oleate to a 10-fold excess of negatively charged acceptor vesicles containing the same amount of cholesterol and 15 mol % POPG and POPC. The dashed line represents the expected value for the percent of label remaining in the donors based on isotopic equilibrium between the two populations. In a different experiment (Δ), [^{3}H]cholesterol was transferred from negatively charged donor vesicles to a 10-fold excess of neutral acceptor vesicles. The two populations were separated after 3 h of incubation (arrow), and the acceptor vesicles were then used as donors in a new exchange experiment (O).

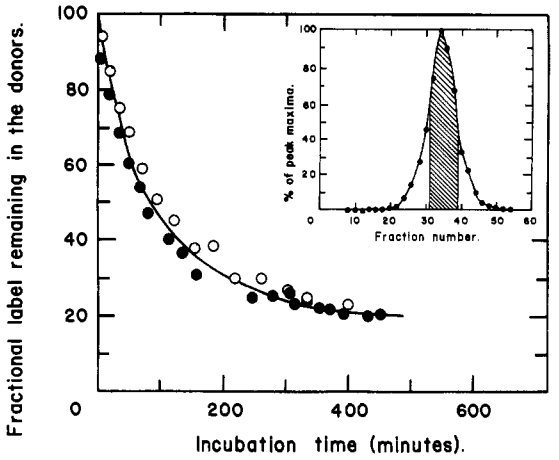

FIGURE 2: Exchange of [^{3}H]cholesterol from unsized (●) and sized (O) vesicles containing POPC and 10 mol % cholesterol at 37 °C. The inset shows the gel filtration pattern of sonicated donor vesicles containing 10 mol % cholesterol in POPC. The fractions were collected and analyzed for ^{3}H, ^{14}C, and P_i. The fraction which had the maximum number of counts was considered to be 100%. The hatched area was used for the transfer experiment.

Table I: Effect of Negatively Charged Phospholipid (POPG) on Cholesterol Transfer Parameters at 37 °C

donor type	phospho-lipid	mol % chol-esterol	$t_{1/2}$ (min)	% nonexchange-able cholesterol
neutral	POPC	10	71 ± 9	23 ± 3
negative	POPC	10	67 ± 9	21 ± 4
negative	egg PC	20	81 ± 11	21 ± 2

equilibration at 37 °C, the donors were then mixed with another 10-fold excess of freshly prepared acceptors, and [^{3}H]cholesterol exchange was followed. No further exchange was detected during the next 8 h, indicating that the nonexchangeable cholesterol is in a pool with an exchange half-time greater than 108 h (4.5 days). The nonexchangeable cholesterol pool in the donor vesicles at the end of the exchange period was determined as [^{3}H]cholesterol counts and by thin-layer chromatography.

Does Vesicle Size Heterogeneity Affect Transfer? Two pools of cholesterol, only one of which is readily exchangeable, could be the result of vesicle size heterogeneity and/or due to the presence of multilamellar liposomes. This possibility was ruled out by the following experiment. The sonicated and centrifuged vesicles were further fractionated by gel filtration on either Sepharose 2B-CL or Sephacryl S-500. The profile of a typical fractionation is shown in the inset of Figure 2. Less than 1% of the lipid phosphorus eluted in the void volume of the column, and 99% of the ^{3}H and ^{14}C counts eluted with the lipid phosphorus. Fractions 31–39 (hatched area) from the included volume peak region were used in an exchange experiment. The results were essentially identical with those obtained with unfractionated vesicles (Figure 2). Therefore, it can be concluded that the nonexchangeable cholesterol pool is not the result of vesicle inhomogeneity or due to the presence of multilamellar liposomes.

Is the Nonexchangeable Pool an Artifact of Vesicle Preparation? Possible preparation artifacts that may give rise to the nonexchangeable pool are as follows: (i) the cholesterol distribution between outer and inner leaflets of the vesicle bilayer differs from the expected value of 2 to 1 based on the outer to inner leaflet mass ratio (Huang et al., 1974); (ii) the production of oxidized derivatives of cholesterol during ultrasonic irradiation; (iii) the presence of cholesterol microcrystals trapped in the vesicles. All of these artifacts were ruled out by the following experiment.

Tritiated cholesterol in negatively charged donors was exchanged with neutral acceptors containing [^{14}C]cholesteryl oleate, a nonexchangeable marker (Figure 1). The exchange was monitored for 3 h (Δ), and the donors and acceptors were separated (arrow in Figure 1) by ion-exchange chromatography. The neutral acceptors containing [^{3}H]cholesterol and [^{14}C]cholesteryl oleate were annealed overnight at 37 °C. These vesicles were then used as donors with a new batch of fresh, negatively charged acceptors added in 10-fold excess. [^{3}H]Cholesterol exchange was then monitored in this system (O). It can be seen in Figure 1 that the data obtained for this exchange system are superimposable on the data obtained for the initial exchange. Both exchange systems have the same half-time ($t_{1/2}$ = 60 min) and both the same value (20 ± 3%) for the nonexchangeable pool. Thus, no artifact due to vesicle preparation was detected.

Does Negative Charge in the Donors Affect Transfer? In this experiment, negatively charged donors and neutral acceptors were used in order to examine the effect of the negatively charged phospholipid on the kinetics of cholesterol exchange. Table I shows the percent of [^{3}H]cholesterol remaining in the donors after 7 h of incubation with vesicles containing 10 and 20 mol % cholesterol in POPC and egg PC at 37 °C, respectively. The transfer half-time ($t_{1/2}$) is unaffected by the presence of the negatively charged lipid in the donors, as is the nonexchangeable cholesterol pool size. In the time frame of the experiment, negative charge in the donor vesicles does not affect the exchange.

Effect of Cholesterol Mole Fraction on Exchange Parameters. The rate constant and the nonexchangeable pool for cholesterol exchange at different mole fractions of cholesterol in the donors containing POPC are given in Table II. The data show a concentration dependence of the rate constants, but the nonexchangeable pool seems independent of the cho-

Table II: Effect of Initial Cholesterol Concentration of Vesicles on Transfer Parameters at 37 °C

mol % cholesterol	k ($\times 10^{-2}$ min^{-1})	$t_{1/2}$ (min)	% nonexchangeable cholesterol
1	1.50 ± 0.10	46 ± 7	18.4 ± 2.3
10	0.97 ± 0.10	71 ± 9	23.0 ± 3.0
20	0.78 ± 0.09	89 ± 11	22.4 ± 2.3

Table III: Temperature Effect on Kinetic Parameters of Cholesterol Transfer at 10 mol % in POPC Vesicles

incubation temp (°C)	$t_{1/2}$ (min)	% nonexchangeable cholesterol
	Equilibrium Exchange[a]	
24	216 ± 40	32.0 ± 6.1
32	137 ± 20	21.4 ± 4.9
37	71 ± 9	23.0 ± 3.0
50	13 ± 1	12.7 ± 1.9
	Net Transfer[b]	
24	213 ± 55	32.4 ± 8.1
37	65 ± 5	19.7 ± 1.5

[a] In an equilibrium exchange experiment, the donor and acceptor vesicles have the same cholesterol concentration. [b] In a net transfer experiment, cholesterol transfer from donor vesicles to acceptor vesicles containing *no* cholesterol.

lesterol concentration within the experimental error of the measurements.

Exchange vs. Net Transfer. Net transfer at 37 °C of 1 mol % cholesterol in pure POPC vesicles to a 10-fold excess of negatively charged acceptor vesicles containing *no* cholesterol was compared with the exchange of [^{3}H]cholesterol between donor and acceptor vesicles both containing 1 mol % cholesterol. The half-times and nonexchangeable pool values for cholesterol net transfer and exchange are very similar: 46 ± 7 min, 18.4 ± 2.3% and 55 ± 8 min, 17.71 ± 1.5% for the equilibrium exchange and the net transfer experiments, respectively.

Effect of Temperature on Kinetic Parameters. The results of equilibrium exchange and net transfer of 10 mol % cholesterol in POPC at different temperatures are summarized in Table III. It can be seen that both the half-times and the nonexchangeable pool size decrease at higher temperatures. There is no significant difference in the kinetic parameters for the net transfer and the equilibrium exchange process obtained under similar conditions.

Discussion

This study provides evidence that both cholesterol exchange and net transfer between phosphatidylcholine bilayers are characterized by a first-order rate constant and a nonexchangeable or nontransferable pool of cholesterol.

The fact that the transfer rate is first order suggests that cholesterol is transferred through the aqueous phase. This has been reported previously for a number of systems (Bruckdorfer & Graham, 1976; Smith & Scow, 1979; McLean & Phillips, 1981, 1982). It is surprising, however, that in none of these studies was a nonexchangeable cholesterol pool reported. In fact, McLean and Phillips (1981), Bloj and Zilversmith (1977), and Backer and Dawidowicz (1979) reported that essentially all of the cholesterol was exchangeable.

To establish firmly the presence of this pool, several control experiments were performed. No effect of the negatively charged phospholipid on the kinetics of cholesterol transfer was observed. This is established by the observation that the exchange rate and nonexchangeable pool size were identical over a 7-h period in paired exchange systems in which the charged lipid (POPG) was in the donors in one system and in the acceptors in the other system. (Table I). For longer periods, the value for the nonexchangeable pool size decreased with the negatively charged donors but remained constant with the neutral ones (data not shown). This behavior is almost certainly due to a change in the operation of the ion-exchange column with time because of the spontaneous transfer of the negatively charged phospholipid during the incubation period (DeCuyper et al., 1983). In the case of charged acceptors, the negatively charged phospholipid transferred to the donor vesicles is not sufficient to perturb significantly the separation between the two populations. When the charge is on the donors, however, POPG transfer to the 10-fold excess of acceptors significantly lowers the donor charge so that at longer periods less donor vesicles are retained on the ion-exchange column. The net result is to make it appear that more cholesterol is exchangeable. It is quite probable that this effect operated to conceal the nonexchangeable pool in certain earlier studies in which charged donors were used [e.g., see McLean & Phillips (1981)].

The possibility of artifacts due to the presence of radioactive impurities in the tritiated cholesterol such as α-cholesterol or oxidation products was ruled out by the following observations: (i) Purification of β-cholesterol by digitonin complex formation did not affect the results, indicating that the sample was free of α-cholesterol. (ii) When oxidation products were present in the sample, two different rates were observed. The faster rate was shown to be due to ^{3}H-labeled products of cholesterol oxidation. Purified cholesterol, free of oxidation products, showed only the slower rate of transfer.

The rate of lipid exchange has been reported to decrease with increasing size in unilamellar vesicles (Fulger et al., 1985). Also, exchange from multilamellar vesicles must necessarily be slower because of the concentric geometry of the lamellae in any one liposome. Thus, the presence of large unilamellar vesicles or multilamellar liposomes in the donor population could give rise to an apparently nonexchangeable pool of cholesterol (Backer & Dawidowicz, 1979). These possibilities were, however, ruled out by our failure to detect the presence of large vesicles or liposomes using molecular sieve chromatography and photon correlation spectroscopy. In addition, donor vesicles prepared by gel filtration gave the same exchange parameters as donor vesicles not subjected to gel filtration (Bloj & Zilversmit, 1977). Photon correlation spectroscopy revealed a narrow size distribution in the donor vesicles with an average diameter of 300 Å that remained constant during the time frame of the exchange experiments. Thus, neither fusion nor aggregation occurred during this period.

The nonexchangeable pool of cholesterol could be formed during preparation of the vesicles by an abnormal distribution of cholesterol between the two leaflets of the vesicles or by the presence of cholesterol microcrystals that remain entraped within the vesicles. Both of these possibilities were eliminated by an experiment in which negatively charged donors and neutral acceptors were separated and the latter were used as donors in a new exchange experiment. The data obtained in the first exchange are indistinguishable from the data obtained in the second exchange as can be seen in Figure 1. Direct determination of the distribution of [3,4-^{13}C]cholesterol between the inner and outer bilayer surfaces by NMR using a shift reagent proved to be impossible because of the lack of resolution of the signals originating from the cholesterol populations in the outer and inner surfaces.

It is possible that the nonexchangeable pool simply reflects the equilibrium value for the distribution of cholesterol between

donor and acceptor vesicles. This possibility was examined by performing an exchange experiment after which the two vesicle populations were separated and the donors exposed to a batch of fresh acceptors. Since no further exchange was detected, this explanation cannot be correct. This conclusion was further strengthened by the observation that the same value for the nonexchangeable cholesterol pool size was obtained in both net transfer and exchange experiments.

It seems clear in the vesicle systems we have examined that two kinetically defined pools of cholesterol exist. The one pool comprising about 80% of the total cholesterol is rapidly transferable to acceptor vesicles; the other remaining 20% of the donor cholesterol has a half-time of transfer greater than 108 h at 37 °C. Our results do not agree with observations of Bloj and Zilversmit (1977) in which cholesterol exchange between vesicles and erythrocytes was studied and of Backer and Dawidowicz (1979), who followed cholesterol exchange between two populations of vesicles.

The difference in results cannot be due to the different methods of vesicle preparation. The data in Figure 2 show that the results obtained with vesicles prepared by ultracentrifugation are the same as those obtained with vesicles prepared by molecular sieve chromatography. A possible cause of the difference in results may rest in the fact that Bloj and Zilversmit as well as Backer and Dawidowicz used albumin to improve the recovery of the vesicles. We did not. Although Bloj and Zilversmit observed that albumin did not appear to affect the extent or initial rate of cholesterol exchange for soy PC and DPPC, Clejan and Bittman (1984) pointed out that in [^{14}C]cholesterol exchange studies from open, unsealed *Mycoplasma gallisepticum* membranes to acceptor vesicles only three-fourths of the cholesterol molecules exchanged in the absence of albumin whereas more than 90% of the radiolabeled cholesterol exchanged when albumin was present.

It seems possible that the two pools of sterol defined by the exchange experiments reflect the phase structure of the sterol-containing phosphatidylcholine vesicles. The phase structure of such bilayers is known to be complex. The complexity, however, is not well understood, and there is considerable disagreement about the interpretation of existing data (Klausner & Kleinfeld, 1984; Yeagle, 1985). For the case of saturated diacylphosphatidylcholines, there is general agreement that below about 20 mol % cholesterol two phases coexist: one cholesterol rich and the other cholesterol poor (Mabrey et al., 1978; Estep et al., 1978; Recktenwald & McConnell, 1981). The situation is much less clear for biologically relevant phosphatidylcholines such as egg phosphatidylcholine and POPC, which have gel to liquid-crystalline phase transitions at lower temperatures. It seems reasonable, however, that two phases may also coexist in these systems at physiological temperatures. If this is indeed the case, then it may be that the two kinetically defined pools of cholesterol described in this paper reflect in some manner this phase structure.

The exchange of radiolabeled cholesterol between phospholipid vesicles and biological membranes has been examined in several systems. For example, the exchange of cholesterol between both *Acholeplasma laidlawii* and *Mycoplasma gallisepticum* intact cells has been shown to be biphasic (Clejan & Bittman, 1984; Davis et al., 1984). These workers suggest that the slow exchange is rate limited by translocation from the inner to outer membrane surface. It is interesting to note, however, that Clejan and Bittman (1984) report that in unsealed plasma membranes derived from *M. gallisepticum* in which both membrane faces are exposed to the aqueous phase, exchange of 75% of the total cholesterol occurred as a first-order process. The remaining 25% of the membrane cholesterol proved to be nonexchangeable. It may be that the nonexchangeable pool of cholesterol in this biological membrane is similar to the pool we have described in this paper.

Registry No. POPG, 10015-87-9; POPC, 6753-55-5; cholesterol, 57-88-5.

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