Physical and chemical changes in isolated chylomicrons: prevention by EDTA

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ABSTRACT When washed chylomicrons obtained from the lymph of rats, which had been fed corn oil, were maintained at 0–4°C in 0.9% NaCl, the following physical and chemical changes were observed to occur: increased polarity of chylomicron lipids, increased ultraviolet absorption of chylomicron lipids at 232 nm, decreased linoleic acid content, and aggregation. These alterations occurred more rapidly at room temperature and were found to be associated with an increased lipid peroxide content. The behavior of isolated olive oil chylomicrons was qualitatively similar. All observed changes in the properties of washed chylomicrons were prevented by 0.25 mM EDTA; when tested, 25 μM EDTA was equally effective. The changes were also prevented by 0.02% hydroquinone. The alterations in chylomicron lipids appear to result from the autoxidation of esterified linoleic acid. Studies with linoleic acid-U-^{14}C-labeled chylomicrons indicated that cleavage of the linoleic acid carbon chain did not accompany the physicochemical changes. These results demonstrate the usefulness of EDTA in preventing these specific and progressive alterations in chylomicrons dispersed in aqueous systems.

SUPPLEMENTARY KEY WORDS triglycerides · corn oil · lymph · autoxidation · linoleic acid · hydroquinone · lipid peroxides · olive oil · polyunsaturated fatty acids

Studies on chylomicron structure and metabolism have been performed with thoracic duct lymph, chylomicron-rich plasma, and washed chylomicrons derived therefrom. Between the time of lymph collection and the subsequent use of the lymph, plasma, and isolated chylomicrons, alterations in the chemical and physical structure of the chylomicrons may occur (1, 2). The prevention of physicochemical changes in chylomicrons during this period is of importance in gaining meaningful information on their chemical, physical, and biological properties. In the present study certain progressive alterations in chylomicrons were observed, and a means for their prevention was found.

Abbreviations: EDTA, ethylenediaminetetraacetic acid; GLC, gas–liquid chromatography; TLC, thin-layer chromatography.

MATERIALS

Palmitic acid-1-^{14}C and linoleic acid-U-^{14}C were obtained from New England Nuclear Corp. (Boston, Mass.) and Applied Science Laboratories Inc. (State College, Pa.), respectively. These fatty acids were over 98% radiochemically pure as determined by TLC and liquid scintillation counting as described below (Methods). The crystallized bovine serum albumin was a product of Pentex Biochemicals, Kankakee, Ill. The corn oil (Mazola) and olive oil (Pompeian) were obtained locally.

METHODS

Isolation and Treatment of Lymph and Chylomicrons

Thoracic duct lymph from rats fed corn oil and either palmitic acid-1-^{14}C (experiments 1 and 2) or linoleic acid-U-^{14}C (experiments 3 and 4) via gastrostomy tube was collected at 0°C by the procedure of Bollman, Cain, and Grindlay (3). Crystallized bovine serum albumin was added to the lymph in order to remove radioactive free fatty acids (4), and chylomicrons were then isolated by flotation at 0–4°C through a discontinuous sodium chloride gradient (5). Tubes were centrifuged at 25,000

rpm (75,465 *g*) for 1 hr in the SW 25.2 rotor of the Beckman model L-2 ultracentrifuge. The chylomicron layers were pooled and dispersed in 0.9% NaCl. This dispersion was overlaid with 0.9% NaCl and centrifuged at 25,000 rpm (75,465 *g*) for 30 min in the SW 25.2 rotor. The resulting layer of chylomicrons was removed and dispersed in 0.9% NaCl. The sodium chloride was of high commercial purity and was dissolved in water redistilled in an all glass system. The chylomicron suspensions in experiment 1 (Tables 1–3), experiment 2 (Table 4), experiment 3 (Table 5), and experiment 4 (Table 6) contained 43, 30, 50, and 76 mg of chylomicron lipid per ml, respectively. In experiment 1 the chylomicron preparation was divided into three equal portions. One portion was untreated and served as a control. The second portion was flushed extensively with nitrogen at 0°C and sealed. A 50 mM solution of sodium EDTA, pH 7.4, was added to the third portion to bring the final concentration of EDTA to 0.25 mM. Whole lymph (31 mg of lipid per ml) was also divided into three equal portions and similarly treated except that the lymph sample which contained EDTA had a final EDTA concentration of 2.0 mM. Because of the higher concentration of divalent ions in lymph, a higher level of EDTA was used. At this time, and after various periods at 0–4°C, 0.2-ml aliquots of the lymph and washed chylomicron preparations were mixed with 2.8 ml of ethanol–diethyl ether 3:1. The chylomicron suspensions contained 5 mg or less of lipid per 100 μl and dissolved completely in this solvent mixture. This extraction procedure was validated in separate experiments, which showed that up to 13 mg of corn oil chylomicrons was completely dissolved by 2.8 ml of ethanol–diethyl ether 3:1 containing 0.2 ml of aqueous 0.9% NaCl. The protein precipitate was removed by filtration. The lipid extracts were analyzed spectrophotometrically and by TLC and GLC as described below. In experiments 3 and 4, with linoleic acid-U-^{14}C-labeled chylomicrons, 100-μl aliquots of the chylomicron suspensions were mixed with 10 ml of ethanol–diethyl ether 3:1 and with 2 ml of chloroform–methanol 2:1 for subsequent analyses. All samples were analyzed in duplicate, and the average values are shown in Tables 1–6. The washed chylomicron and lymph preparations were also examined by phase contrast microscopy.

Absorption of Lipids at 232 nm

Linoleic acid is the major fatty acid in corn oil chylomicrons. When linoleic acid undergoes autoxidation, diene conjugation with strong absorption at 230–236 nm occurs (6). The absorption of the ethanol–diethyl ether extracts at 232 nm was determined immediately after their preparation. If the optical density of the extract exceeded 0.7, an aliquot of the extract was diluted with a measured volume of ethanol–diethyl ether 3:1, and the optical density was remeasured. In experiments 2 and 3 (Tables 4 and 5), the optical density at 232 nm was determined by adding 5-50-μl aliquots of the washed chylomicron preparations directly to cuvettes containing 3.0 ml of ethanol–diethyl ether 3:1. Turbidity was never observed under these conditions. The addition of 0.9% NaCl alone to the solvent mixture did not alter the optical density. The molar extinction coefficient of linoleic acid hydroperoxide in ethanol at 233 nm is 25,250 (7, 8). This value was used to estimate the quantity of hydroperoxide equivalent to the optical densities observed. This may be only an approximation since the molar extinction coefficient of linoleic acid hydroperoxide might be altered when the fatty acid is in esterified form, such as in triglyceride, and when measured in ethanol–diethyl ether 3:1 at 232 nm. Furthermore, it is not established that all of the observed absorption of chylomicron lipids at 232 nm is due to hydroperoxides.

Lipid Peroxide Analysis

Lipid peroxides were measured by a modification of the method of Hartman (9). The chylomicron suspension (5–20 μl) or the isolated chylomicron lipid was dissolved in 6 ml of benzene and then mixed with 3 ml of the $FeSO_4$ reagent (reagent 6 of Hartman [9] diluted with methanol to contain 56 μg of Fe^{++} per ml). After 10 min, 1 ml of dichlorophenolindophenol (reagent 5 of Hartman [9]) was added and after mixing, the optical density was measured at 530 nm without delay. The blank contained no added lipid. The standard contained 2 ml of the $FeSO_4$ reagent and 1 ml of methanol in place of 3 ml of the $FeSO_4$ reagent. The same quantities of peroxide were found whether aliquots of the chylomicron suspensions (5–20 μl) were added directly to the benzene, or whether the chylomicron lipids alone were added. The chylomicron lipids were obtained by extraction of the chylomicron suspension with 20 volumes of chloroform–methanol 2:1 followed by washing according to the procedure of Folch, Lees, and Sloane Stanley (10) and by evaporating the lower phase to dryness with a stream of nitrogen. The lipid residue was immediately dissolved in benzene for peroxide analysis. Since the chylomicron lipids contained the same quantity of peroxides as the parent chylomicron suspensions, the simpler method of addition of 5- to 20-μl aliquots of the chylomicron suspensions directly to benzene was used. The thiobarbituric acid method for determination of lipid peroxides was not used since it reportedly does not measure autoxidation of linoleic acid (11).

Thin-Layer Chromatography

Other aliquots of the lymph and chylomicron extracts were evaporated to dryness under nitrogen, and the residues were dissolved in a small volume of diethyl

ether. A small amount of palmitic acid was added to aid in the subsequent visualization of the free fatty acid band. The lipids were then separated by TLC on Silica Gel G containing the fluorescent agent Ultraphor (12) with a solvent system of petroleum ether (boiling range 40–60°C)–diethyl ether–glacial acetic acid 90:10:1. The total area traversed by the solvent was divided into the following five regions: cholesteryl ester, triglyceride, free fatty acid, partial glyceride, and phospholipid. The TLC region between the phospholipid at the origin and the free fatty acid band contains cholesterol, diglycerides, and monoglycerides. This region is termed the partial glyceride region. The silica gel was scraped into counting vials, and 8 ml of dioxane scintillation fluid (see below) and 0.2 ml of H_2O were added. In the experiments with palmitic acid-1-^{14}C-labeled chylomicrons, the scintillation fluid was prepared by adding 100 g of naphthalene, 7 g of 2,5-diphenyloxazole and 0.3 g of 1,4-bis[2-(5-phenyloxazolyl)]benzene to 1 liter of dioxane. The silica gel sediments in the dioxane system. In the experiments with linoleic acid-U-^{14}C-labeled chylomicrons, the silica gel in the various TLC regions was suspended in the scintillation mixture of Kinard (13), which was slightly modified in that the α-naphthylphenyloxazole was replaced by 1,4-[2-(4-methyl-5-phenyloxazolyl)]benzene and 4% Cab-O-Sil was added as suggested by Gordon and Wolfe (14). Similar results were obtained with the two different liquid scintillation mixtures.

Gas–Liquid Chromatography

Other aliquots of the lymph and chylomicron extracts were evaporated to dryness under nitrogen, and the lipids were dissolved in 0.2 ml benzene and 1.0 ml 5% H_2SO_4 in methanol. The tubes were flushed with nitrogen, sealed, and heated overnight at 65°C. 1.2 ml of water and 1.2 ml of petroleum ether (boiling range 40–60°C) were added and mixed. The petroleum ether layer was removed, and the lower phase was extracted again with 1.2 ml of petroleum ether. Methyl esters of fatty acids in the combined first and second petroleum ether extracts were then separated at 200°C in an F & M 5750 gas chromatograph using 6 ft glass columns containing 14% ethyleneglycol adipate on Chromosorb W. A flame ionization detector was used. Standard mixtures agreed to within 5% of the known composition.

Total Lipid Determination

Lymph and chylomicron preparations were mixed with 20 volumes of chloroform–methanol 2:1, filtered, and washed (10). The lower phase was then diluted to a known volume with chloroform–methanol 2:1, and duplicate aliquots were evaporated to dryness under vacuum to a constant weight. In the experiments with linoleic acid-U-^{14}C-labeled chylomicrons, the radioactivity in the upper-phase washings and the lower-phase lipids was counted.

Ozonolysis

Ozone in oxygen was bubbled for 20 min through 25 ml of chloroform–ethanol 8:1 containing 10 mg of chylomicron lipids labeled with linoleic acid-U-^{14}C. The gas flow rate was 60 ml/min, and the ozone concentration was 4%. 25 ml of ethanol and 3 ml of 30% H_2O_2 were then added. After 2 days at room temperature, 4 ml of water was added. Half the total solution was then evaporated under nitrogen to 2 ml and dissolved in 40 ml of chloroform–methanol 2:1. The solution was then washed (10). Radioactivity in the upper and lower phases was then measured.

RESULTS

Experiment 1

The percentages of radioactivity in the cholesteryl ester, triglyceride, free fatty acid, partial glyceride, and phospholipid regions separated by TLC are shown in Table 1. Radioactivity in the partial glyceride region increased markedly in the untreated chylomicrons maintained at 0–4°C, as did that in the phospholipid region at the origin. These changes were not prevented by a nitrogen atmosphere, but were completely prevented by EDTA, at a final concentration of 0.25 mM. Similar results (Table 1) were observed with the lymph preparations, but the alterations were less marked. After 7 days the percentage of radioactivity in the free fatty acid fraction of the washed chylomicrons and whole lymph was unchanged although it subsequently increased somewhat. The radioactivity in the cholesteryl ester fraction remained constant throughout.

The absorption of the chylomicron lipids at 232 nm is shown in Table 2. A marked increase in absorption was observed in the lipids of the washed chylomicrons which were untreated or kept under nitrogen. EDTA (0.25 mM) completely prevented this increase in absorption. In contrast, the absorption at 232 nm of lipids extracted from untreated whole lymph was not appreciably altered after 7 or 17 days at 0–4°C, and these data are, therefore, not shown in Table 2.

The percentage of linoleic acid in the total lipids of washed chylomicrons gradually decreased (Table 3). The decrease was small after 1 week at 0–4°C, but was much more marked after 17 days. A nitrogen atmosphere did not prevent this alteration, whereas 0.25 mM EDTA completely protected against the loss of linoleic acid.

Phase contrast microscopic examination of the washed chylomicrons immediately after their isolation revealed single dispersed chylomicrons with a large variation in

TABLE 1 PERCENTAGE DISTRIBUTION OF RADIOACTIVITY IN CHYLOMICRON AND LYMPH LIPID FRACTIONS SEPARATED BY TLC (EXPERIMENT 1)

Time at 0–4°C	Lipid Region	Chylomicron Preparations			Whole Lymph Preparations		
		Untreated	Stored under Nitrogen	Containing 0.25 mM EDTA	Untreated	Stored under Nitrogen	Containing 2.0 mM EDTA
days		%	%	%	%	%	%
0	Cholesteryl ester	0.9			0.8		
	Triglyceride	92.3			91.2		
	Free fatty acid	0.1			1.1		
	Partial glyceride*	5.3			4.4		
	Phospholipid	1.4			2.5		
7	Cholesteryl ester	0.8	0.8	0.7	1.0	0.9	0.9
	Triglyceride	78.5	78.9	92.5	89.6	88.8	91.5
	Free fatty acid	0.2	0.2	0.1	1.2	1.1	1.3
	Partial glyceride*	14.1	14.9	5.6	5.1	5.6	4.2
	Phospholipid	6.4	5.2	1.1	3.1	3.6	2.1
17	Cholesteryl ester	0.7	0.7	0.8	0.8	0.8	1.0
	Triglyceride	56.9	49.4	92.1	78.1	81.1	89.4
	Free fatty acid	0.3	0.4	0.3	2.5	3.1	2.0
	Partial glyceride*	23.8	29.9	5.5	10.4	8.7	5.0
	Phospholipid	18.3	19.6	1.3	8.2	6.3	2.6

Washed corn oil chylomicrons, labeled with palmitic acid-1-^{14}C, were suspended in 0.9% NaCl and divided into three equal parts. One portion was untreated, the second was maintained under nitrogen, and the third contained 0.25 mM EDTA. These chylomicron preparations each contained 43 mg of lipid per ml. Three whole lymph preparations were similarly prepared from a sample of rat thoracic duct lymph obtained after feeding corn oil and palmitic acid-1-^{14}C. An EDTA level of 2.0 mM was used in the third lymph preparation. Each lymph preparation contained 31 mg of lipid per ml. All preparations were maintained at 0–4°C.

* This region contains cholesterol, diglycerides, and monoglycerides.

TABLE 2 ABSORPTION OF CHYLOMICRON LIPIDS AT 232 nm (EXPERIMENT 1)*

Time† at 0–4°C	Untreated	Under Nitrogen Atmosphere	Containing 0.25 mM EDTA
days		*absorbance*‡	
0	0.189		
7	2.437	2.932	0.142
17	6.800	6.710	0.263

* The absorbance is the optical density of 1 mg of lipid per ml of ethanol–diethyl ether 3:1.

† The chylomicron preparations are described in Table 1.

‡ For absorbance measurements the chylomicron preparations were diluted 225-fold in ethanol–diethyl ether 3:1.

size as normally seen (15). After 7 days at 0–4°C, the untreated washed corn oil chylomicrons and the preparations flushed with nitrogen showed some clumping of lipid particles. After 17 days the majority of chylomicrons were clumped together, and bulky masses of aggregated chylomicrons were observed. However, the EDTA-treated chylomicrons remained individually dispersed and appeared completely normal. All the lymph preparations, whether examined at 0, 7, or 17 days, appeared to be similar by phase contrast microscopy. Aggregation of the chylomicrons in whole lymph was not observed.

Experiment 2

The rates of increase in 232 nm absorption of washed chylomicrons as a function of EDTA concentration at 0–4°C and at room temperature were measured (Table 4). Antibiotics were added to the preparations kept at room temperature. The increase in absorption was far greater at room temperature than at 0–4°C. This was not due to the added antibiotics which had no effect on chylomicrons maintained at 0–4°C. EDTA at a concentration of 25 μM was as effective as 0.25 mM EDTA in preventing the increased absorption at both temperatures. The ultraviolet absorption spectrum of the chylomicron preparation maintained at 0–4°C for 4 days without EDTA, exhibited an absorption maximum at 230–232 nm typical of the spectrum of ethyl linoleate after 1.6% oxygen uptake (6).

Experiment 3

The observed increase in polarity of chylomicron lipids, the increase in absorption at 232 nm, and the loss of linoleic acid suggest that the esterified linoleic acid in the chylomicrons was undergoing autoxidation. Therefore, lipid peroxides, absorption at 232 nm, and linoleic acid content (determined by GLC), in linoleic acid-U-^{14}C-labeled chylomicrons dispersed in saline, were measured

TABLE 3 FATTY ACID COMPOSITION OF TOTAL LIPIDS OF WASHED CORN OIL CHYLOMICRONS ANALYZED BY GLC* (EXPERIMENT 1)

Time at 0–4°C	Fatty Acid	Washed Chylomicron Preparations		
		Untreated	Under Nitrogen Atmosphere	Containing 0.25 mM EDTA
days		%	%	%
0	16:0	15.0		
	16:1	0.8		
	18:0	3.1		
	18:1	24.3		
	18:2	56.8		
7	16:0	15.5	16.7	14.6
	16:1	0.8	1.0	0.7
	18:0	3.6	3.4	3.3
	18:1	25.6	25.6	24.1
	18:2	54.5	53.3	57.3
17	16:0	22.0	22.1	15.0
	16:1	1.2	1.4	0.8
	18:0	4.8	5.3	3.2
	18:1	34.2	35.0	24.1
	18:2	37.8	36.2	56.9

The chylomicron preparations are described in Table 1.

* Small peaks, which were less than 0.7% of the total fatty acids, were not included.

TABLE 4 EFFECT OF EDTA CONCENTRATION, TEMPERATURE, AND TIME ON THE ABSORPTION OF CHYLOMICRON PREPARATIONS AT 232 nm (EXPERIMENT 2)

EDTA Concentration of Chylomicron Preparation	1 Day	2 Days	3 Days	4 Days	8 Days
μM 0–4°C			*absorbance**		
0	0.158	0.244	0.452	3.014	8.354
250	0.012	0.006	0.026	0.032	0.040
25	0.024	0.020	0.022	0.016	0.028
2.5	0.076	0.132	0.242	0.778	4.454
19–22°C					
0†	0.280	1.078	4.500	7.950‡	
250†	0.078	0.106	0.162	0.106	
25†	0.046	0.090	0.084	0.112	
2.5†	0.214	0.906	3.424	6.860	

* Absorbance of 1 mg of chylomicron lipid per ml of ethanol–diethyl ether 3:1, measured by adding 5–50 μl of the chylomicron preparations, which contained 30 mg of lipid per ml, directly to 3 ml of the solvent mixture. When the measured absorbances exceeded 0.7, the absorption of a smaller quantity of chylomicron lipid was measured. At time zero, the absorbances of the chylomicron suspensions not containing EDTA were 0.178 without and 0.305 with antibiotics. These values were subtracted from the absorbances of all samples measured at later times.

† These preparations contained 0.8 mg of streptomycin sulfate and 600 units of benzyl penicillin per ml.

‡ An aliquot, which did not contain antibiotics, had an optical density of 9.270 after 4 days at 19–22°C.

as a function of time at room temperature (Table 5). An increase in lipid peroxides accompanied the increase in absorption at 232 nm and the decrease in linoleic acid. Although the reaction between ferrous ions and lipid peroxides (see Methods) is not stoichiometric and high values are obtained, the method is both sensitive and highly reproducible (9, 16), and also provides a reliable comparison of the lipid peroxides present in the chylomicron preparations at various times. The lipid peroxide analyses indicated 7- to 9-fold more peroxides than calculated from the absorption at 232 nm and 6- to 7-fold more peroxides than calculated from the GLC data. It, therefore, appears that six to nine ferrous ions were oxidized per lipid peroxide molecule in the peroxide analysis. EDTA completely prevented the accumulation of lipid peroxides. Hydroquinone (0.02%) was equally effective. The specific radioactivity of the chylomicron lipids and the total lipid radioactivity were unaltered as a function of time. No radioactivity was found at any time in the washings (upper phase) of the lipid extract (10). However, 17% of the radioactivity was found in the upper phase after cleavage of the double bonds of lipids of linoleic acid-U-^{14}C-labeled chylomicrons with ozone and hydrogen peroxide. The ozonolysis mixture was evaporated to about 4% of its volume before extraction and washing. The 17% recovery of the activity in the upper phase was expected since the three carbon acid (malonic acid) would readily be extracted into the aqueous upper phase. The volatile six carbon acid (caproic acid) was removed during the evaporation of the ozonolysis mixture. This was consistent with the recovery of 30% of the linoleic acid-U-^{14}C radioactivity in the distillate after ozonolysis, treatment with H_2O_2, and distillation in a stream of nitrogen.

Experiment 4

The migration of lipids from palmitic acid-1-^{14}C-labeled chylomicrons on TLC showed no difference in the cholesteryl ester radioactivity, while the triglyceride radioactivity was markedly decreased (Table 1). Since alteration of cholesteryl esters containing polyunsaturated fatty acids would not be observed in chylomicrons labeled with palmitic acid-1-^{14}C, washed corn oil chylomicrons labeled with linoleic acid-U-^{14}C were subsequently obtained of sufficiently high specific activity to determine whether cholesteryl linoleate in chylomicrons undergoes alteration. These chylomicrons were maintained at 25°C. The TLC plates were developed with hexane–diethyl ether–glacial acetic acid 91:9:1, and wide separation of the triglycerides and cholesteryl esters was achieved. The alterations in the chylomicron cholesteryl esters closely paralleled the changes in the triglyceride radioactivity (Table 6). Sufficiently high counting rates were obtained to make the observed decrease in the cholesteryl ester radioactivity significant. The rate of increase in absorption at 232 nm was considerably slower than ob-

TABLE 5 EFFECT OF EDTA (0.125 mM) ON CORN OIL CHYLOMICRONS (EXPERIMENT 3)

	Chylomicron Preparation	Time (hr)						
		0	26	45	70	94	119	142
Hydroperoxides*	Untreated	0.030	0.046	0.081	0.142	0.218	0.305	0.339
(*μeq/mg lipid*)	EDTA	0.029	0.028	0.030	0.027	0.033	0.034	0.031
Lipid peroxides†	Untreated	0.26	0.34	0.78	1.10	1.50	2.18	2.96
(*μeq/mg lipid*)	EDTA	0.28	0.23	0.34	0.29	0.25	0.35	0.34
Loss of linoleic acid‡	Untreated			0.068	0.193		0.382	0.554
(*μeq/mg lipid*)	EDTA			−0.017§	0.025		0.000	0.008
Lipid ^{14}C	Untreated	2080		2060	2050		2130	2020
(*dpm/mg lipid*)‖	EDTA	2060		2040	2080		2160	2090

The chylomicrons were suspended in 0.9% NaCl (50 mg of chylomicrons per ml) containing 0.8 mg of streptomycin sulfate and 600 units of benzyl pencillin per ml. The temperature was 23–27°C.

* Using a molar extinction coefficient of 25,250 (7, 8).

† By the method of Hartman (6) as modified.

‡ Calculated from GLC.

§ Calculated increase in linoleic acid.

‖ The total lipid, measured gravimetrically, was not detectably altered in the untreated and the EDTA-containing chylomicron preparations during the experimental period.

TABLE 6 PERCENTAGE DISTRIBUTION OF RADIOACTIVITY IN LIPID FRACTIONS OF LINOLEIC ACID-U-^{14}C-LABELED CHYLOMICRONS SEPARATED BY TLC (EXPERIMENT 4)

Days at 25°C	Lipid Region	Chylomicron Preparations	
		Untreated	Containing 0.25 mM EDTA
		%	%
0	Cholesteryl ester	0.52	
	Triglyceride	92.15	
	Free fatty acid	0.22	
	Partial glyceride	1.78	
	Phospholipid	5.22	
6	Cholesteryl ester	0.49	0.51
	Triglyceride	80.17	92.31
	Free fatty acid	0.51	0.25
	Partial glyceride	7.46	1.68
	Phospholipid	11.17	5.04
10	Cholesteryl ester	0.43	0.51
	Triglyceride	61.98	92.15
	Free fatty acid	0.55	0.24
	Partial glyceride	15.21	1.90
	Phospholipid	21.60	5.00

The absorption at 232 nm indicated the formation of 0.126 and 0.262 μeq of hydroperoxide per mg of lipid in the untreated chylomicrons after 6 and 10 days, respectively. The absorption at 232 nm of the chylomicron preparation containing EDTA remained unchanged.

served in previous chylomicron preparations. EDTA, 0.25 mM, prevented these changes throughout the entire period at 25°C.

Olive Oil Chylomicrons

To determine if these observations apply as well to chylomicrons of lower polyunsaturated fatty acid content, chylomicrons were also isolated from the thoracic duct lymph of rats fed olive oil and palmitic acid-1-^{14}C. These chylomicrons contained 12.8% palmitic, 1.5% stearic, 73.6% oleic, and 12.0% linoleic acid, and were maintained at 25°C in 0.9% NaCl. After a lag period, the absorption at 232 nm of olive oil chylomicrons increased markedly, and the migration of lipid radioactivity on TLC plates decreased. These alterations were prevented by 0.25 mM EDTA.

DISCUSSION

The free fatty acid radioactivity in washed chylomicrons did not increase after 7 days at 0–4°C (Table 1). This suggests that the increased radioactivity in the partial glyceride and phospholipid fractions (Table 1) was not a result of monoglyceride and diglyceride formation by partial hydrolysis of triglyceride. The magnitude of the decrease in radioactivity in the triglyceride region cannot be accounted for by partial hydrolysis of triglyceride and subsequent exchange of the liberated free fatty acids with phospholipid fatty acids. The progressive increase in the chylomicron lipid radioactivity, which migrated in the partial glyceride and phospholipid regions (Table 1), clearly indicates that the chylomicron lipids gradually became more polar. The lipids of washed chylomicrons concurrently exhibited an increased absorption at 232 nm (Table 2) characteristic of the formation of conjugated double bonds in linoleic acid (6). These alterations, together with the decrease in linoleic acid content (Table 3), suggest that the esterified linoleic acid in the chylomicron lipids gradually autoxidized. Autoxidation of the linoleic acid in triglycerides would cause the triglyceride to become more polar and migrate more slowly in agreement with the observed shift in the TLC pattern of lipid radioactivity (Table 1). Autoxidation of linoleic acid gives rise to polar products, which should be adsorbed strongly on the GLC column and correspond-

ingly diminish the proportion of linoleic acid methyl ester as observed (Table 3). The observed increase in lipid peroxides, as measured by the ferrous ion procedure, paralleled the loss in linoleic acid measured by GLC and the increased hydroperoxide content calculated from the absorption at 232 nm (Table 5). It is, therefore, evident that chylomicron lipids suspended in saline, undergo gradual alterations characterized by autoxidation of the polyunsaturated fatty acids. Lack of radioactivity from linoleic acid-U-^{14}C in the upper phase after extraction and washing (10), indicates that the double bonds in linoleic acid did not undergo cleavage. Oxidative cleavage of the double bonds with ozone and hydrogen peroxide led to significant water-soluble radioactivity. Schauenstein (17) has reported the formation of a variety of water soluble products from the autoxidation of methyl linoleate in water after prolonged periods at 40°C. The absence of detectable water-soluble products in the present study is probably a result of the maintenance of the chylomicrons at a lower temperature. The decomposition of methyl linoleate hydroperoxide is markedly temperature dependent and very slow below 30°C (18).

After 7 days at 0–4°C, about 14% of the chylomicron triglyceride became more polar (Table 1), the absorption of chylomicron lipids at 232 nm increased 13-fold (Table 2), and the relative percentage of linoleic acid in chylomicrons decreased 2.3% (Table 3). Close inspection shows that these results are in agreement. A 2.3% loss in the percentage of linoleic acid represents an actual loss of 50 μg of linoleic acid per mg of chylomicron fatty acids. About 570 μg of linoleic acid is present in each mg of corn oil chylomicron fatty acids. Therefore, about one in 11 linoleic acid esters was altered. Depending on the extent of autoxidation of the linoleic acid in the chylomicron phospholipids, which contain about 3% of the total linoleic acid in corn oil chylomicrons, between 10 and 15 of every 100 triglyceride molecules should, therefore, be altered. Indeed it was shown that 14% of the chylomicron triglyceride became more polar (Table 1). Assuming a molar extinction coefficient of 25,250 (see Methods), the change in absorbance at 232 nm represents a conversion of 70 μg linoleic acid to the hydroperoxide form per mg of lipid. In view of the assumptions involved in the use of this extinction coefficient and the limits of accuracy of the methods employed, the GLC, TLC, and spectrophotometric data are in good agreement. Calculations on the changes in the lipids of chylomicrons stored under nitrogen (Tables 1–3) are also in agreement.

The increased polarity, increased absorption at 232 nm, and the decreased linoleic acid content of washed chylomicrons were not prevented by a nitrogen atmosphere (Tables 1–3). It is probable that some oxygen was still present in the preparations flushed with nitrogen. The nitrogen replacement was done at 0°C. At this temperature the solubility of oxygen in water is much greater than at room temperature. Furthermore, the concentration of oxygen in the chylomicrons was probably greater than in the water phase since oxygen is more soluble in organic solvents than in water (19). Removal of 95% of the oxygen decreased the rate of autoxidation of ethyl linoleate at 45°C only about 25% (20).

Autoxidation of polyunsaturated fatty acids can be catalyzed by trace metal ions (6, 21, 22). The preventive effects of EDTA in the present study (Tables 1–6) are consistent with the view that the physicochemical alterations of chylomicron lipids resulted from autoxidation catalyzed by trace metals. EDTA has also been reported to decrease lipid peroxide formation in tissue homogenates (23–25). Peroxide formation in the chylomicrons was also prevented by hydroquinone, the antioxidant action of which has long been recognized (6). This finding further implicates autoxidation as the cause of the observed alterations in chylomicron lipids.

The results obtained in experiments 1–4 were based upon analyses of chylomicrons isolated from four samples of thoracic duct lymph obtained from four separate rats at different times. The rates of change in absorption at 232 nm of the chylomicrons varied accordingly. Such variation might be related to different amounts of antioxidant in the chylomicrons and to variable amounts of trace ions present in the aqueous phases of the chylomicron preparations. The presence of antioxidant in the chylomicrons is suggested by the lag phase both in the increased 232 nm absorption and in the formation of lipid peroxides measured with ferrous ions (Tables 4 and 5) (26).

The absence of increased 232 nm absorption of whole lymph lipids, even after 17 days of storage, does not exclude the possibility that lipid autoxidation caused the changes in the polarity of lymph lipids (Table 1). Molecular species absorbing at 232 nm may react with other lymphatic substances to form products which do not absorb appreciably at this wavelength. Many substances interact with linoleic acid hydroperoxide to form products with decreased ultraviolet absorption (27). Also, secondary reactions which alter newly formed conjugated double bonds may occur in early stages of autoxidation, thereby decreasing ultraviolet absorption (22).

The possibility that microorganisms are responsible for the observed alterations in distribution of radioactivity, ultraviolet absorption, linoleic acid, and lipid peroxide content does not seem likely for the following reasons. A substantial increase in absorption at 232 nm was observed after only 24 hr at 0–4°C (Table 4). When the chylomicrons were kept at room temperature for 4 days, antibiotics did not markedly alter the increase in 232 nm absorption (Table 4). Also, at 0–4°C alterations in

the lipids were less marked in whole lymph (Table 1), which would seem to be a far more ideal environment for growth of microorganisms.

Corn oil chylomicrons were mainly used in this study since they have a lower freezing point and are less likely to be damaged by cooling to 0–4°C than are cream chylomicrons. Zilversmit (28) observed that when cream and corn oil chylomicrons were cooled to 4°C and then heated to 60°C, the cream chylomicron preparation liberated oil, whereas the corn oil preparation did not. The autoxidation of corn oil chylomicrons and its prevention by EDTA (Tables 1–6) are also characteristic of chylomicrons containing a lower content of polyunsaturated fatty acids, as evidenced by the observed behavior of olive oil chylomicrons (Results).

Degradation studies on chylomicrons (28, 29) have revealed the presence of a membrane fraction containing free cholesterol, phospholipid, and protein, and an oil phase containing triglyceride and cholesteryl ester. The simultaneous autoxidation of chylomicron triglycerides and cholesteryl esters provides added evidence that these lipids are localized in the same compartment of intact chylomicrons. It is likely that autoxidation occurs near the surface of chylomicrons dispersed in aqueous systems, since trace metal ions in the water phase are involved in the process. The parallel changes in the triglycerides and cholesteryl esters of intact chylomicrons, therefore, suggest that these lipids in the chylomicron interior diffuse continuously, and to similar extents, to sites in or adjacent to the chylomicron surface membrane.

The greater stability of the chylomicron lipids in whole lymph, as compared with the lipids of washed chylomicrons (Table 1), is consistent with the unchanged flotation pattern of serum lipoproteins after the storage of whole serum and the progressive alteration in flotation soon after the lipoproteins were isolated (30). Ultracentrifugal analysis indicated that the degradation of serum lipoproteins which occurred during dialysis could be prevented by serum dialysate (30). Serum and lymph, therefore, apparently contain one or more substances, possibly antioxidants and (or) metal-binding proteins, which aid in the maintenance of normal lipoprotein and chylomicron structure. This suggests that the previous use of whole lymph in metabolic studies (2, 31, 32) might have been more physiological, in terms of normalcy of chylomicron structure, than the use of washed chylomicrons. Gradual alterations in the flotation of β-lipoproteins were enhanced by added copper and prevented by agents which complex copper; these observations are suggestive of an oxidative change (30). Such alterations in isolated lipoproteins may have been caused by the autoxidative process characterized in the present study to occur in the lipids of isolated chylomicrons.

Although the observed alterations in chylomicrons were completely prevented by EDTA (Tables 1–6), it is possible that other unknown changes in the structure and composition of chylomicrons occur after their isolation. However, the EDTA-protected corn oil chylomicrons appear to be very stable even for prolonged periods at 0–4°C. The use of EDTA in the isolation and storage of chylomicrons should, therefore, be of value in studies on their composition, structure, and biological properties.

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