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Complete Exchangeability of Cholesterol in Phosphatidylcholine/Cholesterol Vesicles of Different Degrees of Unsaturation†

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ABSTRACT: [^{14}C]Cholesterol/phosphatidylcholine vesicles (molar ratio 0.8:1) prepared by sonication were fractionated on agarose columns. Cholesterol exchange between vesicles and erythrocytes is 13-fold faster than phosphatidylcholine exchange. Vesicles of labeled cholesterol and phosphatidylcholine of different degrees of unsaturation were incubated with an excess of erythrocytes. The half-lives for cholesterol exchange, for cholesterol incorporated in dipalmitoyl-, dimyristoyl-, dioleyl-, egg, and soy phosphatidylcholine vesicles are 4.7, 3.9, 2.6, 3.1, and 1.8 h, respectively. Nearly all the cholesterol in the sonicated vesicles appears to be exchangeable. These results indicate: (1) that the rate of cholesterol exchange is faster in the presence of unsaturated phosphatidylcholines and (2) that the inside–outside transposition of cholesterol in the sonicated vesicles is at least as fast as its removal from the vesicles. Thus, for soy phosphatidylcholine vesicles the half-time of transposition is less than 2 h. The exchange of cholesterol from the sonicated vesicles eluted in the void volume of the agarose column is 1.8 to 2.5 times slower than the exchange from unilamellar vesicles of the same composition. The rate is also dependent upon the unsaturation of the phosphatidylcholine, and all of the cholesterol is exchangeable as well. The presence of 5% lysophosphatidylcholine in dipalmitoylphosphatidylcholine/cholesterol vesicles does not alter the cholesterol exchange rate in large or small vesicles.

The transmembrane movement of phospholipids in single-walled sonicated vesicles appears to be a very slow or nonexistent process. Johnson et al. (1975) showed that the transmembrane migration of PC[1] from rat liver has a half-life of 4 days or more. Similar results have been found by Rothman and Dawidowicz (1975) in vesicles of dioleylphosphatidylcholine. Roseman et al. (1975) measured the transmembrane migration of phosphatidylethanolamine in phosphatidylethanolamine/PC vesicles and found that the process exhibits a half-life of the order of weeks.

The rate of transmembrane migration of cholesterol, however, has not been clearly established. Bruckdorfer et al. (1968) incubated sonicated vesicles, formed by egg PC and [^{3}H]cholesterol, with rat erythrocyte ghosts and found that after 24 h at 37 °C complete equilibration of the label had taken place, with no net transfer of cholesterol in either direction. On the other hand, Poznansky and Lange (1976) found that in vesicles of dipalmitoylphosphatidylcholine and cholesterol only 70% of the latter is available for exchange with erythrocyte ghosts. The remaining 30% was thought to be part of the inner monolayer and not subject to significant transmembrane movement. Smith and Green (1974), using a fluorescent analogue of cholesterol, sterophenol, concluded that in egg PC vesicles, the half-life for transmembrane migration of sterophenol is between 1 and 1.5 h.

In this paper, we have examined the movement of cholesterol in sonicated vesicles of PC of different degrees of unsaturation.

Materials and Methods

Lipids. Glycerol tri[9,10-^{3}H]oleate (specific activity 142 mCi/mmol) was from New England Nuclear Corp. (Boston, Mass.). It was purified by silica gel H thin-layer chromatography with hexane–diethyl ether–acetic acid (60:40:1, v/v) (Bloj and Zilversmit, 1976). [4-^{14}C]Cholesterol and [1,2-^{3}H]cholesterol (specific activities 61 and 43 mCi/mmol, respectively) were from Amersham/Searle (Arlington Heights, Ill.). They were purified by silica gel H thin-layer chromatography with hexane–diethyl ether (50:50, v/v). The cholesterol band was eluted with chloroform, diluted with unlabeled cholesterol to a specific activity of approximately 2000 dpm/μg, and stored at −20 °C in the dark. The purity was greater than 99%.

^{32}P-labeled rat liver PC and soy PC (Nattermann, Köln,

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[1] Abbreviations used are: PC, phosphatidylcholine; Na_2EDTA, disodium (ethylenedinitrilo)tetraacetate.

Germany) were purified by neutral alumina chromatography (Johnson and Zilversmit, 1975). Egg PC (grade I) was obtained from Lipid Products Inc. (England); dipalmitoyl-, dimyristoyl-, and dioleyl-PC were from Sigma Chemical Co. (St. Louis, Mo.). The purity was checked by thin-layer chromatography and found to be greater than 99%, except for dioleyl-PC which contained 3% lyso-PC. This amount of lyso-PC does not affect cholesterol exchange (see Results).

Phospholipids were stored in chloroform (1–2 mg/mL) at −20 °C in the dark. Unlabeled cholesterol (Sigma Chemical Co.) was purified as the dibromide derivative and crystallized from methanol. Butylated hydroxytoluene (Nutritional Biochemical Corp., Cleveland, Ohio) was used without further purification.

1,2-[1-^{14}C]Dipalmitoyl-*sn*-glycero-3-phosphatidylcholine (specific activity 80 mCi/mmol) from Applied Science Laboratories Inc., State College, Pa., was purified by thin-layer chromatography with chloroform–methanol–acetic acid–water (25:15:4:2, v/v). The PC was eluted with chloroform–methanol–water (80:35:5, v/v) and stored under N_2 in chloroform–methanol (2:1) at −20 °C. Lyso-PC derived from egg PC was obtained from Avanti Biochemicals, Inc., Birmingham, Ala.

Buffer Solution. The same buffer was used for the preparation of sonicated vesicles, gel filtration through Bio-Gel A-50, washing of red blood cells, and incubation of sonicated vesicles with erythrocytes or erythrocyte membranes. The buffer was prepared by adding (in millimoles) 1.2 $MgCl_2$, 2.4 $CaCl_2$, 4.2 NaH_2PO_4, 1.7 Na_2HPO_4, 5 KCl, 118 NaCl, 13.5 $NaHCO_3$, and 10 glucose to 1 L of distilled water. The pH was adjusted to 7.4 with NaOH and a small precipitate removed by filtration. Streptomycin sulfate (10 mg %) and benzylpenicillin (sodium salt, 6 mg %) were added to the solution.

Preparation of Sonicated Vesicles and Fractionation by Gel Filtration. For preparation of sonicated vesicles, aliquots containing 10 mg of PC, 4.2 mg of [^{14}C]cholesterol, 0.10 mg of butylated hydroxytoluene, and a small amount of [^{3}H]triolein (0.2 mol %) were evaporated under a stream of N_2 in a test tube. The dried lipids were redissolved in approximately 4 mL of diethyl ether (in the case of dipalmitoyl- and dimyristoyl-PC, chloroform–ether, 50:50, v/v, was used), and slowly redried under N_2 to form a thin film on the walls of the tube. Two milliliters of buffer was added; the tube was flushed with N_2 and capped. After agitation in a vortex mixer for 5–10 min, the lipid suspension was sonicated in a sonicating bath (Laboratory Supply Co., Hicksville, N.Y.) for 2 h. The water bath was maintained between 0 and 10 °C for dioleyl-, rat liver, egg, and soy PC. Dimyristoyl-PC was sonicated between 25 and 35 °C, and dipalmitoyl-PC between 40 and 50 °C. After sonication, the vesicles were centrifuged for 15 min at 40 000*g* and the pellet was discarded. The supernatant contained 70 to 95% of the radioactivity without selective loss of radioisotope. No increase in the lyso-PC content was observed during sonication. Except when otherwise stated, the sonicated vesicles were fractionated by gel filtration through agarose columns (Bio-Gel A-50 100–200 mesh, Bio-Rad Laboratories, Richmond, Calif.). The columns (45 × 2.5 cm) were operated at room temperature when the vesicles contained saturated PC and at 4 °C when the PC in the vesicles were unsaturated.

Isolation of Red Blood Cells. Blood was obtained by heart puncture from 400–600 g fed, male albino rats of the Sprague Dawley strain (Blue Spruce Farms, Altamont, N.Y.). Na_2EDTA 100 mM (1 mL/10 mL of blood) was used as anticoagulant. The blood was centrifuged for 15 min at 2500*g* and the plasma and white cells were discarded. The red blood cells were washed three or four times with 2–3 volumes of buffer. After each centrifugation, the top portion of the red cells was discarded. The cells were finally suspended in an equal volume of buffer and used within 24 h. The cholesterol content averaged 1.4 mg/mL of packed cells. Human and rat erythrocyte ghosts were prepared according to Steck (1974). Human erythrocyte ghosts incubated for 24 h at 37 °C with dipalmitoyl-PC/cholesterol vesicles containing [^{3}H]triolein showed no evidence of lipase activity.

Incubations. Typically, vesicles containing 65 μg of [^{14}C]cholesterol, the appropriate PC, and [^{3}H]triolein were incubated by gentle agitation at 37 °C with red blood cells (2100 μg of cholesterol) in a final volume of 10 mL. The incubation mixture contained 5 mg/mL of bovine serum albumin (fraction V powder, fatty acid poor, Miles Laboratories Inc., Kankakee, Ill.). At zero time, and different times thereafter, 0.7-mL portions of the incubation mixture were centrifuged for 2 min at 8000*g* (Eppendorf, centrifuge 3200) and 0.5 mL of the supernatants were removed. These aliquots were adjusted to 1 mL with distilled water and 20 μg of nonlabeled triolein in 0.1 mL of chloroform–methanol, 2:1 (v/v), was added as carrier. The lipids were partitioned with 5 mL of chloroform–methanol, 2:1 (v/v). After centrifugation (10 min, 3000*g*), two clear phases were separated by a compact interphase of denatured protein. The lower phase was removed, dried down, and counted in 10 mL of liquid scintillation counting medium (Gordon and Wolfe, 1960) in a Packard Tri Carb Scintillation spectrophotometer (Model 3375). Appropriate overlap and efficiency corrections were made. The amount of labeled cholesterol removed from the vesicles was calculated as the diminution in the $^{14}C/^3H$ ratio of the supernatant, and expressed as percentage decrease from the zero time ratio. Hemolysis of the red blood cell was less than 5% after 24 h of incubation.

Results

Gel Filtration of PC/Cholesterol Sonicated Vesicles. When the PC/cholesterol sonicated vesicles were fractionated by gel filtration through agarose columns, two lipid peaks were observed. A profile of one of the fractionations is shown in Figure 1. In this experiment, the vesicles were prepared from rat liver [^{32}P]PC and [^{14}C]cholesterol. The elution profile for both radioisotopes is shown as well as the $^{32}P/^{14}C$ ratio. The PC and cholesterol were distributed in a constant ratio throughout the fractions. The recovery from the column was 80% in this experiment, and ranged between 76 and 100% for the different preparations. In similar experiments, with [^{14}C]dipalmitoyl-PC and [^{3}H]cholesterol a constant ratio was also obtained.

In the experiment shown in Figure 1, the first peak accounted for 20% of the lipid recovered, and its size varied according to the kind of PC used: about 40% for egg and dioleyl-PC, about 20% for soy and dimyristoyl-PC and about 9% for dipalmitoyl-PC. In the exchange experiments, the pooled fractions from the leading half of the first peak and the trailing half of the second peak were used. When sonicated vesicles, containing [^{3}H]triolein, were subjected to gel filtration, we observed that, except for soy PC vesicles, the marker was not homogeneously distributed, being associated preferentially with the vesicles of the first peak and decreasing monotonically as the vesicle size decreased.

Intact Erythrocytes and Erythrocyte Ghosts as Acceptors of Labeled Cholesterol from Sonicated Vesicles. Preliminary experiments were performed to choose between intact erythrocytes and erythrocyte ghosts as acceptors of labeled cholesterol from sonicated vesicles. The recovery of sonicated vesicles in the supernatant after centrifugation was measured

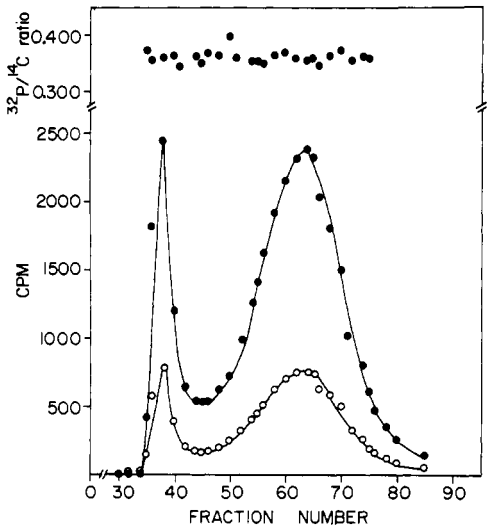

FIGURE 1: Elution pattern of sonicated [^{32}P]phosphatidylcholine and [^{14}C]cholesterol vesicles fractionated through a column (43 × 2.5 cm) of Bio-Gel A-50. Fractions of 2.8 mL were collected each 15 min: (●-●) ^{14}C; (○-○) ^{32}P.

by the recovery of [^{3}H]triolein, a nonexchangeable marker (Zilversmit and Hughes, 1976). When sonicated vesicles were incubated with intact erythrocytes, about 90% of the labeled triolein remained in the supernatant during 4 h of incubation (Table I). On the other hand, when vesicles were incubated with erythrocyte ghosts, the recovery of vesicles decreased steadily with the length of incubation, and after 4 h only 19% of the vesicles remained in the supernatant. The presence of hemolysate or albumin in the medium substantially improved the recovery. In the case of soy PC vesicles, the exchange of cholesterol seems to be 2-3 times faster when intact erythrocytes are used. This was not found to be the case for dipalmitoyl-PC vesicles.

The exchange of cholesterol between sonicated vesicles and intact red blood cells was investigated in longer term incubations, as shown in Table II. One set of experiments was performed with sonicated vesicles of PC of different degrees of unsaturation in the presence of very low concentrations of albumin. Experiments 1-5 show that after 28-32 h of incubation more than 90% of the radioactive cholesterol in the vesicles has been exchanged. The recovery of the sonicated vesicles was variable. In experiments 6 and 7, the incubations were carried out with unilamellar vesicles obtained by gel filtration after sonication. The results show that the extent of cholesterol exchange is independent of the presence of albumin in the incubation medium, but that albumin is essential to ensure near quantitative recovery of the vesicles. The effect of albumin on the initial rate of cholesterol exchange was also evaluated. A 10% increment was seen in one experiment, no effect in another experiment, and 10% inhibition in a third experiment. In subsequent incubations we have incorporated 5 mg/mL of bovine serum albumin, which not only improved recoveries but also prevented hemolysis of the red blood cells during prolonged incubations.

Comparison of the Exchange of PC and Cholesterol between Red Blood Cells and Sonicated Vesicles. In the following experiments, the exchanges of PC and of cholesterol between sonicated vesicles and red blood cells are compared in vesicles containing both labeled components. In the experiment depicted in Figure 2, the sonicated vesicles consisted of [^{32}P]phosphatidylcholine from rat liver and of [^{14}C]cholesterol. Since the presence of albumin ensured quantitative recovery of the vesicles in the supernatants, the decrease of label from the supernatant after centrifugation was used as a measure of exchange. After 1.5 h of incubation, 45% of the labeled cholesterol had been removed from the vesicles, without a detectable diminution in the label of the PC. After 3 h, when 60% of the labeled cholesterol had been removed from the vesicles, only 7% of the PC had been exchanged. A similar

TABLE I: Interaction of Sonicated Vesicles with Erythrocytes and Erythrocyte Ghosts.[a]

	Recovery (%)			Exchange (%)		
	Time (min)			Time (min)		
Acceptor	45	90	240	45	90	240
Erythrocytes	89	87	89	14	29	66
Erythrocyte ghosts	51	39	19	−10[b]	3	−44[b]
Erythrocyte ghosts + hemolysate	88	97	72	7	5	11
Erythrocyte ghosts + bovine serum albumin (5 mg/mL)	99	97	91	2	11	33

[a] Soy PC/cholesterol vesicles (25 μg of [^{14}C]cholesterol) with a trace of [^{3}H]triolein were incubated with intact erythrocytes or erythrocyte ghosts (700 μg of cholesterol) in a final volume of 2.5 mL. At different times, aliquots of the mixture were centrifuged (2 min, 8000*g* for erythrocytes, 15 min 35 000*g* for erythrocyte ghosts) and 0.4 mL of the supernatants was extracted and counted. The transfer of [^{14}C]cholesterol was measured as the diminution of the $^{14}C/^3H$ ratio in the supernatant. The recovery of vesicles was monitored by the recovery of ^{3}H in the supernatant. [b] In two separate experiments, in which sonicated vesicles were incubated with erythrocyte ghosts, poor recovery of the vesicles paralleled negative values for exchange. Extensive fusion or adsorption of the vesicles with the membranes might be involved. When the recovery of unfractionated vesicles is low, biased results may be obtained (DiCorleto and Zilversmit, 1977).

TABLE II: Influence of Bovine Serum Albumin on the Exchange of Cholesterol between Red Blood Cells and Sonicated Vesicles[a]

Type of vesicle	PC[b]	Time of incubation (h)	BSA (mg/mL)	Cholesterol exchanged (%)	Recovery of vesicles (%)
Nonfractionated					
1	DPPC	28	0.01	92	96
2	DMPC	30	0.01	90	36
3	egg PC	28	0.01	95	91
4	DOPC	32	0.01	93	54
5	soy PC	30	0.01	99	21
Fractionated					
6	DPPC	27	0	97	73
			5	94	91
7	soy PC	7	0	84	29
			5	92	92

[a] PC/cholesterol vesicles (6 μg of [^{14}C]cholesterol/mL) were incubated with red blood cells (210 μg of cholesterol/mL) at 37 °C. Where indicated, bovine serum albumin was also present. The red blood cells were replaced twice during the incubations. At different times, aliquots were centrifuged and the supernatants extracted as described under Materials and Methods. The sonicated vesicles contained [^{3}H]triolein as nonexchangeable marker. The exchange of cholesterol was calculated from the diminution in the $^{14}C/^3H$ ratio. The recovery of vesicles was monitored by the recovery of ^{3}H in the supernatant. In the experiments with fractionated vesicles, the trailing half of the second peak eluted from the agarose columns was used. [b] Phosphatidylcholine (PC) used: DP, dipalmitoyl; DM, dimyristoyl; DO, dioleyl.

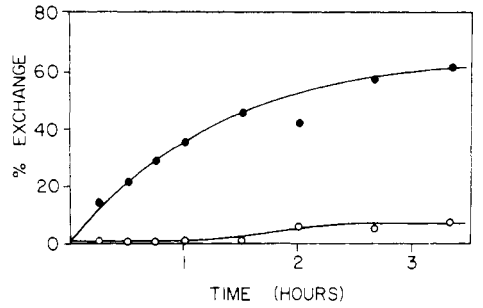

FIGURE 2: Exchange of PC (O-O) and cholesterol (●-●) between sonicated fractionated small vesicles and red blood cells. ^{32}P labeled phosphatidylcholine/^{14}C cholesterol vesicles (50 μg of cholesterol) were incubated with red blood cells (700 μg of cholesterol) in a final volume of 4.25 mL in the presence of bovine serum albumin (5 mg/mL).

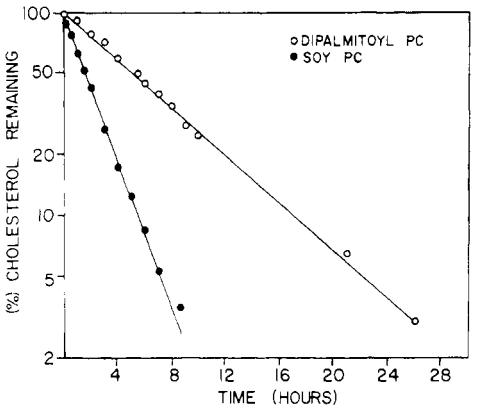

FIGURE 3: Extensive exchange of cholesterol between sonicated vesicles and red blood cells. The details are described under Materials and Methods. The data points are averages obtained on two different preparations.

experiment was performed with vesicles of [^3H]cholesterol and [^{14}C]dipalmitoyl-PC. The half-lives for removal of both labels from the vesicles were 4.6 h for cholesterol and 58 h for dipalmitoyl-PC.

Effect of Phosphatidylcholines of Different Degrees of Unsaturation on Cholesterol Exchange. Cholesterol/PC vesicles (molar ratio 0.8:1) were prepared with synthetic or with natural phosphatidylcholines of different degrees of unsaturation. Unilamellar vesicles were isolated by gel chromatography and subjected to extensive exchange with intact red blood cells. Figure 3 shows two typical experiments in which the vesicles consisted of dipalmitoyl- or soy PC and cholesterol. The ratio of total cholesterol in vesicles and red blood cells was 1:32. The red blood cells were replaced twice during the incubations (after 4 and 8 h for dipalmitoyl-PC and after 1 and 3 h of incubation in the case of soy PC) to ensure that no return of radioactive cholesterol from the erythrocyte to the vesicles occurred. After 26 h of incubation, 97% of the labeled cholesterol had been removed from the dipalmitoyl-PC vesicles. Similar amounts of label were removed from the soy PC vesicles in about 8 h. These experiments suggest that the rate of cholesterol exchange is dependent upon the unsaturation of the PC used in the preparation of the sonicated vesicles. Similar experiments were carried out with vesicles formed by dimyristoyl-PC, egg PC, and dioleyl-PC. The erythrocytes were changed twice during the incubations. The single-exponential curves fell between the two depicted in Figure 3. The half-lives for cholesterol exchange from the different vesicles are given in Table III.

In a parallel set of experiments we have measured the rate

TABLE III: Half-Lives for the Transfer of [^{14}C]Cholesterol from Phosphatidylcholine/Cholesterol Liposomes to Red Blood Cells.

PC[c]	Half-lives (h)	
	Single-walled vesicles[a]	Multilamellar vesicles[b]
DPPC	4.7 (4.5-5.0)	9.6
DMPC	3.9 (4.0-3.8)	9.8
egg PC	3.1 (3.0-3.2)	6.5
DOPC	2.6 (2.6-2.6)	4.8
soy PC	1.8 (1.8-1.8)	
DPPC + lyso-PC (5%)	4.1	10.9

[a] Values are the average of two to three experiments, with the range between parentheses. Two preparations of dipalmitoyl-, egg, and soy PC were made and tested immediately after sonication. In each instance the second preparation was tested again 3-5 days later. One preparation of dimyristoyl- and dioleyl-PC was tested immediately after sonication and again 4-7 days later. [b] Vesicles appeared in the leading half of the first peak of Bio-Gel A-50 columns and are probably multilamellar (Newman and Huang, 1975). Values are from single experiments. [c] Phosphatidylcholine (PC) used: DP, dipalmitoyl; DM, dimyristoyl; DO, dioleyl.

of exchange of cholesterol between red blood cells and sonicated vesicles eluted in the first peak of the agarose columns. The process was found to be slower than the exchange from single-walled vesicles. In all cases the experimental points fitted a single exponential curve. The experiments were continued up to 90% exchange. The half-lives of cholesterol exchange shown in Table III are 1.8 to 2.5 times larger than the ones found for the single-walled vesicles of the same composition, and depend upon the degree of phospholipid unsaturation.

The effect of adding 5% lyso-PC to dipalmitoyl-PC/cholesterol liposomes was evaluated. After fractionation through agarose, the cholesterol exchange was measured with vesicles from the first and second peaks. The presence of this amount of lyso-PC seems to have a very minor effect, as seen from the half-life values in Table III.

Discussion

Bruckdorfer et al. (1968) showed that when sonicated vesicles, composed of [^3H]cholesterol and egg PC (molar ratio 0.8:1), are incubated with rat erythrocyte ghosts almost complete equilibration of label occurs after 24 h, without net transfer of cholesterol in either direction. These results have been corroborated by Cooper et al. (1975) using dipalmitoyl-PC/cholesterol vesicles and intact red blood cells. In the experiments presented here, we have used sonicated vesicles with the same molar ratio, so as to avoid a net cholesterol transfer. We have found that the exchange of cholesterol is 2-3 times faster with intact red blood cells as acceptors than with erythrocyte ghosts. The addition of hemolysate equivalent to that contained in the intact erythrocytes did not restore the cholesterol exchange efficiency of the ghosts to that of the red blood cells. This indicates that a change in membrane structure due to hypotonic hemolysis was responsible for the difference in cholesterol exchange rates.

Complexes of different stoichiometry between PC and cholesterol have been postulated (Bruckdorfer and Graham, 1976; Demel and De Kruyff, 1976). If these complexes should exchange as a unit, the exchange rates of the two lipids would be similar. However, the rate of cholesterol exchange in our vesicles was several times faster than the exchange of PC. In addition, the rate of cholesterol exchange was affected greatly by differences in the degree of PC unsaturation.

The transmembrane movement of PC in red blood cell

ghosts is considerably faster than that in sonicated unilamellar PC vesicles (Bloj and Zilversmit, 1976). It is interesting, therefore, that in a different biological membrane, i.e., the influenza viral membrane, the transposition of cholesterol is much slower (Lenard and Rothman, 1976) than that reported for sonicated vesicles in the present study.

When sonicated PC vesicles contain more than 30 mol % cholesterol, cholesterol appears to concentrate in the inner monolayer (Huang et al., 1974; Newman and Huang, 1975; De Kruyff et al., 1976). During incubations with an excess of red blood cells, the loss of labeled cholesterol from the vesicles can be represented by a single exponential function, which indicates that the cholesterol in the vesicles behaves as a single pool. Since it is unlikely that the red blood cells could interact with the inner monolayer of the vesicles, we conclude that transposition of cholesterol between inner and outer monolayers is fast compared to the exchange reaction. Although the results indicate that the rate of cholesterol exchange between red blood cells and vesicles is higher for the more unsaturated PC, no conclusion can be drawn about the effect of unsaturation on the rate of transmembrane movement of the sterol.

Preliminary exchange experiments were performed with sonicated vesicles, which had not been fractionated by gel filtration through agarose columns. Most of the curves obtained were biphasic. This is in agreement with the observation that, under our sonication conditions, not all of the PC and cholesterol was present in single-walled vesicles. The vesicles present in the void volume of the agarose column probably are multilamellar (Huang, 1969; Newman and Huang, 1975) and were observed to exchange cholesterol at a slower rate. The biphasic curves were not observed with the dipalmitoyl-PC/cholesterol liposomes, which is consistent with the finding that for these vesicles only 8% of the labeled cholesterol was present in the void volume of the agarose column.

Our results, showing complete exchange of the cholesterol from dipalmitoyl-PC/cholesterol vesicles, do not agree with the observations of Poznansky and Lange (1976) who observed that 30% of the cholesterol in such vesicles is nonexchangeable. We have attempted to discover the basis for this difference by introducing a number of procedural changes which would tend to equalize the techniques used in the two laboratories. Accordingly, we have repeated the exchange experiments with dipalmitoyl-PC vesicles under the following conditions in which in each comparison the first condition is the one used by us and the second one resembles as closely as possible those of Poznansky and Lange: (1) phospholipid cholesterol mixtures evaporated either from chloroform/diethyl ether (1:1) or lyophilized from benzene (personal communication); (2) vesicles with or without BHT; (3) bath sonicator or probe sonicator; (4) sonication at 40 or at 4 °C; (5) use of sized vesicles or of unsized ones; (6) 8×10^{-5} and 10^{-6} M albumin concentrations in the medium; (7) use of intact rat erythrocytes or of human or rat erythrocyte ghosts as acceptors; (8) replacing the acceptor membranes during the exchange experiments or not replacing them; (9) cholesterol/PC molar ratio of 0.8/1.0 or of 0.9/1.0.

Although the various procedural alterations affected the rates of cholesterol exchange slightly, none of them gave rise to a nonexchangeable cholesterol fraction comparable to that found by Poznansky and Lange. The two conditions that seemed to increase the apparently nonexchangeable fraction slightly were: nonreplacement of acceptor membranes and use of the heterogeneous (nonsized) vesicle preparation. However, when all the procedural changes were combined into one experiment 86% of the cholesterol had exchanged in 30 h, without showing any tendency of reaching a plateau. In the experiments of Poznansky and Lange, [^{14}C]dipalmitoyl PC was used as nonexchangeable marker, whereas we used [^{3}H]triolein. We have observed that dipalmitoyl-PC exchanges slowly with red blood cells, although this difference can probably not account for the differences in exchangeable cholesterol observed by Poznansky and Lange and by us.

In our experiments, the movement of cholesterol between sonicated vesicles and red blood cells was faster when a more unsaturated PC was used. This resembles the finding (Spritz, 1965) that cholesterol of very low density lipoproteins from rabbits fed safflower oil exchanged significantly faster than cholesterol in very low density lipoproteins from rabbits fed coconut oil. Similarly, Yeh et al. (1974) observed that the exchange of cholesterol between plasma and red blood cells from pigs fed hydrogenated soybean oil (which contained 50% trans fatty acids) was slower than the exchange in the control animals. These results suggest that the mechanism by which cholesterol is exchanged between red blood cells and plasma lipoproteins might be similar to the mechanism of exchange between sonicated vesicles and red blood cells. Perhaps the phospholipid fatty acid composition of cell membranes is a determinant for the rate at which cholesterol is transported between and within cells.

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References

Bloj, B., and Zilversmit, D. B. (1976), *Biochemistry 15,* 1277.

Bruckdorfer, K. R., Edwards, P. A., and Green, C. (1968), *Eur. J. Biochem. 4,* 506.

Bruckdorfer, K. R., and Graham, J. M. (1976), *Biol. Membr. 3,* 103.

Cooper, R. A., Arner, E. C., Wiley, J. S., and Shattil, S. J. (1975), *J. Clin. Invest. 55,* 115.

De Kruyff, B., Cullis, P. R., and Radda, G. K. (1976), *Biochim. Biophys. Acta 436,* 729.

Demel, R. A., and De Kruyff, B. (1976), *Biochim. Biophys. Acta 457,* 109.

DiCorleto, P. E., and Zilversmit, D. B. (1977), *Biochemistry 16,* 2145.

Gordon, C. F., and Wolfe, A. L. (1960), *Anal. Chem. 32,* 574.

Huang, C. (1969), *Biochemistry 8,* 344.

Huang, C., Sipe, J. P., Chow, S. T., and Martin, R. B. (1974), *Proc. Natl. Acad. Sci. U.S.A. 71,* 359.

Johnson, L. W., Hughes, M. E., and Zilversmit, D. B. (1975), *Biochim. Biophys. Acta 375,* 176.

Johnson, L. W., and Zilversmit, D. B. (1975), *Biochim. Biophys. Acta 375,* 165.

Lenard, J., and Rothman, J. E. (1976), *Proc. Natl. Acad. Sci. U.S.A. 73,* 391.

Newman, G. C., and Huang, C. (1975), *Biochemistry 14,* 3363.

Poznansky, M., and Lange, Y. (1976), *Nature (London) 259,* 420.

Roseman, M., Litman, B. J., and Thompson, T. E. (1975), *Biochemistry 14,* 4826.

Rothman, J. E., and Dawidowicz, E. A. (1975), *Biochemistry*

14, 2809.
Smith, R. J. M., and Green, C. (1974), *FEBS Lett. 42*, 108.
Spritz, N. (1965), *J. Clin. Invest. 44*, 339.
Steck, T. L. (1974), *Methods Membr. Biol. 2*, 245.
Yeh, S. C., Mizuguchi, T., and Kummerow, F. A. (1974), *Proc. Soc. Exp. Biol. Med. 146*, 236.
Zilversmit, D. B., and Hughes, M. E. (1976), *Methods Membr. Biol. 7*, 211.

Phosphatidylcholine ^{13}C-Labeled Carbonyls as a Probe of Bilayer Structure†

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ABSTRACT: Dipalmitoyl- and dihexanoylphosphatidylcholine have been synthesized using fatty acids which have the acyl carbonyl carbons enriched with carbon-13. The chemical shifts of these carbonyl carbons, which are known to be sensitive to intermolecular interactions, have been measured in a variety of solvents, including aqueous dispersions. The use of dihexanoylphosphatidylcholine permits the observation of molecules in both monomer and micelle forms in aqueous solutions. Carbon-13–proton two- and three-bond coupling constants have also been measured. From these data, it can be concluded that, when the molecules are in bilayers, the observed shifts are determined by hydrogen bonding of the carbonyl oxygens with the water, even though there is partial exclusion of water molecules from this region of the bilayer. The extent of water exclusion can be quantified and taken as a measure of molecular packing. The hydration difference between carbonyls of molecules on the the inside and outside of small single-walled vesicles is found to be 0.05. Furthermore, the relative shifts of the two carbonyl carbon-13's indicate that the fatty acid esterified to the 1-carbon of the glycerol is less accessible to water than that esterified to the 2-carbon of glycerol.

Nearly all of the nuclear magnetic resonance (NMR)[1] studies of aqueous phospholipid dispersions done to date have been concerned with the various motional parameters. Aside from the obvious reason for focusing on the dynamic structure of phospholipids—that the motions are important in determining bilayer properties—there are several reasons why the more traditional types of NMR studies, i.e., measurement of chemical shifts and spin–spin coupling constants, from which detailed information about conformations and intermolecular interactions can be obtained, have not been carried out. Most of these reasons stem from the fact that long acyl chain phospholipid molecules exist in an aggregated state at all accessible concentrations. The aggregation produces line broadening, which makes coupling constants unresolvable, and it also tends to make the chemical shifts insensitive to the presence of other substances, with the exception of paramagnetic metal ions. Aggregation also makes dilution studies impossible, and although mixtures of different kinds of phospholipids have been studied, the use of small single-walled vesicles, in order to obtain the highest resolution, necessitates the characterization of the vesicles over the entire mole fraction range (Gent and Prestegard, 1974).

In a modest attempt to overcome some of the problems mentioned above, as well as that of the low signal-to-noise ratio for all natural abundance ^{13}C studies done at low concentrations, we have combined two experimental approaches. One is the use of 1,2-dihexanoyl-3-*sn*-phosphatidylcholine (DHPC),[1] which has a critical micelle concentration of about 10 mM (Hershberg et al., 1976; Tausk et al., 1974, and references therein), so that molecules can be studied in both the monomeric and micellar forms in aqueous solution. The other is the use of DHPC and 1,2-dipalmitoyl-3-*sn*-phosphatidylcholine (DPPC) synthesized from fatty acids which have the acyl carbonyl carbons enriched in ^{13}C. The increased signal-to-noise ratio makes it possible to obtain spectra for millimolar phospholipid dispersions in a reasonable (~12 h) amount of time. The use of ^{13}C-labeled carbonyls also allows the assignment of the resonances from the two acyl chains. Furthermore, information about bond conformations in the vicinity of the carbonyls can be obtained for both the aggregated and monomeric forms of DHPC, because of the combination of enhanced signal and narrow line width. And while this study provides information about only one functional group, it is one that has long been known to be particularly sensitive to intermolecular interactions (Maciel and Ruben, 1963). In addition, the region of the molecule where the carbonyl is located can be thought of as an interface between the zwitterionic head group and the hydrophobic methylene chain (Huang, 1976). It has been the subject of few direct studies (Gally et al., 1975; Seelig and Seelig, 1975), but it is a crucial one for the forces that determine the packing of the molecules in micelles and bilayers (Tanford, 1973; Israelachvilli et al., 1976).

Materials and Methods

Preparation of Phosphatidylcholines. 1,2-[1′-^{13}C]Palmitoyl-3-*sn*-phosphatidylcholine (α,β-DPPC) was synthesized using 90% enriched [1-^{13}C]palmitic acid (Merck, Sharpe and Dohme) using the method of Cubero Robles and Van den Berg (1969), and purified as previously described (Suurkuusk et al.,

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[1] Abbreviations used: DPPC and DHPC, 1,2-dipalmitoyl- and 1,2-dihexanoyl-3-*sn*-phosphatidylcholine, respectively; α denotes that the ^{13}C-enriched fatty acid is esterified to the 1-carbon of the glycerol, and β denotes esterification to the 2-carbon, e.g., β-DPPC is 1-palmitoyl,2-[1′-^{13}C]palmitoyl-3-*sn*-phosphatidylcholine; lyso-PPC, 1-[1′-^{13}C]palmitoyl-3-*sn*-phosphatidylcholine; NMR, nuclear magnetic resonance.