J. Lipid Research, October 1963
Volume 4, Number 4

Behavior of dog lymph chylomicron lipid constituents during incubation with serum*

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[Manuscript received March 15, 1963; accepted July 24, 1963.]

SUMMARY

Dog lymph chylomicrons incubated with serum showed a progressive gain in free cholesterol whereas no change took place in the cholesterol ester and triglyceride fractions. Chylomicron phospholipids were observed to suffer a net loss while at the same time exchanging with serum phospholipids. Before incubation, lymph chylomicron phospholipids contained a higher proportion of noncholine phospholipids than serum; during incubation, this fraction was largely transferred to serum. The noncholine fraction showed comparatively little exchange. Lecithin was by far the largest component of both chylomicron and serum phospholipids. In absolute amounts, the largest transfer and exchange could be accounted for by lecithin. The percentages of sphingomyelin and lysolecithin in chylomicron phospholipids were lower than in those of serum. During incubation, the sphingomyelin content of chylomicrons changed little, but active exchange of sphingomyelin took place. The amount of lysolecithin increased both in chylomicrons and in serum. Almost all the phospholipid lost from the chylomicrons appeared in the high-density lipoprotein fraction. Evidence is presented that the extra phospholipid is taken up by preexisting high-density lipoprotein. Exchange of phospholipids between chylomicrons and serum lipoprotein fractions took place about in proportion to the relative phospholipid contents of these fractions. The bearing of these results on the mechanisms whereby chylomicrons are removed from the circulation is discussed.

Exchange of phospholipids between chylomicrons and blood serum has been studied in only fragmentary ways, and the conclusions obtained by various authors appear to be in conflict. Eder (1) reported in 1957 that virtually no exchange of P^{32}-labeled phospholipids took place between chylomicrons and serum. In the following year, Havel and Clarke (2) found a marked transfer of chylomicron phospholipid P^{32} to serum, whereas McCandless and Zilversmit (3) observed a small but significant exchange between these lipid fractions. Preliminary experiments designed to resolve these disparities revealed that the extent of exchange of phospholipids depended greatly on the relative amounts of chylomicron and serum that were incubated together (4). These studies showed, for example, that in a given incubation of 30 min, a 36% decrease in the specific activity of P^{32}-labeled chylomicrons was observed, but that by decreasing the relative amount of chylomicrons by a factor of 20, the decrease in specific activity in the same time interval became 81%.

It is the purpose of the present paper to report in greater detail the movement of various phospholipid fractions between chylomicrons and serum lipoproteins. Some data on simultaneous changes in cholesterol distribution will also be given.

EXPERIMENTAL METHODS

Collection of Lymph and Serum. Mongrel male dogs (13–24 kg), fasted for 12 hr, were fed 100 ml whipping cream 3–4 hr before cannulation. The dogs were then anesthetized with intravenously administered 0.5 ml of 5.9% Na-pentobarbital per kg body weight. A polyethylene tube, 0.05–0.062 in. o.d., was inserted into

* Supported in part by PHS Grants HTS-5279 and H-1238 from the National Heart Institute, National Institutes of Health, U.S. Public Health Service.

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the thoracic duct, and lymph was collected in an ice-cold Erlenmeyer flask for 12–20 hr.

During the collection of lymph, 50 ml of cream was fed every 3 hr by stomach tube to keep the lymph milky, and 0.5–1 ml of saline (0.9% NaCl) per min was given intravenously by continuous drip.

For the collection of P^{32}-labeled lymph, 10 mc of phosphate-P^{32} was injected intravenously soon after the cannulation. The specific activity of chylomicron phospholipid in lymph was greatest 4–8 hr after administration of P^{32}.

Blood was collected from the femoral artery of a similarly fasted anesthetized dog through a cannula. The blood was allowed to clot for about 2 hr at 15° and was centrifuged at 1500 × *g* for 20 min for the separation of serum. For P^{32}-labeled serum, 10 mc of phosphate-P^{32} was injected intravenously 6 hr before collection of the blood.

Preparation of Chylomicron Suspensions. The lymph was allowed to clot at 4° and filtered through thick gauze with vigorous stirring. The filtered lymph was diluted about 2- to 3-fold with 0.9% saline to prevent clotting and was then centrifuged in polyethylene tubes at 25,000 × *g* for 3–4 hr at 4°.

After centrifugation, the compact chylomicron layer was scooped off with a stainless steel spatula. The chylomicrons were resuspended with about 1/3 to 1/2 the original volume of 0.9% saline, forced through a 20-gauge needle, and centrifuged as before. The resuspension and centrifugation were performed four times. The last time, the washed chylomicrons were resuspended with a 22-gauge needle. The amount of chemical phosphorus or P^{32} in the subnatant portion of the last washing was negligible. The content of lipid phosphorus in the chylomicron suspension was determined and was adjusted with 0.9% saline to the same content per milliliter as that of the serum used for incubation. This suspension was stored in a polyethylene container for up to two weeks in the dark at 4°. It appeared stable under these conditions as judged by lipid phosphorus analysis and the lack of creaming.

Incubation of Chylomicrons with Serum. Various amounts of chylomicron suspension (5, 4, 3, 2, and 1 ml) were mixed with 5, 6, 7, 8, and 9 ml of serum, respectively, in a lusteroid tube with a rubber stopper covered with Saran Wrap and were gently shaken at 37° for 0, 15, 30, 60, 120, and 180 min.[1] As a blank test, 5, 3, and 1 ml of the chylomicrons were incubated with 5, 7, and 9 ml of 0.9% saline, respectively, for 0 or 180 min. Also 5, 7, and 9 ml of serum were shaken with 5, 3, and 1 ml of saline for the same time. In this way, the initial proportions of phospholipids in chylomicrons and serum were about 50:50, 40:60, 30:70, 20:80, and 10:90 (indicated in Tables and Figures as C:S = 50:50, etc.). As soon after incubation as possible, the mixtures were subjected to the following procedure for the separation of the chylomicron fraction.

Separation of Chylomicrons from Incubation Mixture. Five milliliters of a salt solution (d = 1.006) was placed in the bottom of a 1 x 3 in. lusteroid centrifuge tube, and 10 ml of a salt solution (d = 1.019) was pipetted under the first layer. Then 5 ml of a salt solution (d = 1.041) was delivered under the second layer. Finally, a 10-ml aliquot of the incubation mixture adjusted to a solvent density of 1.063 (2 ml of salt solution, d = 1.35, and 10 ml of the incubation mixture) was layered at the bottom of the tube, which was then spun in a swinging bucket rotor for 1 hr at 90,000 × *g*. After centrifugation, the tube was cut at about 1 cm under the surface with a tube slicer (Spinco No. 384) to separate the top (chylomicron) fraction from the subnatant (serum) fraction.

Compact chylomicron layers were scooped off, and dispersed chylomicrons were transferred quantitatively to an Erlenmeyer flask with a small syringe and needle, with saline for washing fluid. The subnatant fractions were transferred quantitatively into a graduated cylinder by washing the tubes with saline and were made up to a known volume with saline.

Extraction and Separation of Lipids. An aliquot of the original serum and chylomicron suspensions as well as the centrifuged chylomicron and serum fractions were extracted by Folch's method (5). For the determination of lipids and protein in lipoprotein fractions, we employed the extraction procedure of Hillyard et al. (6), in which ethanol–ether–chloroform 6:2:1 (v/v), ethanol–ether 3:1, and ether are used. The solvents were evaporated from the combined extracts in vacuo at temperatures below 50°. To the residue was added a small amount of water to dissolve salts, and lipids were reextracted with chloroform–methanol 2:1 and washed by Folch's method. A comparison of the amount of lipid phosphorus and cholesterol in serum before and after incubation with chylomicrons determined by this method and by the method of Folch et al. showed good agreement.

An aliquot of the washed chloroform–methanol extract containing less than 20 mg of total lipid was evaporated in vacuo at less than 50°, and the residue

[1] Lusteroid tubes were used because the shaking of chylomicrons with saline or serum in a glass vessel caused clumping of chylomicrons and adherence to the vessel wall. Zero-time incubations were in practice more nearly 5-min incubations since it took about 5 min to prepare the sample for centrifugation.

was reextracted with about 10 ml of chloroform. A mixture of 0.5 g of nonactivated silicic acid[2] with 0.5 g of Supercel was slurried with about 3–4 volumes of chloroform and introduced into a glass tube, 8 mm i.d. x 10 cm, which had a solvent reservoir at the top and a constriction with a small plug of glass wool at the bottom. The column was first washed with 5 ml of methanol and then with 20 ml of chloroform. The chloroform extract of the sample was applied to the column quantitatively with additional chloroform and the nonphospholipid fraction was eluted with chloroform to a total eluate of 50 ml. The phospholipid fraction was eluted with 50 ml of methanol.

An aliquot of the nonphospholipid fraction described above was evaporated in vacuo at less than 50° and reextracted with 5–10 ml of Skelly B (petroleum ether, bp 60–80°). A column of 1 g nonactivated silicic acid–Supercel prepared as before was washed with 20 ml of Skelly B instead of with methanol and chloroform. The sample was put on with a small amount of Skelly B, and the column was eluted with 60 ml of chloroform–Skelly B 1:9 (v/v) and then with 60 ml of chloroform. The first fraction contained cholesterol esters and the second free cholesterol, glycerides, and free fatty acids. The fractionation was checked by silicic acid thin-layer chromatography with a developing solvent of ethyl ether–Skelly B–glacial acetic acid 10:90:1 (7).

For the fractionation of individual phospholipids, the procedure of Newman, Liu, and Zilversmit (8) was used, except that fractions were collected batchwise as follows: 30 ml chloroform (nonphospholipids), 50 ml 20% (v/v) methanol in chloroform (noncholine phospholipids), 150 ml 40% methanol in chloroform (lecithin), 80 ml 60% methanol in chloroform (sphingomyelin), and 50 ml methanol (lysolecithin). Each fraction was checked qualitatively by silicic acid thin-layer chromatography (developing solvent chloroform–methanol–conc. ammonia–water 66:33:1:5), which showed that satisfactory separations had been achieved. In the case of the chylomicron fraction, the excess neutral lipid was first removed by silicic acid–Supercel chromatography as described above.

Fractionation of Lipoproteins. To a 10-ml aliquot of the serum fraction (solvent density = 1.040) was added 2 ml of an NaCl solution (283.3 g/liter, d = 1.18) to obtain a solvent density of 1.063. Ten milliliters of this mixture was fractionated into low-density lipoprotein ($d < 1.063$), high-density lipoprotein ($1.063 < d < 1.21$), and bottom fraction ($d > 1.21$) by ultracentrifugation at 105,000 × *g* for 24 or 48 hr, essentially as described by Bragdon, Havel, and Boyle (9) but employing NaBr instead of KBr. Each fraction was checked for purity by paper electrophoresis.

[2] The nonactivated silicic acid was kept in a desiccator maintained at 30% relative humidity by a saturated aqueous solution of $CaCl_2$ in contact with excess salt.

TABLE 1. SEPARATION OF CHYLOMICRON AND SERUM PHOSPHOLIPIDS*

Fractions	Thickness of Layer	Distribution of Lipid Phosphorus in % of Total	
		I	II
	mm		
1	10	15.3	15.4
2	5	0.15	0.27
3	"	0.39	0.56
4	"	0.52	0.81
5	"	2.21	3.03
6	"	6.35	6.35
7	"	9.72	8.82
8	"	65.0	63.2
Total Recovery (%)		99.7	98.5

* Amounts of lipid phosphorus of original chylomicrons and serum in both experiments were 111 μg and 558 μg or 16.6 and 83.4%, respectively.

Other Methods. Triglycerides contained in the nonphospholipid fraction were determined by the procedure of Van Handel and Zilversmit (10) with the glycerol determined by the modification of Newman et al. (8). Liquid scintillation counting described previously (8) was used to determine P^{32}. Total, free, and esterified cholesterol fractions were saponified and extracted with petroleum ether according to Abell et al. (11). To the dried residue were added 0.6 ml of glacial acetic acid and 0.4 ml of $FeCl_3$ color reagent (12), and the color intensity was determined in a Coleman Junior Spectrophotometer. A standard of 10 μg cholesterol was used. Protein nitrogen was determined by direct Nesslerization of a Kjeldahl digest as described by Minari and Zilversmit (13).

RESULTS

Adequacy of Separating Chylomicrons from Incubation Mixtures. Table 1 shows the results of two experiments (I and II) in which chylomicrons mixed with serum were centrifuged immediately after mixing as described above. After centrifugation, each lusteroid tube was cut into eight sections by means of a tube slicer. The top 1-cm fraction contained about 15% of the total phospholipid phosphorus, whereas the next fraction contained only about 0.2% of the lipid phosphorus. In subsequent experiments, therefore, it was decided to use the top 1-cm of each tube for "chylomicron fraction" and the rest of the solution for "serum fraction." Perhaps an even more sen-

TABLE 2. CHYLOMICRON AND SERUM TRIGLYCERIDES DURING INCUBATION

Expt.	C:S*	Incubation Time	Chylomicron Fraction	Serum Fraction	Total Recovery†	Original Amount in Chylomicrons	Original Amount in Serum
		min	*% of original*		%	*mg*	*mg*
A	50:50	0	91.5	126	91.8	235	1.51
		15	91.4	132	91.7		
		30	97.6	130	97.8		
		60	91.1	132	91.3		
		120	96.1	139	96.4		
		180	98.0	129	98.1		
Blank‡		0	91.2	0.10§	91.3	235	0
"		180	95.6	0.14§	95.7		
A	30:70	0	91.2	96.3	91.2	141	2.11
		15	90.1	101	90.2		
		30	95.1	106	95.3		
		60	98.4	105	98.5		
		120	90.3	102	90.4		
		180	90.6	104	90.8		
Blank‡		0	95.8	0.18§	96.0	141	0
"		180	98.0	0.15§	98.2		
A	10:90	0	90.1	99.6	90.6	46.9	2.72
		15	91.9	110	92.9		
		30	92.7	105	93.4		
		60	91.9	112	93.0		
		120	93.2	104	93.8		
		180	93.8	102	94.0		
Blank‡		0	—	0.19§	—	46.9	0
"		180	—	0.12§	—		

* Approximate ratio of chylomicron to serum phospholipid at the beginning of incubation.
† Sum of triglyceride in separated fractions as percentage of that originally incubated.
‡ Chylomicrons were incubated with saline.
§ Percentage of original chylomicron triglyceride.

sitive test of separation is given in Table 2, which summarizes results of experiments in which the 1-cm top fraction and the rest of the solution in the tube after centrifugation were analyzed for triglyceride. In experiment A (C:S = 50:50), for example, 235 mg of chylomicron triglyceride was incubated with serum containing 1.51 mg triglyceride. After centrifugation, the excess amount of triglyceride remaining in the serum fractions constituted only 0.2% of the chylomicron triglyceride. In other experiments in which relatively less chylomicron triglyceride was present, the separations were even better. Similarly, in the blank incubations in which chylomicrons were incubated with saline instead of serum, the total amount of triglyceride remaining in the bottom fractions was less than 0.2%.

Behavior of Triglyceride and Cholesterol During Incubation. Table 2 shows that when washed lymph chylomicrons are incubated with dog serum from fasted animals at 37° for as long as 3 hr, they do not undergo appreciable lipolysis. However, certain other changes take place under these conditions, as is evident from Table 3 in which are presented the total cholesterol of chylomicrons and serum during incubation. In experiment A, 1,680 μg of chylomicron cholesterol initially present was augmented by 35% during the 3-hr incubation. At the same time, the initial serum cholesterol of 4,700 μg decreased by 15%. No changes in the distribution of cholesterol were observed when chylomicrons or serum were incubated with saline (C. blank and S. blank, respectively).

In order to find out whether the transfer of cholesterol from serum into the chylomicrons involved both ester and free cholesterol, the total cholesterol was fractionated by silicic acid chromatography. Table 4 and Fig. 1 give the results for cholesterol ester and free cholesterol respectively. From these three experiments, it is evident that the migration of cholesterol from serum to chylomicron involved only the free cholesterol fraction. Apparently, the transfer of free cholesterol to the chylomicron fraction continued during the entire 3-hr period. In the blank incubations

TABLE 3. TOTAL CHOLESTEROL DURING INCUBATION OF CHYLOMICRONS AND SERUM*

Expt.	Incubation Time	Chylomicron Fraction	Serum Fraction	Total Recovery†	Original Amount in Chylomicrons	Original Amount in Serum
	min	*% of original*		%	*μg*	*μg*
A	0	91.8	97.5	95.8	1680	4700
	15	99.4	95.1	96.1		
	30	105	93.1	96.1		
	60	113	89.8	95.6		
	120	123	87.6	96.8		
	180	135	85.4	98.3		
C Blank‡	0	96.2	0	96.2		
"	180	96.2	0	96.6		
B	0	101	100	99.1	2010	8800
	15	104	97.1	98.6		
	30	113	96.8	101		
	60	119	95.9	101		
	120	137	94.0	104		
	180	141	88.1	100		
C Blank‡	0	92.8	0	92.8		
"	180	91.0	0	91.0		
S Blank§	0	0	99.8	99.8		
"	180	0	94.3	94.3		
C	0	98.2	95.7	96.2	2650	8410
	15	105	94.5	97.1		
	30	113	93.3	98.0		
	60	117	91.1	97.1		
	120	125	90.4	98.7		
	180	132	85.1	96.3		
C Blank‡	0	100	0	101		
"	180	98.2	0	101		
S Blank§	0	0	101	100		
"	180	0	101	98.2		

* Approximate ratio of chylomicron to serum phospholipid at the beginning of incubation (C:S) was 50:50.
† Sum of cholesterol in the two fractions as percentage of that originally incubated.
‡ C Blank: chylomicrons incubated with saline.
§ S Blank: serum incubated with saline.

in which chylomicrons were incubated with saline, no transfer of cholesterol was observed.

Behavior of Chylomicron Phospholipids During Incubation. While the free cholesterol was observed to transfer into the chylomicron fraction during the incubation of lymph chylomicrons with serum, the phospholipids showed a quite different behavior. Figure 2 shows the decrease of chylomicron phosphorus during the 3-hr incubation period. The percentage loss of phospholipid appeared to depend on the relative amounts of phospholipid present in chylomicron and serum; the greatest percentage losses were observed when the ratio of chylomicron to serum phospholipid (C:S) was the smallest. After 1 hr of incubation, the loss of chylomicron phospholipid was 25% when 436 μg chylomicron phospholipid P was incubated with 427 μg of serum phospholipid P, and rose to 42% when 87 μg of chylomicron phospholipid P was incubated with 769 μg of serum phospholipid P. This increase in percentage transfer of phospholipid from chylomicron to serum should not obscure the fact that at relatively low chylomicron concentrations the absolute transfer of phospholipid increased nearly linearly with the amount of phospholipid initially present in the chylomicron fraction. In 1 hr, for example, the transfer of phospholipid P in the experiments illustrated in Fig. 2 was 34, 68, 93, 103, and 108 μg as the initial amounts of chylomicron phospholipid increased from 87 to 174, 262, 349, and 436 μg, respectively. It is interesting to note that the amount of phospholipid transferred reaches an upper limit as the amount of chylomicron phospholipid, relative to the amount of serum phospholipid, diminishes. This may point to some limiting factor such as the amount of phospholipid acceptor in the serum.

Table 5 shows that when chylomicrons were in-

TABLE 4. ESTERIFIED CHOLESTEROL DURING INCUBATION OF CHYLOMICRONS AND SERUM*

	C:S†	Incubation Time	Chylomicron Fraction	Serum Fraction	Total Recovery	Original Amount in Chylomicrons	Original Amount in Serum
		min	*% of original*		%	*μg*	*μg*
	50:50	0	90.8	97.3	96.2	595	3460
		15	94.5	96.2	95.9		
		30	91.1	95.8	95.1		
		60	94.2	94.5	94.4		
		120	95.1	96.8	96.5		
		180	96.2	96.8	96.7		
Blank‡		0	94.6	0	94.6	595	0
"		180	95.8	0	95.8		
	30:70	0	94.4	96.0	95.9	357	4840
		15	94.6	96.2	96.1		
		30	95.9	101	101		
		60	95.6	103	102		
		120	97.8	104	103		
		180	98.1	104	104		
Blank‡		0	93.4	0	93.4	357	0
"		180	92.2	0	92.2		
	10:90	0	104	102	102	119	6230
		15	101	105	105		
		30	109	105	105		
		60	108	107	107		
		120	105	106	106		
		180	108	109	109		
Blank‡		0	104	0	104	119	0
"		180	98.2	0	98.2		

* Experiment A

† Approximate ratio of chylomicron to serum phospholipid at beginning of incubation.

‡ Chylomicrons were incubated with saline.

cubated with saline, no appreciable loss of chylomicron phospholipid took place. In addition, it shows that the loss of lipid phosphorus from the chylomicron fraction was balanced by an equal gain of phospholipids in serum. Thus, no destruction of phospholipids during incubation had taken place.

In experiments with P^{32}-labeled chylomicrons, it was observed that not only a net loss but also exchange of chylomicron phospholipid took place. In Fig 3, for example, are given the specific activities of chylomicrons and serum during the incubation of these fractions in various proportions. In the experiment in which initially equal amounts of chylomicron and serum phospholipid were present, the specific activity of the chylomicron fraction fell from 180 cpm/μg to 123 cpm/μg in 3 hr. During that time the serum phospholipid specific activity rose from 0 to 72 cpm/μg. The changes in chylomicron specific activities were even more pronounced when the ratio of chylomicron to serum phospholipid was small. In the experiment in which 87.2 μg chylomicron phospholipid P and 769 μg serum phospholipid P were initially present (C:S = 10:90), the chylomicron specific activity fell from 180 to 55 cpm/μg, a drop of 70%. In Table 6, two additional experiments (B and C) can be compared to the data of experiment A (C:S = 50:50), which was also given in Fig. 3. The percentage decreases of specific activity in 3 hr were respectively 32, 33, and 40%. Another interesting comparison may be made between Fig. 2 and 3. While the net loss of phospholipid from the chylomicrons took place primarily in the first 15 min (C:S = 10:90), and was virtually complete in 30 min, the exchange of phospholipids appeared to continue during the 3-hr incubation period and possibly beyond.

Although the decrease in chylomicron specific activity already points to the presence of an exchange reaction of phospholipids between serum and chylomicrons, more direct proof of such a mechanism was obtained from an experiment in which unlabeled chylomicrons were incubated with serum containing labeled phospholipids. Figure 4 shows that in such an experiment radioactive phospholipids rapidly accumulate in the chylomicron fraction. In the experiment in which equal amounts

TABLE 5. LIPID PHOSPHORUS DURING INCUBATION OF CHYLOMICRONS AND SERUM

Expt.*	Incubation Time	Chylomicron Fraction	Serum Fraction	Total Recovery†	Original Amount in Chylomicrons	Original Amount in Serum
	min	*% of original*		*%*	*μg*	*μg*
B	0	97.9	100	99	556	531
	15	88.7	117	103		
	30	80.4	120	102		
	60	78.7	128	103		
	120	78.2	129	103		
	180	71.2	132	101		
C Blank‡	0	100	0.9‖	101	556	0
"	180	95.5	1.5‖	97		
S Blank§	0	Trace	101	101	0	531
"	180	Trace	99.9	99.9		
C	0	89.4	106	97.4	608	563
	15	79.5	123	100		
	30	75.6	128	101		
	60	69.1	128	97.6		
	120	66.7	130	97.3		
	180	67.2	133	98.9		
C Blank‡	0	97.8	Trace	97.8	608	0
"	180	100	Trace	100		
S Blank§	0	Trace	102	102	0	563
"	180	Trace	101	101		
D_1	0	99.1	103	101	575	550
	15	83.5	119	101		
	30	80.9	121	100		
	60	80.9	124	102		
	120	80.3	126	103		
	180	78.3	128	103		
C Blank‡	0	100	Trace	100	575	0
"	180	96.0	Trace	96.0		
S Blank§	0	Trace	98.4	98.4	0	550
"	180	Trace	100	100		
D_2	0	91.3	101	99.9	115	990
	15	66.8	103	99.0		
	30	66.8	103	99.6		
	60	67.8	103	99.6		
	120	67.3	103	99.9		
C Blank‡	0	99.1	Trace	99.1	115	0
"	180	99.1	Trace	99.1		
S Blank§	180	Trace	96.5	96.5	0	990

* Approximate ratio of chylomicron to serum phospholipid at the beginning of incubation was 50:50 for expts. B, C, and D_1, and 10:90 for expt. D_2.

† Sum of lipid phosphorus in separate fractions as percentage of that originally incubated.

‡ C Blank: chylomicrons incubated with saline.

§ S Blank: serum incubated with saline.

‖ Percentage of lipid phosphorus in original chylomicron.

of phospholipid were present in the serum and chylomicron fractions, the specific activity of the serum phospholipid decreased from 10.8 to 6.1 while the specific activity of the chylomicron fraction rose from 0 to 3.6. Because the serum used in these experiments contained phosphate-P^{32} in addition to phospholipid-P^{32}, we repeated the experiment with serum dialyzed against Ringer's solution. The results are shown in Table 7. Apparently the removal of dialyzable constituents did not influence the exchange of phospholipid-P^{32} between serum and chylomicrons.

Exchange of Individual Phospholipids. Since there appeared to be a relatively rapid exchange of phospholipids between lymph chylomicrons and serum, it

TABLE 6. SPECIFIC ACTIVITIES OF THE CHYLOMICRON PHOSPHOLIPID DURING INCUBATION WITH SERUM*

Incubation Time		Experiment A	Experiment B	Experiment C
	min	*% of original specific activity*		
	0	100	95.8	99.0
	15	93.9	85.9	83.9
	30	88.2	80.1	75.9
	60	82.6	75.9	71.4
	120	73.8	69.3	65.9
	180	67.7	66.5	59.6
Blank†	0	102	97.5	97.8
"	180	100	99.5	94.8

* P^{32}-labeled chylomicrons of different batches A, B, and C were incubated with different sera. Specific activity (cpm/μg lipid phosphorus) of original chylomicrons were: A = 179, B = 289, C = 180; and the approximate ratio of chylomicron to serum phospholipid at the beginning of incubation (C:S) was 50:50.

† Chylomicrons incubated with saline.

became interesting to investigate the fate of various subfractions of the total phospholipids. Fractionation of the phospholipids from washed chylomicrons and comparison with the fractions obtained from serum made it clear that there is a considerable difference in the distribution of phospholipids. Although lecithin was the major phospholipid fraction in both samples, 75% of the total lipid phosphorus in chylomicrons and 80% in serum, there were large differences in the other fractions. Chylomicrons showed, for example, a considerable noncholine phospholipid content, 14% of the total lipid phosphorus, whereas in serum this fraction made up only 4%. Sphingomyelin was 4.5% of lipid phosphorus in the chylomicrons and 7.5% in serum. Finally lysolecithin, which, in plasma, made up 8% of the total phospholipid phosphorus, contributed only 3% to the lipid phosphorus of the chylomicrons.

TABLE 7. RADIOACTIVITIES OF CHYLOMICRON AND SERUM FRACTION AFTER INCUBATION OF NONLABELED CHYLOMICRONS WITH DIALYZED AND UNDIALYZED P^{32}-LABELED SERUM*

C:S†	Dialysis	Incubation Time	Chylomicron Fraction	Serum Fraction	Original Amounts of Lipid Phosphorus: Chylomicrons	Original Amounts of Lipid Phosphorus: Serum
		min	*% of original serum P^{32}*		*μg*	*μg*
50:50	−	180	27.3	72.7	575	550
	+	180	25.1	71.5	505	478
10:90	−	120	4.5	88.5	115	990
	+	180	4.3	92.7	101	860

* Radioactivities (cpm) of original undialyzed and dialyzed sera were 5,917 and 2,806 (C:S 50:50) and 10,651 and 5,051 (C:S 10:90), respectively.

† Approximate ratio of chylomicron to serum phospholipid at beginning of incubation.

Also, in the transfer of phospholipids between chylomicrons and serum, the individual fractions differed markedly. Figure 5 shows a semilogarithmic plot of the amounts of individual phospholipids in chylomicrons during incubation with serum. Although the absolute loss of lecithin from the chylomicrons exceeded the losses in other fractions, the percentage loss of lipid phosphorus was the greatest in the noncholine fraction. Although most of the noncholine loss occurred early in the experiment, the loss continued during the entire period of incubation. In contrast, the sphingomyelin fraction showed only a relatively small loss of phospholipid phosphorus and it occurred entirely during the first 30 min of incubation. The only chylomicron phospholipid fraction that showed a gain throughout the period of incubation was the 100% methanol eluate, which contains lysolecithin. An occasional

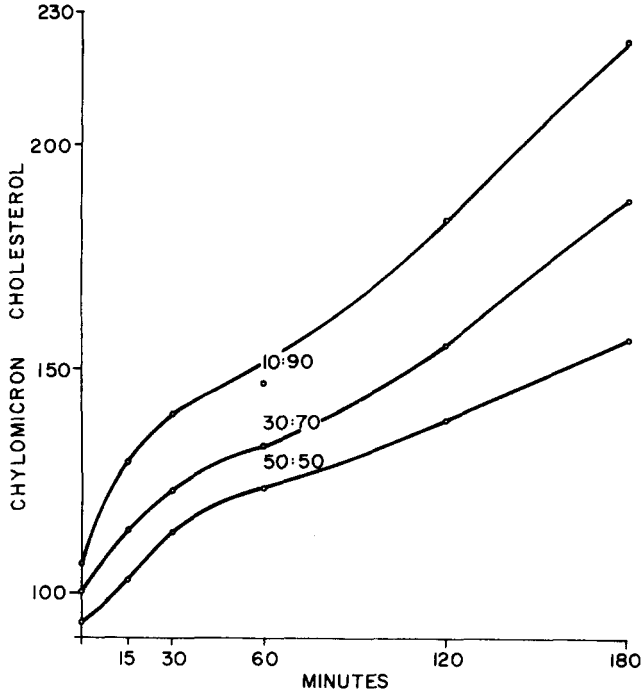

FIG. 1. Free cholesterol in chylomicron fraction during incubation of chylomicrons with serum (experiment A). The ordinate shows percentage of original free cholesterol of chylomicrons. Ratios shown on each curve are the approximate ratio of chylomicron to serum phospholipid (C:S) at the beginning of incubation. The amounts of free cholesterol (μg) in the original chylomicron and serum and in the chylomicron fractions incubated with saline (blank) were as follows:

	C:S 50:50	30:70	10:90
Original serum	1240	1737	2233
Original chylomicrons	1087	652	217
Blank 0 min	1055	631	218
180 min	1055	641	217

The total recoveries of free cholesterol in whole incubation mixtures were 95.5 to 101%.

TABLE 8. PHOSPHOLIPID SPECIFIC ACTIVITIES OF SERUM LIPOPROTEIN FRACTIONS AFTER INCUBATION WITH P^{32}-LABELED CHYLOMICRONS*

	Fractions					
	LDL		HDL		Bottom	
	d<1.063		1.063<d<1.21		d>1.21	
	Experiment					
Incubation Time	B	C	B	C	B	C
min	*cpm/μg phospholipid P*					
0	—	7.3	—	7.4	—	14.2
15	53.8	31.7	67.7	45.6	35.1	37.3
30	67.6	40.7	92.3	58.1	46.5	37.1
60	81.7	44.2	104.3	—	49.6	—
120	103.7	51.3	126.8	73.0	60.1	41.7
180	108.2	56.2	132.6	74.8	60.9	42.9
Original lipid phosphorus, μg/ml serum	4.7	8.1	56.8	89.5	40.2†	10.1

* The initial proportion of chylomicron to serum phospholipid in these experiments was C:S 50:50.

† This sample was centrifuged for only 24 hr and the bottom fraction appeared contaminated with HDL. In experiment C, 48-hr centrifugation was employed.

TABLE 9. RATIO OF PHOSPHOLIPID TO PROTEIN IN HDL FRACTION*

Incubation Time	Experiment	
	B	C
min		
0	0.585	0.888
15	0.617	0.934
30	0.692	0.923
60	0.725	—
120	0.729	1.013
180	0.759	1.195

* Phospholipid calculated as lipid P × 25; protein calculated as N × 6.25. The ratios in the serum HDL before incubation were 0.472 and 0.846 for experiments B and C, respectively.

check of this fraction by thin-layer silicic acid chromatography revealed an apparent increase in the spot representing lysolecithin.[3]

The serum fractions were also analyzed for individual phospholipid fractions after various times of incubation. For the most part, the increase in the amount of phospholipid in the serum corresponded to the loss of that fraction by the chylomicrons. In most experiments, the lipid phosphorus of the methanol-eluted fraction showed an increase parallel to that observed in chylomicrons.

In contrast to the observations with cholesterol and total phospholipid, the individual phospholipid fractions showed some changes during blank incubations. In the case of chylomicrons incubated with saline, there occurred slight decreases in the phosphorus of the 20 and 40% methanol eluates, whereas increases in the 60 and 100% methanol eluates were observed. Similarly, the methanol eluate of the serum incubated with saline increased somewhat, whereas the 20% methanol eluate showed a decrease. These changes might be accounted for either by the formation of lysophosphatides or by oxidative changes during incubation. The behavior of P^{32}-labeled lipids was also characteristic for the different fractions, as may be seen in Fig. 6. Again the lecithin fraction showed the largest decrease in total P^{32} although the percentage decrease in the specific activity of the sphingomyelin and lysolecithin fraction, particularly during the first hour, was greater. The specific activity of the noncholine fraction showed practically no change during the incubation procedure except for a slight decrease during the first 30 min. That the decrease in specific activity of this fraction is relatively small is due in part to the small pool of noncholine phospholipids in serum, but also, to a con-

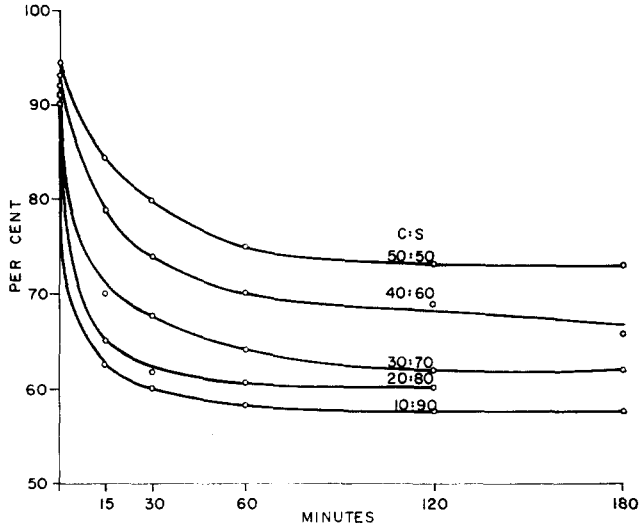

FIG. 2. Lipid phosphorus in the chylomicron fraction during incubation (experiment A) in percentage of initial chylomicron lipid phosphorus. C:S, approximate ratio of chylomicron to serum phospholipid at the beginning of incubation. The amounts of lipid phosphorus (μg) in the original chylomicron and serum were as follows:

	C:S 50:50	40:60	30:70	20:80	10:90
Chylomicron	436	349	262	174	87.2
Serum	427	512	598	683	769

The total recoveries of lipid phosphorus in the whole incubation mixture were 95.0 to 98.6%.

[3] An increase in the column methanol eluate might represent the formation of highly polar constituents other than lysolecithin; e.g., oxidation products. Thin-layer chromatography with chloroform–methanol–water 140 : 50 : 7 (by volume) showed an increased fluorescence in the lysolecithin position and the absence of fluorescence in the areas of lower R_F. Increases in lysolecithin were not observed in all serum or chylomicron samples.

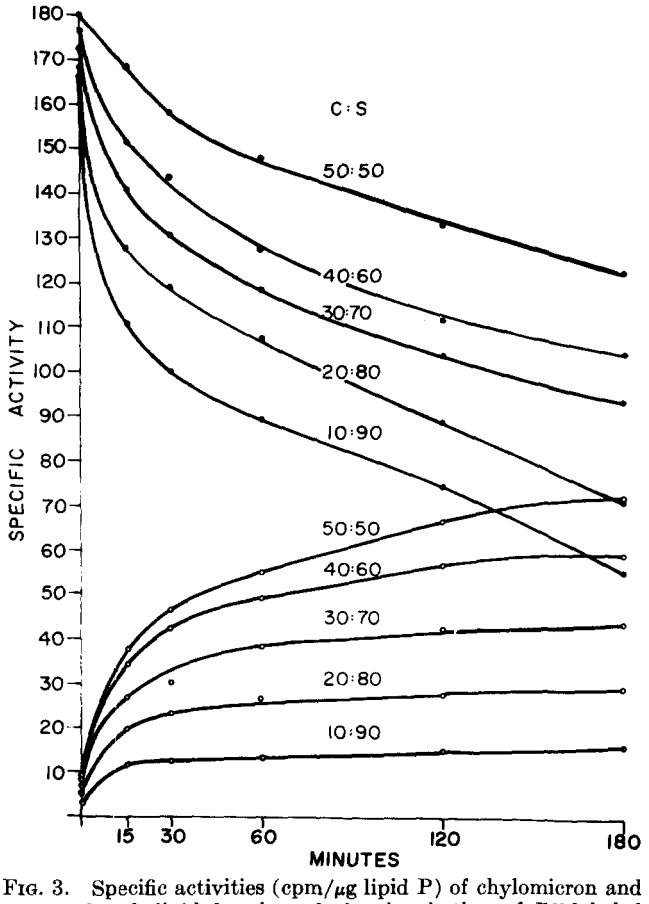

FIG. 3. Specific activities (cpm/μg lipid P) of chylomicron and serum phospholipid fractions during incubation of P^{32}-labeled chylomicrons with serum (experiment A). –·–, Chylomicron fraction; –○–, serum fraction; C:S, approximate ratio of chylomicron to serum phospholipid at the beginning of incubation. Specific activity of original chylomicrons was 178, and of chylomicron fractions incubated with saline (blank) as follows:

	C:S 50:50	30:70	10:90
0 min	182	180	176
180 min	180	183	178

siderable extent, to the fact that little exchange appears to take place.[4]

Transfer of Chylomicron Phospholipid to Serum Lipoprotein Fractions. After it had been shown that the transfer and exchange of individual phospholipid fractions differed considerably, it became interesting to investigate whether the participation of various serum lipoprotein fractions showed any marked differences. Therefore, lipoproteins of a density less than 1.063 (LDL), between 1.063 and 1.21 (HDL), and the fraction of density greater than 1.21 (bottom) were separated after the usual incubation of P^{32}-labeled chylomicrons and nonlabeled whole serum. Table 8 shows the results of two experiments. In experiment B, the HDL fraction was separated after 24 hr of centrifugation; in experiment C, after 48 hr. Otherwise, the experiments were carried out in the same manner. In confirmation of similar findings by others, the amount of phospholipid in the LDL fraction of dog serum was less that $^1/_{10}$ as great as in the HDL. In experiment C in which the bottom fraction was separated after a 48-hr centrifugation, the amount of phospholipid in the bottom fraction was of the same order of magnitude as that in the LDL. The specific activities of the three fractions shown in Table 8 are quite similar. This would indicate then that in dog serum, which is rich in HDL, most of the phospholipid released from the chylomicron either by a net transfer or by an exchange mechanism ends up in the HDL fraction.

The net transfer of chylomicron phospholipid to the HDL fraction might take place in two ways: the phospholipid could be added on to pre-existing HDL, or the phospholipid could be attached to some other

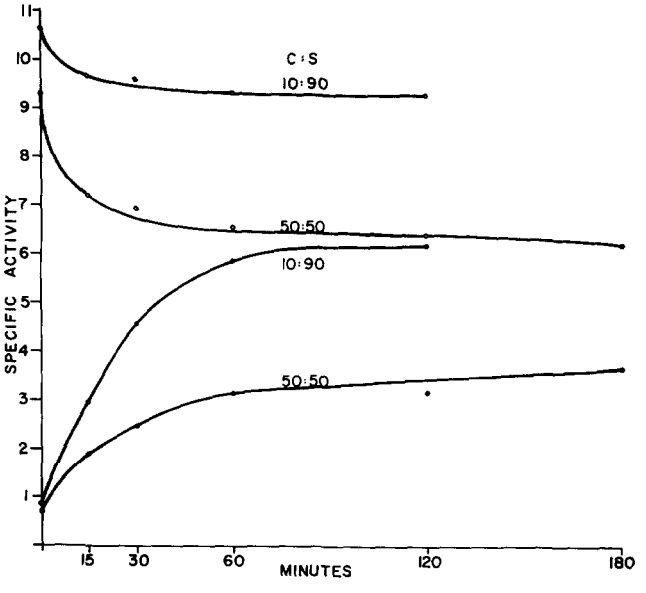

FIG. 4. Specific activities (cpm/μg lipid P) of chylomicron and serum phospholipid fractions during incubation of chylomicrons with P^{32}-labeled serum (experiment D). –·–, Chylomicron fraction; –○–, serum fraction; C:S, ratio of chylomicron to serum phospholipid at the beginning of incubation. Specific activity of original serum was 10.8, and of lipid phosphorus in the serum fractions incubated with saline (blank) as follows:

	C:S 50:50	10:90
0 min	10.9	–
180 min	10.6	11.2

The total recoveries of lipid phosphorus in the whole incubation mixture were 99.0 to 103%.

[4] It may be pointed out that a random net loss of phospholipid from the chylomicron fraction would not change the specific activity of the chylomicron phospholipid. An exchange with a nonlabeled pool of serum phospholipid will, however, decrease the specific activity of phospholipids. Thus a comparison of total loss of phospholipid-P^{32} and a decrease in specific activity gives a rough measure of net loss and exchange.

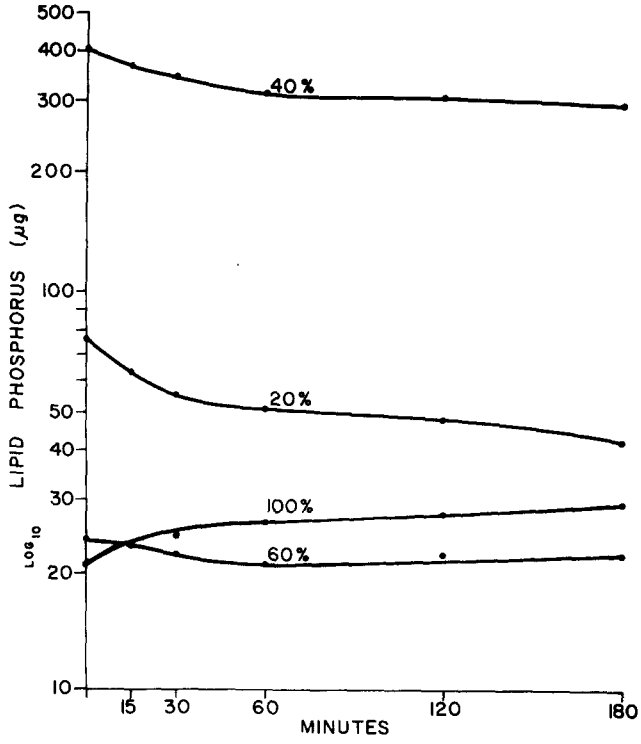

FIG. 5. Amounts of individual phospholipid phosphorus in the chylomicron fraction during incubation. The results were obtained from experiment B in which approximate ratio of chylomicron to serum phospholipid was C:S 50:50. % in figure refers to % methanol (by volume) in chloroform used for elution. Total recoveries were 90.4–96.6%. The amount (μg) of phospholipid phosphorus in the original chylomicron, serum, and chylomicron fractions incubated with saline (blank) were as follows:

Methanol in Eluate: Main Component:	20% Noncholine	40% Lecithin
Original serum	19.6 (3.7%)	425 (80.0%)
Original chylomicrons	76.4 (13.7%)	417 (74.9%)
Blank 0 min	76.4	409
180 min	63.5	355

Methanol in Eluate: Main Component:	60% Sphingomyelin	100% Lysolecithin
Original serum	40.0 (7.5%)	42.5 (8.0%)
Original chylomicrons	25.2 (4.5%)	16.3 (2.9%)
Blank 0 min	24.2	17.3
180 min	28.1	33.7

protein and thereby form new HDL. The data in Table 9 might give some evidence for the first mechanism since it shows that in both experiments the ratio of phospholipid to protein in the HDL fraction increases steadily during the incubation. This increase in the phospholipid content is caused entirely by the addition of phospholipid since the amounts of HDL protein during incubation were quite constant. In additional data not presented here, it was also interesting to note that the increase of phospholipid phosphorus in the LDL fraction took place immediately; i.e., the zero time sample already showed the full increase in phospholipid P although the P^{32} continued to mount during the 3-hr period. In the HDL fraction, there appeared to be a more gradual increase in both phospholipid P and P^{32}. Another interesting observation was that the shifts in cholesterol:phospholipid ratio in the LDL and HDL during the incubation ran parallel. In both fractions, this ratio decreased about 35% during the 3-hr incubation period.

DISCUSSION

In previous experiments from this laboratory (3), it was observed that when P^{32}-labeled chylomicrons were injected intravenously into dogs the triglyceride portion of the chylomicron disappeared from the bloodstream at a much faster rate than the labeled phospholipids. Incubation in vitro of P^{32}-labeled chylomicrons with unlabeled serum showed that some exchange of phospholipids took place.

If this exchange process were very rapid, one might explain the observed differences in rate by the fact that serum lipoprotein phospholipids disappear from the blood relatively slowly compared to chylomicron triglyceride. In more recent experiments on the exchange of chylomicron phospholipids with serum (4), it was found that the extent of exchange depends greatly on the ratio of chylomicron and serum phospholipid present in the incubation mixture. In the earlier exchange experiments (3), the ratio of chylomicron to serum phospholipid was about 50:50. Since this is probably far removed from the ratio at which chylomicron and serum phospholipids occur in the intact animal during fat absorption, the present experiments were designed to investigate the behavior of chylomicron lipid constituents under a variety of conditions.

In order to compare our results with those of others, it is essential to stress the conditions of our experiments that might affect their outcome. Our chylomicrons were obtained from the thoracic duct of dogs fed cream, they were washed with saline repeatedly, and they were used fresh or for periods of up to two weeks. As far as the chemical composition of the chylomicrons is concerned, it is noteworthy that there was nearly twice as much free cholesterol as cholesterol in the esterified form. This appears to agree with the results reported by Hillyard et al. (14) on one dog fed cream. In a different dog fed corn oil plus cholesterol (14), there was much more cholesterol ester than free cholesterol.

Our finding of a net transfer of serum cholesterol into the chylomicron fraction seems to have been observed

also by Chevallier and Philippot in the rat (15). These investigators reported, however, that both free cholesterol and cholesterol ester accumulated in the chylomicron fraction whereas we found a migration of the free cholesterol fraction only. Glomset et al. observed (16) that when rat and human plasma were incubated for 18 hr or more at 37°, an increase in cholesterol ester took place. Such an increase was not observed during our 3-hr incubations of dog serum either alone or with chylomicrons.

Exchange of phospholipids between chylomicrons and serum lipoproteins has not been studied much. In contrast to our earlier work showing a very limited exchange of phospholipids, Havel and Clarke reported more extensive exchange (2). In the present studies, we found exchange of phospholipids to be quite a complex process. The percentage exchange per unit of time appeared to increase greatly as the relative amount of serum was increased. The exchange of phospholipids was not the same for individual phospholipid fractions, but, in the serum, the high- and low-density lipoprotein phospholipids appeared to be involved in proportion to their concentration. As was observed for the exchange of phospholipids between α- and β-lipoproteins (17) or between high-density and low-density lipoproteins (18), the exchange, although initially quite rapid, did not achieve equilibrium within the 3-hr period of incubation. For all phospholipid fractions, the specific activities of the initially labeled chylomicrons remained 2–3 times as high as the final serum phospholipid specific activity.

In addition to the exchange described previously (2, 3), we also noted in these experiments a net transfer of phospholipids from the lymph chylomicron to the serum. The extent of this transfer also appeared to depend on the relative amounts of lymph chylomicrons and serum present and differed considerably for the individual phospholipid fractions. From these data, one might postulate that the following things happen when a lymph chylomicron comes in contact with blood in the intact animal. In the dog, lymph chylomicrons circulate on the average for about 15 min (3). During that time, they pick up free cholesterol from the serum and lose a considerable amount of phospholipid to the serum high-density lipoproteins so that the chylomicron composition changes. During this time, phospholipids also exchange between the chylomicrons and the serum lipoproteins. A calculation based on the assumption that the amount of serum phospholipid present at any one time is ten times as great as the phospholipids of the chylomicrons, and that exchange in vivo, takes place at the same rate as exchange in vitro, shows that during the first 15 min after injection

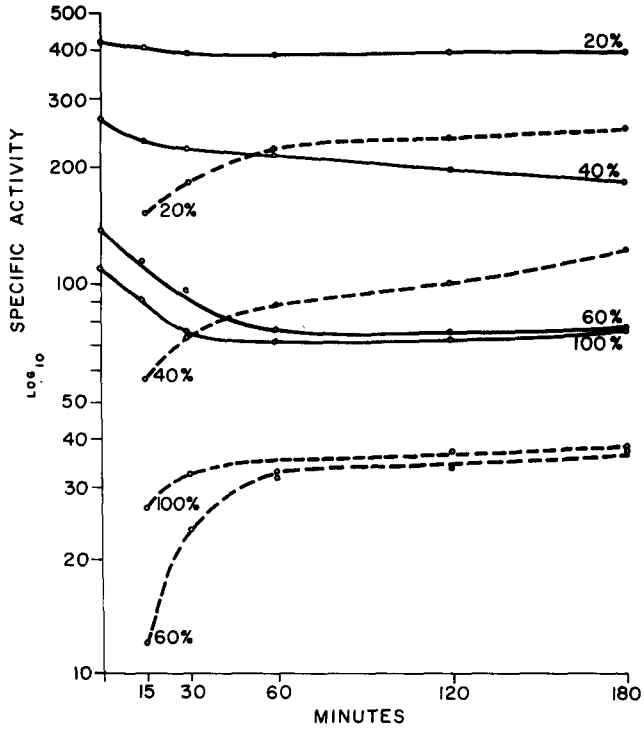

FIG. 6. Specific activities of individual phospholipids in chylomicrons and serum during incubation. Solid line, chylomicron fraction; broken line, serum fraction. The results were obtained from experiment B in which the approximate ratio of chylomicron to serum phospholipid was C:S 50:50. Specific activity is represented by cpm/μg lipid phosphorus. % in figure refers to % methanol (by volume) in chloroform used for elution. Specific activities of individual phospholipids in the original chylomicron and the chylomicron fraction incubated with saline (blank) were as follows:

Methanol in Eluate: Main Component:		20% Non-choline	40% Lecithin	60% Sphingomyelin	100% Lysolecithin
Original		431	285	145	141
Blank	0 min	437	284	144	140
	180 min	424	289	148	162

of lymph chylomicrons as much as 60% of their P^{32} could be lost by exchange and net transfer reactions if no triglyceride had disappeared from the bloodstream. During this time, about 65% of the triglyceride leaves the circulation (3), and it becomes quite difficult to calculate the amount of phospholipid released by exchange from the chylomicrons of a continually shrinking pool of triglyceride. It would appear, therefore, that the conclusion of McCandless and Zilversmit (3) that exchange of phospholipid cannot account for the slower disappearance of chylomicron phospholipid-P^{32} than that of chylomicron triglyceride should be modified. It seems now that exchange might account for this differential rate and that the previous experiments do not prove that in the intact animal the chylo-

micron triglyceride is removed separately and at a faster rate than the chylomicron phospholipid. On the other hand, these experiments do not lend any strength to the conclusion of Havel and Fredrickson (19) that triglyceride and phospholipid of chylomicrons are removed simultaneously. The observation of Havel and Clarke (2) that some increase in the high-density lipoprotein phospholipid was observed when large amounts of chylomicrons were infused into a dog could be accounted for by our observation that a net transfer of phospholipids occurs from lymph chylomicrons to serum during incubation of the two components.

Finally, it may be observed that the exchange of chylomicron components with those of serum involves a set of independent reactions and not the exchange of intact lipoprotein molecules. This conclusion is substantiated by the observation that while phospholipids migrate from the lymph chylomicron to the serum lipoproteins, the free cholesterol moves in the opposite direction and the cholesterol ester fraction does not move at all. Such behavior could not be easily explained by the net transfer or exchange of intact lipoproteins.

The authors wish to thank Dr. H. A. I. Newman for his helpful suggestions and advice, Mrs. Rita Russell for her technical assistance, and Dr. Akira Yokoyama for the thin-layer chromatography of phospholipid fractions.

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