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Dependence on Phospholipid Composition of the Fraction of Cholesterol Undergoing Spontaneous Exchange between Small Unilamellar Vesicles†

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ABSTRACT: Spontaneous cholesterol exchange between small unilamellar vesicles comprised of different phospholipids and their binary mixtures has been studied in order to understand the factors involved in the establishment and maintenance of intracellular cholesterol distributions. Exchange was performed from neutral donor vesicles containing different cholesterol concentrations, traces of [^{3}H]cholesterol, and [^{14}C]cholesteryl oleate as a nonexchangeable marker. The acceptor vesicles, in 10-fold excess, had the same composition, but 15 mol % phosphatidylglycerol was included to permit chromatographic separation. Data were best fitted by a single exponential and a base value. In donor vesicles containing only one phospholipid, the kinetic rate constants agreed with data reported previously; however, the base values were larger than the expected equilibrium value of 9.09%. The size of this nonexchangeable pool and the exchange rate were found to depend on the type of phospholipid. In binary phospholipid donor systems, well above the transition temperatures of the lipid components, the exchange parameters were preferentially closer to those of one component according to the order POPC > DMPC > DPPC > bovine brain SPM.

It is well-known that the distribution of cholesterol among the various subcellular membranes of any specific type of cell is not uniform. For example, in the rat hepatocyte the plasma membrane contains 28.0 wt % cholesterol while in the endoplasmic reticulum, the site of cholesterol biosynthesis, the concentration is only 6 wt %. In the inner mitochondrial membrane the concentration is less than 1% (Thompson & Huang, 1986).

The process of spontaneous cholesterol exchange between phospholipid vesicle bilayers has been studied in an attempt to understand some of the factors controlling the establishment and maintenance of this cholesterol distribution. Several studies in small unilamellar phosphatidylcholine vesicles have shown that cholesterol moves between vesicles by transfer through the aqueous phase (Backer & Dawidowicz, 1981; McLean & Phillips, 1981, 1982). The data also suggest that the rate-limiting step is the desorption of cholesterol from the donor membrane. The same results have been obtained in cholesterol-transfer studies between biological membranes (Lange et al., 1979, 1983). The ultimate cholesterol concentration in the various membranes could be a result of a partitioning equilibrium, which depends on membrane composition. In fact, Wattenberg and Silbert (1983) showed that the differences in cholesterol concentration between various membrane fractions were retained when any two fractions were incubated together.

In a recent study we reexamined the kinetics of the spontaneous exchange of [^{3}H]cholesterol between small unilamellar vesicles of 1-palmitoyl-2-oleoylphosphatidylcholine and found two kinetically defined cholesterol pools (Bar et al., 1986). About 80% of the total cholesterol exchanges with first-order kinetics ($t_{1/2}$ = 90 min), in good agreement with previous studies, while the remaining 20% was found to be nonexchangeable in the time frame of the experiments (8 h). This nonexchangeable pool of cholesterol together with differences in the rates of exchange may contribute to the unequal distribution of cholesterol between membranes in the same cell. We now report studies on the kinetics of [^{3}H]cholesterol exchange between small unilamellar vesicles comprised of different phospholipids and binary mixtures of these phospholipids.

Materials and Methods

Materials. Cholesterol was obtained from Nu-Chek-Prep, Inc. (Elisian, MN). Bovine brain sphingomyelin, 1,2-diacyl-*sn*-glycero-3-phosphocholines, and phosphatidylglycerols were purchased from Avanti Polar Lipids, Inc. (Birmingham,

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AL). 1-Palmitoyl-2-oleoyl- (POPC, POPG),[1] dipalmitoyl- (DPPC, DPPG), and dimyristoylphosphatidylcholine and -phosphatidylglycerol (DMPC, DMPG) and egg yolk and bovine erythrocyte sphingomyelin were supplied by Sigma Chemical Co. (St. Louis, MO). L-β,γ-Dihexadecylphosphatidylcholine (DHPC) was purchased from Bachem Feinchemikalien AG (Bubendorf, Switzerland). [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).

The purity of the labeled and unlabeled lipids was assayed by TLC on silica gel plates. Usually, the compounds were found to be more than 99% pure. A percentage of the label in the [1,2-^{3}H]cholesterol preparation was associated with cholesterol oxidation products. The actual percent of these impurities depended upon the specific preparation and the time of storage. These impurities were removed immediately before use by preparative TLC of the labeled cholesterol using hexane/ether/acetic acid (50:50:1 v/v/v) as the solvent system. At the end of an exchange experiment, about 95% of the purified [1,2-^{3}H]cholesterol cochromatographed with a cholesterol standard at all temperatures. All lipids were stored under nitrogen in chloroform stock solutions at −20 °C.

Gel filtration and ion-exchange chromatography were performed with Sephacryl S-500 and DEAE-Sepharose CL-6B (Pharmacia Fine Chemicals, Piscataway, NJ).

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, above the main transition temperatures of the phospholipids. The sonication was performed in a Heat System Ultrasonics Model W 350 at setting 2 with a 50% duty cycle in 5-min intervals separated by 2-min resting periods for a total period of 20–30 min. After differential centrifugation, the clear supernatant was allowed to equilibrate overnight under N_2 at the temperature of the exchange experiment before use in kinetic studies.

Analytical Procedures. In order to estimate the homogeneity, size, and stability of the vesicles, the dispersions were analyzed by gel filtration chromatography and photon correlation spectroscopy by procedures described elsewhere (Bar et al., 1986).

Phospholipid concentrations were determined by phosphorus analysis using the method of Barlett (1959).

Cholesterol Exchange. The equilibrium exchange of the radiolabeled [1,2-^{3}H]cholesterol from neutral donors to a 10-fold excess of negatively charged acceptors was monitored by a modification of the method of McLean and Phillips (1981) as previously described (Bar et al., 1986). The donor vesicles were comprised of neutral phospholipid, 1–10 mol % nonlabeled cholesterol, a trace of [^{3}H]cholesterol, and a trace of [^{14}C]cholesteryl oleate as a nonexchangeable marker. Acceptor vesicles had the same unlabeled cholesterol concentration, but 15 mol % of the negatively charged 1,2-diacylphosphatidylglycerol with a corresponding lower concentration of the same neutral phospholipid or mixture of phospholipids. Phosphatidylglycerol was selected to impart a negative charge to the acceptor vesicles because it does not phase separate in the phospholipid matrices used. It is worth noting that no effect of the negatively charged phospholipid on the kinetics of cholesterol transfer was observed (Bar et al., 1986).

[1] Abbreviations: POPC, 1-palmitoyl-2-oleoylphosphatidylcholine; POPG, 1-palmitoyl-2-oleoylphosphatidylglycerol; DPPC, dipalmitoylphosphatidylcholine; DPPG, dipalmitoylphosphatidylglycerol; DMPC, dimyristoylphosphatidylcholine; DMPG, dimyristoylphosphatidylglycerol; SPM, sphingomyelin; DHPC, dihexadecylphosphatidylcholine.

Table I: Effect of the Phospholipid Matrix on the Cholesterol-Transfer Parameters at 37 °C

	initial cholesterol concentration			
	1 mol %		10 mol %	
phospholipid matrix	$t_{1/2}$[a] (min)	% nonexchangeable cholesterol	$t_{1/2}$ (min)	% nonexchangeable cholesterol
POPC	46 ± 7	18.4 ± 2.3	71 ± 9	23.0 ± 3.0
DMPC	121 ± 30	18.0 ± 5.0	153 ± 10	20.1 ± 3.0
SPM (bovine brain)	202 ± 70	88.0 ± 3.0	190 ± 100	90.0 ± 2.0

[a] The $t_{1/2}$ and percent of nonexchangeable cholesterol values are the means ± SE of different vesicle preparations.

During the incubation period, 0.2-mL aliquots of the mixed vesicle dispersion were applied to a preequilibrated ion-exchange column (DEAE-Sepharose CL-6B). After the mixture had entered the column completely, 2.0 mL of PIPES buffer, pH 7.0, was added. Fractions (1 mL) of the eluate were collected and counted in 8 mL of scintillation fluid. Preequilibration of the ion-exchange column and elution were carried out at the temperature of the experiment. This procedure resulted in an improved chromatographic recovery of donors (90–95%) based on the counts of [^{14}C]cholesteryl oleate, at all time intervals examined.

The fraction of the label that remains in the uncharged donors vesicles at time t is given by

$$x_t = (^3H/^{14}C)_t/(^3H/^{14}C)_{mix}$$

where $(^3H/^{14}C)_t$ and $(^3H/^{14}C)_{mix}$ represent the ratio of [^{3}H]cholesterol to [^{14}C]cholesteryl oleate in the eluate at time t and in the incubation mixture, respectively.

Kinetic data were fitted to a one- or two-exponential function and one equilibrium value by an iterative nonlinear least-squares analysis program on a Control Data Corp. CYBER 730 computer (Johnson & Frasier, 1985).

Results

Effect of the Phospholipid Matrix on [^{3}H]Cholesterol Exchange. [^{3}H]Cholesterol transfer from neutral donors containing a single phospholipid component to negatively charged acceptor vesicles is best described by a one-exponential function and an equilibrium value, as shown in our previous study (Bar et al., 1986).

The half-lives ($t_{1/2}$) and the cholesterol fractions remaining in the donor vesicles at infinite time at 37 °C are listed in Table I for vesicles comprised of three different phospholipids with initial cholesterol concentrations of 1 and 10 mol %. It can be seen that the values of both parameters depend upon the phospholipid matrix. At each cholesterol concentration, the rate of [^{3}H]cholesterol exchange *decreases* in the order POPC > DMPC > SPM while the nonexchangeable fraction of cholesterol *increases* in the same order.

The exchange rates from POPC and DMPC donor vesicles decrease with increasing cholesterol concentration. A similar trend has been reported by McLean and Phillips (1982) for egg phosphatidylcholine vesicles.

Random distribution of the labeled cholesterol in a mixture of donor vesicles and a 10-fold excess of acceptors is expected to lead to an equilibrium cholesterol concentration of 9.09% of the initial mole percent of cholesterol in the donor vesicles.

Table II: [^{3}H]Cholesterol-Transfer Parameters from Neutral Donor Vesicles Containing 10 mol % Cholesterol and Sphingomyelin from Different Natural Sources at 50 °C

SPM source	$t_{1/2}$ (min)	% nonexchangeable cholesterol
bovine brain	135 ± 20	60 ± 7
egg yolk	373 ± 100	64 ± 3
bovine erythrocyte	424 ± 100	38 ± 4

Table III: Effect of Temperature on Kinetic Parameters of [^{3}H]Cholesterol Exchange from Neutral Donor Vesicles Containing 10 mol % Cholesterol

phospholipid	incubation temp (°C)	$t_{1/2}$ (min)	% nonexchangeable cholesterol
POPC	37	71 ± 9	23.0 ± 3.0
	50	13 ± 4	12.7 ± 1.9
DMPC	37	153 ± 10	20.1 ± 3.0
	50	38 ± 2	20.0 ± 4.0
SPM (bovine brain)	37	190 ± 100	90.0 ± 2.0
	50	135 ± 20	60.0 ± 7.0

However, as can be seen from Table I, this value was not attained in any of the phospholipid systems examined. The percent of [^{3}H]cholesterol remaining in the donor vesicles at 37 °C was about 20% of the total cholesterol for POPC and DMPC and about 90% for SPM. These values were found to be essentially independent of the initial cholesterol concentration, in contrast to the exchange half-lives which do depend on the cholesterol concentration.

The excess of cholesterol remaining in the vesicles could be the result of strong hydrogen bonding between the carbonyl group in the phospholipid and hydroxyl group in the cholesterol molecule. In order to verify this possibility, cholesterol transfer experiments were done with SUV's comprised of either DHPC or DPPC as well as mixtures of the two phospholipids at 50 °C. DHPC, the ether homologue of DPPC, has no carbonyl group. The two kinetic parameters, namely, the half-life and the percentage of cholesterol remaining in the donors, were found to be similar in all the systems and comparable to the corresponding values in vesicles of pure DPPC ($t_{1/2}$ = 62 ± 6 min and 31.1 ± 1.5%, respectively). Thus, the contribution of the carbonyl hydrogen bonding to the existence of the nonexchangeable cholesterol pool appears to be insignificant.

Table II shows the half-life and the fraction of nonexchangeable [^{3}H]cholesterol in vesicles comprised of sphingomyelin mixtures derived from three different natural sources. In these systems, the donor vesicles contained 10 mol % cholesterol, and transfer experiments were performed at 50 °C, which is above the transition temperature of the sphingolipids (Barenholz & Gatt, 1982). It can clearly be seen that both parameters are strongly dependent on the compositions of these natural sphingomyelin mixtures (Barenholz & Gatt, 1982).

Effect of Temperature on [^{3}H]Cholesterol Exchange. Summarized in Table III are the [^{3}H]cholesterol exchange parameters for neutral donor vesicles containing 10 mol % cholesterol in different phospholipid matrices at 37 and 50 °C. Again, all phospholipids were above their phase transition temperatures. As expected, the half-time for exchange decreases with increasing temperature. The activation energies for cholesterol exchange were found to be 20 and 26 kcal/mol in POPC and DMPC, respectively, in good agreement with previous studies (McLean & Phillips, 1982). The value for bovine brain SPM is 8 kcal/mol.

[^{3}H]Cholesterol Exchange from Vesicles Containing a Binary Mixture of Phospholipids. Figure 1 gives the half-lives

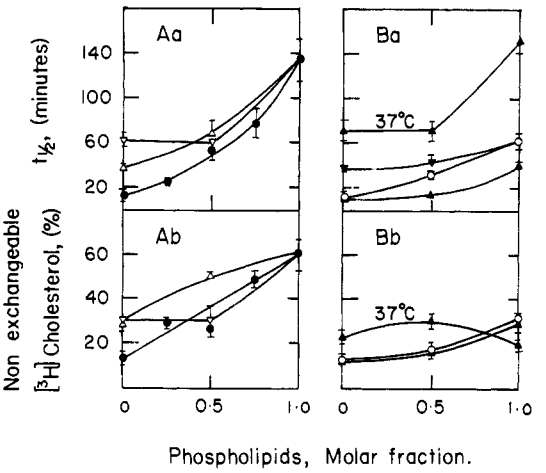

FIGURE 1: Half-life values (Aa and Ba) and percentages of nonexchangeable [^{3}H]cholesterol (Ab and Bb) remaining in neutral donor vesicles comprised of 10 mol % cholesterol in pure phospholipids and their binary mixtures at 50 °C (unless specified to 10-fold excess of negatively charged acceptor vesicles as calculated by a nonlinear least-squares analysis: POPC/bovine brain SPM (●); DPPC/bovine brain SPM (▽); DMPC/bovine brain SPM (△); POPC/DMPC (▲); POPC/DPPC (○); DMPC/DPPC (▼). The *x* axis represents the molar fraction of the second component in the binary phospholipid mixture.

(Aa and Ba) and the percent of nonexchangeable [^{3}H]-cholesterol (Ab and Bb) from liquid crystalline neutral donor vesicles containing 10 mol % cholesterol in pure phospholipids and equimolar binary mixtures of phospholipids. The kinetic data for the mixtures were fitted with a single exponential and a base value as were the pure phospholipids since this was found to give the best fit. The exchange parameters for an ideal mixture might be expected to be the mole fraction weighted average of the values for the pure phospholipid components. However, deviations from this situation can clearly be seen in the data in Figure 1. For systems containing bovine brain SPM (Aa and Ab), the exchange parameters are much closer to the corresponding values of the vesicles containing only phosphatidylcholine. For example, in [^{3}H]-cholesterol exchange from an equimolar mixture of bovine brain SPM and DPPC at 50 °C (▽) the exchange parameters ($t_{1/2}$ = 70 ± 20 min) and the percentage of nonexchangeable cholesterol (36 ± 6%) are similar to the values for [^{3}H]-cholesterol exchange from DPPC vesicles ($t_{1/2}$ = 62 ± 6 min, 31.5 ± 1.5%). Although the deviations from ideality are less pronounced for POPC/bovine brain SPM (●) and DMPC/bovine brain SPM (△) mixtures, they seem to be real as judged by the results obtained for half-life values of [^{3}H]cholesterol from vesicles containing molar ratios of 1:3 and 3:1 of POPC/bovine brain SPM (●).

For vesicles comprised of mixed phosphatidylcholines (Figure 1, Ba and Bb), the exchanged parameters appeared to be closer to those of one component according to the order POPC > DMPC > DPPC. For example, in an equimolar mixture of POPC/DMPC (▲), the exchange parameters for [^{3}H]cholesterol transfer at 37 °C (70 ± 8 min and 30 ± 3%) are very similar to the values obtained for cholesterol exchange in POPC-containing vesicles (71 ± 9 min and 23.0 ± 3.0%). The same behavior was observed for [^{3}H]cholesterol exchange from an equimolar mixture of POPC/DMPC at 50 °C (▲).

All of the experiments discussed above were performed at temperatures well above the transition temperature of the phospholipids involved. Vesicles comprised of DMPC/bovine brain SPM at different molar ratios at 37 °C will be in the liquid crystalline and gel phase depending on the amount of

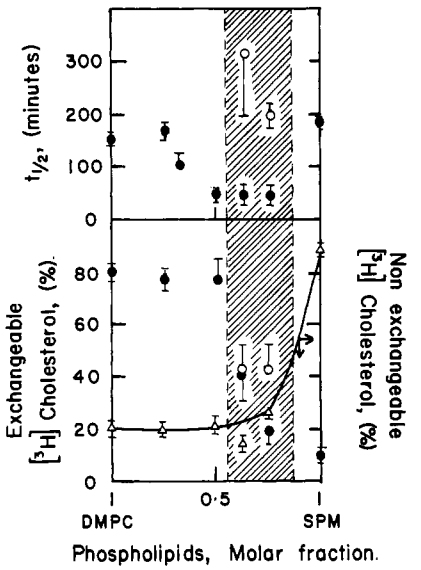

FIGURE 2: Half-life values and the percentages of exchangeable [^{3}H]cholesterol associated with them (●, ○) from neutral donor vesicles comprised of 10 mol % cholesterol in DMPC and bovine brain SPM and their binary mixtures at 37 °C. The open triangles (△) represent the percentage of nonexchangeable [^{3}H]cholesterol.

sphingomyelin (Calhoun & Shipley, 1979). The exchange parameters for [^{3}H]cholesterol from donor vesicles containing 10 mol % cholesterol at 37 °C in four different molar ratios of DMPC/bovine brain SPM and the pure components are presented in Figure 2. In vesicles comprised of pure DMPC and molar ratios of 3:1 and 2:1 of DMPC/SPM, the exchange parameters were found to be close to those of vesicles of DMPC alone, within experimental error (153 ± 10, 173 ± 15, and 104 ± 30 min, respectively) while for an equimolar ratio of the phospholipids the exchange rate was found to be 3 times larger ($t_{1/2}$ = 48 ± 12 min). Increasing the amount of sphingomyelin, the best fit was found to be a two-exponential function and an equilibrium value regardless of the amount of sphingomyelin (162 ± 131 and 48 ± 20 min) (hatched area in Figure 2). The slower rate is closer to the rate of cholesterol exchange in vesicles comprised of sphingomyelin alone (190 ± 100 min). The amount of nonexchangeable cholesterol remains constant at 21 ± 3% in all the mixtures examined.

Discussion

In a recent study of cholesterol exchange between POPC bilayers we showed the exchange to be characterized by a first-order rate constant and a small nonexchangeable pool of cholesterol (Bar et al., 1986). In this paper we examine the exchange of [^{3}H]cholesterol from other phospholipids and their mixtures.

Similar to cholesterol exchange in POPC vesicles, the transfer kinetics of both pure phospholipids and their binary mixtures was well described by a first-order model (Tables I–III). This suggests that cholesterol is transferred through the aqueous phase, as has been reported previously for a number of lipid systems (Bruckdorfer & Graham, 1976; Smith & Scow, 1979; Roseman & Thompson, 1980; McLean & Phillips, 1981, 1982).

At a given temperature the rates of [^{3}H]cholesterol exchange depend on the cholesterol concentration in vesicles containing DMPC or POPC (Table I). Similar behavior has been reported for cholesterol exchange from egg phosphatidylcholine vesicles over a concentration range of 1–40 mol % cholesterol (McLean & Phillips, 1982). A model in which cholesterol separates together with phospholipid in clusters of definite stoichiometry in equilibrium with liquid crystalline phospholipid has been proposed to describe this behavior (Phillips, 1972). However, in sphingomyelin-containing vesicles cholesterol exchange rates appear to be virtually independent of the cholesterol concentration. The reason for this is not clear. It is possible, however, that the low precision of these measurements, due to the small size of the exchangeable pool, obscures a dependence of the rate on cholesterol concentration.

The rate of [^{3}H]cholesterol exchange appears to be dependent on both the head group and the acyl chain composition of the matrix phospholipids as it can be seen from Tables I and II. At a given temperature and concentration, the exchange rates are slower in sphingomyelin- than in phosphatidylcholine-containing vesicles. The exchange of cholesterol appears to be a function of the fatty acid composition (Phillips, 1972). Thus, cholesterol molecules exchange more rapidly from unsaturated phosphatidylcholine bilayers, as can be seen in Table I. The same behavior is observed with cholesterol exchange from sphingomyelin derived from different sources. Thus, in vesicles made from bovine brain sphingomyelin, which contains up to 27% of saturated fatty acid (Barenholz & Gatt, 1982), spontaneous cholesterol exchange is faster than it is in vesicles made from bovine erythrocyte sphingomyelin and egg yolk sphingomyelin, which contain 90% and 70% of saturated fatty acids, respectively.

As in the previous kinetic study of cholesterol exchange from small unilamellar POPC vesicles (Bar et al., 1986), a nonexchangeable pool of [^{3}H]cholesterol was found in DMPC and sphingomyelin vesicles in the time frame of the experiments (Tables I–III). However, the size of the nonexchangeable pool in sphingomyelin system proved to be quite large. A similar result has been very recently reported by Yeagle and Young (1986). The percent of nonexchangeable cholesterol in all systems examined was found to be independent of the initial cholesterol concentration up to 10 mol % (Table I).

Previous calorimetric studies of liposome dispersions of mixtures of cholesterol with phosphatidylcholines (Estep et al., 1978; Mabrey et al., 1978) and with both synthetic (Estep et al., 1979; Calhoun & Shipley, 1979) and natural (Demel et al., 1977) sphingomyelins have demonstrated the coexistence of cholesterol-rich and cholesterol-free domains in these bilayer systems. The cholesterol-rich domains are gellike over the entire available temperature range while the cholesterol-free domains are liquid crystalline above and gel below the T_M of the phospholipid. Estep et al. (1978, 1979) have further suggested that certain characteristics of the heat capacity vs. temperature functions of these systems are best explained by the existence of disorganized interfacial region between these two coexisting phases.

Snyder and Freire (1980), using a Monte Carlo analysis and heat capacity data, calculated the relative abundance of the interfacial lipid in several gel phase systems containing cholesterol and phospholipids. They report that at a given concentration of cholesterol below 22 mol % the nonideality of mixing of cholesterol in the system increases in the order DMPC = *N*-palmitoylsphingomyelin < DPPC < *N*-lignoceroylsphingomyelin, and as a result the fraction of interfacial lipid decreases in the reverse order (Snyder & Freire, 1980). If we assume that cholesterol desorption in our systems proceeds primarily from the interfacial regions, then the size of the nonexchangeable cholesterol pool should increase as the fraction of lipid in the interfacial regions decreases. This would be the case, however, only if desorption of cholesterol from cholesterol-rich domains and the exchange of cholesterol from

the interior of these domains and the interfacial regions are very slow processes. There is experimental evidence that suggests that such a situation does in fact exist for desorption of a neutral glycosphingolipid from liquid crystalline phosphatidylcholine bilayers (Brown et al., 1985). The data we have presented show that the size of the nonexchangeable pool does increase in the order POPC < DMPC < DPPC < SPM, in rough qualitative agreement with the ordering of the increasing fractional content of interfacial lipid determined by Snyder and Freire (1980). Although the natural sphingomyelin preparations used in our study are heterogeneous in their *N*-acyl groups, both bovine brain and bovine erythrocyte sphingomyelins have substantial proportions of acyl chains of 20 carbons or more in length (Barenholz & Gatt, 1982). However, egg yolk sphingomyelin has a very high content of amide-linked palmitic acid (Barenholz & Gatt, 1982) and thus rather closely resembles the synthetic DL-*erythro*-*N*-palmitoylsphingomyelin discussed by Snyder and Freire (1980). On the basis of the analysis of the heat capacity data, egg yolk sphingomyelin would be expected to show a nonexchangeable cholesterol pool at 50 °C of about the same size as DMPC. It, however, has a pool size twice as large (Tables II and III). We have no ready explanation for this discrepancy.

The results for [^{3}H]cholesterol exchange from binary mixtures of phospholipids shown in Figure 1 are characterized by a single first-order rate constant. It seems reasonable to conclude from this that the desorbing molecules are contained in a single type of domain. The one exception to a single-exponential fit is for the DMPC/bovine brain sphinomyelin system at high sphingomyelin concentration. Discussion of this case is reserved until later.

Our interpretation of the results in binary phospholipid mixtures is based on the simple idea that the cholesterol desorption parameters in an ideal mixture of the phospholipids should be mole fraction weighted averages of the values of the parameters obtained for cholesterol exchange from systems containing the individual phospholipids alone plus cholesterol. Thus under our experimental conditions there would be two phases, one liquid crystalline comprised of the ideal mixture of the two phospholipids but no cholesterol and the other a gellike phase comprised of the phospholipid mixture plus cholesterol. Deviations from simple averaging of the kinetic parameters would reflect nonideality of mixing of the phospholipid components with a concomitant preferential interaction of cholesterol with one of the phospholipids. If under these circumstances only cholesterol-free and cholesterol-rich phases coexist, then by analogy to cholesterol desorption from a system of one phospholipid, desorption from the interface between these phases would resemble most closely desorption from that phospholipid that interacts less strongly with cholesterol. This would be the case since this phospholipid would be in higher concentration in the interface between the phases and in the cholesterol-free phase. The situation could, of course, be much more complex with more than two coexisting phases.

The data presented in Figure 1 (Ba and Bb) show that the kinetic parameters for the system of POPC/DMPC with 10 mol % cholesterol are much closer to the corresponding values obtained in POPC alone at 37 and 50 °C. A similar situation exists for the POPC/DPPC system. Thus, our conclusion based on the assumptions outlined above is that cholesterol preferentially interacts with DMPC and DPPC relative to POPC. Differential scanning calorimetric data for DPPC/POPC mixtures show the formation of a gel phase rich in DPPC at temperatures below 40 °C (Davis et al., 1980). Formation of a gel phase rich in DMPC probably occurs also in POPC/DMPC systems below about 20 °C since the acyl chain length mismatch is even greater than in the POPC/DPPC system. It is thus not difficult to believe that a gellike phase rich in the diacyl-saturated phosphatidylcholine and in cholesterol coexists with a cholesterol-free phase rich in POPC in these two cholesterol/mixed-phospholipid systems.

In the case of cholesterol exchange from binary phospholipid systems containing sphingomyelin, this line of argument leads to the conclusion that cholesterol preferentially interacts with sphingomyelin relative to the phosphatidylcholines. This conclusion is in agreement with calorimetric studies on binary mixtures of phospholipids containing cholesterol (Demel et al., 1977). It is also in agreement with conclusions based on the equilibrium partition of cholesterol between a reference system and single lamellar vesicles containing only one phospholipid (Lange et al., 1979).

The results obtained for [^{3}H]cholesterol transfer from mixtures containing DMPC and bovine brain SPM are compatible with the phase diagram for mixtures of these two phospholipids at the same temperature (Calhoun, & Shipley, 1979). Moreover, their thermal analysis of an equimolar mixture of DMPC and *N*-palmitoyl-SPM containing 10 mol % cholesterol shows the sharp and the broad components associated with the cholesterol-rich and cholesterol-free domains. Up to a molar ratio of 1 between the phospholipids, the cholesterol rate of exchange and the nonexchangeable pool are similar to the values obtained for DMPC vesicles. Again, cholesterol shows a higher affinity for the sphingomyelin molecules than for DMPC. In accordance with the phase diagram, at high ratios of SPM the coexistence of two phases is expected, the liquid crystalline phase rich in DMPC molecules and the gel phase rich in SPM. Consequently, two rate constants were found to be the best fit for the [^{3}H]cholesterol exchange in this region. The slower rate is similar to that obtained for cholesterol in SPM vesicles, in agreement with the existence of a phase rich in SPM. Interestingly, the faster rate is now 3 times the value observed for DMPC vesicles at this temperature.

The cholesterol desorption data presented in this paper clearly reflect differences in the molecular interactions between cholesterol and various phospholipids. We believe in addition that the kinetic parameters can be interpreted in terms of the phase structures of the individual systems. It will be of considerable interest to examine cholesterol desorption parameters in more complex phospholipid bilayer systems containing a membrane protein and/or glycosphingolipids.

Registry No. POPC, 6753-55-5; POPG, 81490-05-3; DPPC, 2644-64-6; DPPG, 4537-77-3; DMPC, 13699-48-4; DMPG, 61361-72-6; cholesterol, 57-88-5.

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Analysis of a Specific Oxygenation Reaction of Soybean Lipoxygenase-1 with Fatty Acids Esterified in Phospholipids†

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ABSTRACT: Soybean lipoxygenase was reacted with phosphatidylcholine (at pH 9, with 10 mM deoxycholate), and the oxygenation products were analyzed by high-pressure liquid chromatography, UV, gas chromatography–mass spectrometry (GC–MS), and NMR. The structures of the intact glycerolipid products were established by GC–MS of diglycerides recovered by phospholipase C hydrolysis and by proton NMR of the intact phosphatidylcholine. These analyses, together with analyses of the transesterified fatty acids, indicated that arachidonyl and linoleoyl moieties in the phosphatidylcholine were converted exclusively to the 15(*S*)-hydroperoxy-5(*Z*),8(*Z*),11(*Z*),13(*E*)-eicosatetraenoate and 13(*S*)-hydroperoxy-9(*Z*),11(*E*)-octadecadienoate analogues, respectively. Control experiments proved that the intact phospholipid (and not hydrolyzed/reesterified fatty acid) was the true substrate of the oxygenation reaction. Phosphatidylethanolamine and phosphatidylinositol lipids were also substrates for specific oxygenation by the soybean lipoxygenase. The results provide concrete evidence that fatty acids esterified in phospholipid can be subject to highly specific oxygenation by a lipoxygenase enzyme.

There are two main isoenzymes in soybeans, and these are classified on the basis of their substrate specificities, pH optima, and the specificity of the oxygenation reaction they catalyze with polyunsaturated lipids. The classic enzyme originally isolated by Theorell, Holman, and Akeson (1947) is now called soybean lipoxygenase-1. This enzyme is known to react with polyunsaturated fatty acids with optimal activity around pH 9. The enzyme is quite discriminating in terms of the range of acceptable fatty acid substrates, and characteristically, a single major hydroperoxide is formed as product (Hamberg & Samuelsson, 1966; Holman et al., 1969). Typically, the hydroperoxide group is introduced into the free fatty acid substrate in the ω6 position and in the Ls steric configuration (Hamberg & Samuelsson, 1967). By contrast, the soybean lipoxygenase-2 will react with free acid or ester substrates with the optimal activity being around pH 6.5. This isoenzyme will accept a wide range of polyunsaturated lipid substrates, and it will catalyze oxygenation with little or no positional and stereo specificity. In effect, the soybean lipoxygenase-2 is a catalyst of enzyme induced autoxidation of polyunsaturated lipids. Although there are several reports of oxygenation of esterified polyunsaturated lipids by soybean lipoxygenase(s) [e.g., Koch et al. (1958), Guss et al. (1968), and Christopher et al. (1970)], these would generally be considered as reactions of the nonspecific soybean lipoxygenase-2 isoenzyme. Invariably, the reactions that have been subjected to product analysis have been shown to be lacking in specificity of oxygenation [e.g., Christopher et al. (1972) and Roza and Francke (1978)].

The dogma stemming from these findings is that positional-specific and stereo-specific oxygenation is associated with reaction of a lipoxygenase and a free acid substrate. However, this paper is concerned with establishing that this need not be the case. A recent report by Eskola and Laasko (1983)

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