Oxidized lipids in the diet are incorporated by the liver into very low density lipoprotein in rats

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Abstract Previous studies have shown that the quantity of oxidized lipids in the diet directly correlates with the level of oxidized chylomicrons in mesenteric lymph and the level of oxidized lipids in endogenous lipoproteins such as very low density lipoprotein (VLDL) and low density lipoprotein (LDL). The aim of the present study was to determine whether oxidized fatty acids in the diet are delivered via chylomicrons to the liver and whether these lipids are repackaged and secreted in VLDL. In these experiments, oxidized [^{14}C]linoleic acid was utilized as a marker for oxidized dietary fats. When we determined the metabolism of nonoxidized and oxidized [^{14}C]linoleic acid-labeled chylomicrons, we found that hepatic uptake was similar with 13.57 ± 0.84% of nonoxidized and 13.40 ± 0.96% of oxidized linoleic acid delivered to the liver 30 min after chylomicron administration. Additionally, uptake by the extrahepatic tissues was also similar. When the hepatic secretion of VLDL was determined in an in vitro perfusion system after the administration of nonoxidized and oxidized linoleic acid-labeled chylomicrons to intact animals, we found that oxidized linoleic acid was utilized for the formation and secretion of VLDL. After the administration of labeled nonoxidized and oxidized linoleic acid, 0.86 ± 0.07% and 0.70 ± 0.09% of the administered label was found in the liver perfusate at 2 h, respectively. The presence of oxidized linoleic acid in oxidized VLDL was confirmed by demonstrating the presence of hydroperoxide-derived hydroxy octadecanoic acid. Thus, our findings demonstrate that oxidized dietary lipids are delivered to the liver via chylomicrons where they are utilized for synthesis of endogenous lipoproteins such as VLDL.—**Staprans, I., J. H. Rapp, X-M. Pan, and K. R. Feingold.** Oxidized lipids in the diet are incorporated by the liver into very low density lipoprotein in rats. *J. Lipid Res.* 1996. **37:** 420–430.

Supplementary key words oxidized dietary lipids • oxidized linoleic acid • hydroxy octadecanoic acid • hepatic VLDL secretion

While the etiology of atherosclerosis is multifactorial, studies have suggested that oxidized lipoproteins play a major role (1–3). The mechanisms by which lipoproteins are oxidized in vivo have not been precisely defined but a potential site for lipoprotein oxidation has been postulated to be the microenvironment within the arterial wall (1–3). Recent studies in our and other laboratories have suggested that oxidized lipids in the diet may play a significant role in generating oxidized lipoproteins in the circulation. In rats, the levels of oxidized lipids in chylomicrons isolated from mesenteric lymph were directly affected by the quantity of oxidized lipids in the diet (4–6). Similarly, in humans, feeding diets high in oxidized lipids also results in an increase in oxidized lipids in the postprandial serum chylomicron fraction (7, 8). Moreover, in rats, the quantities of oxidized lipids in the diet directly correlated with the levels of oxidized lipids in the serum VLDL plus LDL fraction (6). These results suggest that oxidized lipids in the diet may make a major contribution to the levels of oxidized lipids in intestinally derived lipoproteins and also in endogenous lipoprotein particles such as VLDL. One explanation that could account for this relationship is that oxidized lipids in the diet are absorbed by the small intestine, packaged and secreted in chylomicron particles, transported to the liver, and then repackaged and secreted in VLDL particles.

Our previous studies have shown that oxidized and nonoxidized control chylomicrons are metabolized similarly, with approximately 15% of the fatty acids in chylomicrons delivered to the liver (4). In these experiments [^{3}H]oleic acid label was used to monitor the metabolism of the oxidized and nonoxidized control chylomicron particles. As it is the linoleic acid and not oleic acid that is oxidized in these particles (7), these results do not unequivocally prove that oxidized dietary fatty acids in chylomicrons are delivered to the liver. Moreover, whether the oxidized lipids delivered to the liver are then utilized for the formation and secretion of VLDL is unknown.

Abbreviations: VLDL, very low density lipoprotein; LDL, low density lipoprotein; HODE, hydroxy octadecadienoic acids; OH-18, hydroxy octadecanoic acid; TLC, thin-layer chromatography; GLC, gas–liquid chromatography; HPLC, high pressure liquid chromatography.

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The aim of the present study was to determine whether oxidized fatty acids in the diet are delivered via chylomicrons to the liver and whether these lipids are repackaged and secreted in VLDL. In these experiments, oxidized ^{14}C-labeled linoleic acid was utilized as a source for oxidized dietary fats, and hydroxy octadecanoic acids (OH-18) derived from hydrogenated oxidized linoleic acid were markers for the oxidized fatty acids in VLDL secreted by the liver.

METHODS

Materials

[4-^{14}C]linoleic acid and [^{3}H]retinol were purchased from Amersham Corp. (Arlington Heights, IL) and linoleic acid, platinum(IV) oxide, and Sepharose 2B were from Sigma Chemical Co., (St. Louis, MO). Hydroxy octadecadienoic acids (9-HODE, 13-HODE) were from Cayman Chemical Co. (Ann Arbor, MI). Standard corn oil and vitamin E (alpha-tocopherol)-depleted corn oil were obtained from ICN Biochemicals (Irvine, CA). LPO-FF kit (FF-004) for the determination of lipid peroxides was purchased from Kamiya Biomedical Co. (Thousand Oaks, CA). Silicic acid (BIO-SIL HA, 325 mesh) was from Bio-Rad Laboratories, Richmond, CA. Bio-Safe II counting cocktail was from Research Products International Corp. (Mt. Prospect, IL) and silica gel 60A plates for thin-layer chromatography were from Analtech, Inc. (Newark, DE).

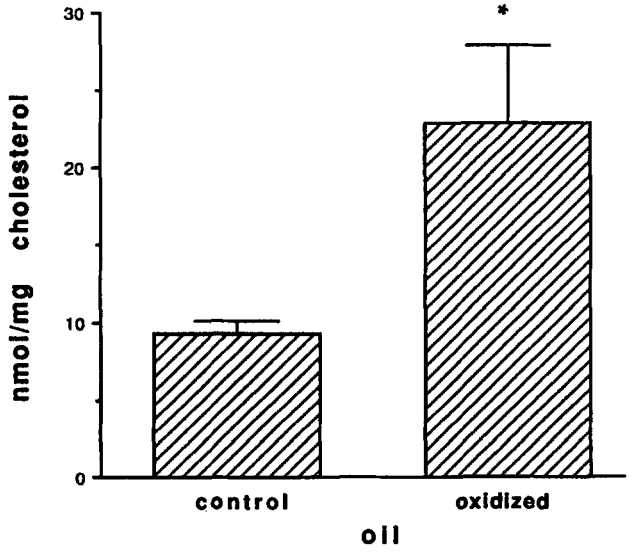

Fig. 1. Levels of oxidized lipids in the serum postprandial chylomicron fraction. Rats were administered intragastrically either control or oxidized oil and serum samples were obtained 2 h later. The chylomicron fraction was isolated by centrifugation. There were 5 rats in each group. All data are expressed as nanomoles conjugated dienes per mg cholesterol and represent means ± SEM; $*P < 0.05$.

Animals

Male Sprague-Dawley rats (180–200 g) were purchased from Simonsen Laboratories (Gilroy, CA) and were maintained on Purina rat chow and water ad libitum. Before all experiments, rats were fasted overnight but drinking water was available. All animals were housed in a temperature-(70°F) and humidity-(60%) controlled animal facility at the San Francisco Veterans Affairs Medical Center and all studies were performed in accordance with institutional policies. All animal procedures were approved by the Subcommittee on Animal Studies.

Dietary lipids

Two kinds of oils were administered to rats intragastrically prior to lymph collection or obtaining postprandial serum. Nonoxidized (control) oil was commercially available corn oil containing no detectable lipid peroxides and only a trace of conjugated dienes (< 5 nmol/mg oil). Oxidized oil was vitamin E-depleted corn oil containing 20–40 nmol lipid peroxides and 60–80 nmol conjugated dienes/mg oil that had accumulated during storage. When we examined the oxidized oil for thiobarbituric acid reactive substances (TBARS) (9), no significant amount was detected, indicating that there were no detectable fatty acid breakdown products in oxidized oil, and the only indication of linoleic acid oxidation was the rearrangement of double bonds producing detectable conjugated dienes and very low levels of lipid peroxides. Thus, the oxidized oil used in our experiments had a low levels of oxidized linoleic acid. Both oils were adjusted to have a similar vitamin E concentration (0.14 mg/ml oil).

Determination of oxidation

Oxidation in oils and chylomicrons was determined by measuring conjugated dienes and lipid peroxides. Conjugated dienes were measured using second derivative UV spectroscopy in a Perkin-Elmer 555 Spectrophotometer (Cupertino, CA) as described by Corongiu, Banni, and Dessi (10) and by us previously (7). Lipid peroxides were determined directly with a LPO-FF kit (Kamiya Biomedical Co) using a colorimetric method utilizing a leukomethylene blue derivative as indicated by the manufacturer (11).

Oxidation of dietary linoleic acid

Unless stated otherwise, [^{14}C]linoleic acid was oxidized for 2–3 h in methanol in the presence of 50 μM Cu_2SO_4 and 20 μg linoleic acid carrier as described by Boeynaems et al. (12). The oxidation was estimated by measuring the disappearance of linoleic acid peak in GLC (13).

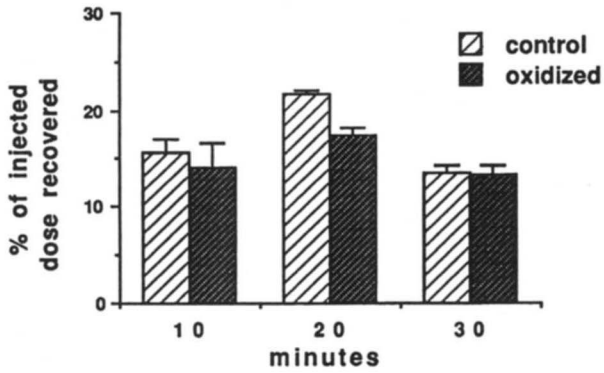

Fig. 2. Recovery of [^{14}C]linoleic acid label in whole livers at 10, 20, and 30 min after the intravenous injection of either nonoxidized [^{14}C]linoleic acid-labeled control or oxidized [^{14}C]linoleic acid-labeled oxidized chylomicrons (10 mg triglyceride). There were 5 rats in each group. All data are expressed as means ± SEM.

Chylomicron isolation from mesenteric lymph

Two chylomicron preparations were prepared using nonoxidized (control) and oxidized dietary lipids. Control (nonoxidized) chylomicrons were prepared from nonoxidized corn oil and labeled with a mixture of 50 μCi of nonoxidized [^{14}C]linoleic acid and 0.02 mg linoleic acid carrier from a freshly opened vial. Oxidized chylomicrons were prepared from corn oil containing lipid peroxides and labeled with 50 μCi oxidized [^{14}C]linoleic acid together with 0.02 mg oxidized linoleic acid carrier. Thus, control chylomicrons contained nonoxidized linoleic acid label and no oxidized fatty acids. Oxidized chylomicrons contained oxidized linoleic acid label and low levels of oxidized fatty acids derived from oxidized fatty acids in the oil.

Chylomicrons were isolated from the mesenteric lymph duct using a procedure described by us previously (14). Rats were administered intragastrically a single bolus of either nonoxidized or oxidized [^{14}C]linoleic acid in a lipid emulsion. The lipid emulsion consisted of 3 ml of either nonoxidized or oxidized corn oil and non-fat milk (2:1) dispersed with a Polytron homogenizer (Brinkmann Instruments, Westbury, NY). Some chylomicron preparations were labeled with 100 μCi [^{3}H]retinol.

The rats were restrained and chyle was collected on ice for periods up to 8 h. During this period rats were allowed to drink water and were injected every 2 h with 0.15 M NaCl subcutaneously to maintain hydration. Chylomicrons were isolated from lymph by flotation in an SW-40 rotor for 5×10^6 *g* at 14°C and were stored in the presence of 0.2 mM EDTA. As our preliminary experiments indicated that chylomicrons do not oxidize easily in the absence of metal ions, no additional antioxidants were used to prevent further oxidation of chylomicrons during their collection and storage.

The specific activity of isolated chylomicrons was 0.1–0.2 μCi/mg triglyceride. Almost all of the radioactivity (99%) was found in triglyceride portion of chylomicrons. Control and oxidized chylomicrons contained 6.0 and 60.5 μmol conjugated dienes/mmol triglyceride, respectively. Lipid peroxides, when measured by a direct peroxide assay (11), were not detected in control chylomicrons. Oxidized chylomicrons contained 0.23 μmol lipid peroxides/mmol triglyceride. Because linoleic acid is modified or degraded during oxidation, fatty acid composition analysis indicated that in oxidized chylomicrons linoleic acid was reduced by 2–3% (7, 13).

Chylomicron isolation from postprandial serum

Rats were fasted overnight and were administered nonoxidized or oxidized oil as a single bolus as described above for lymph collection. Two h after administration, blood was obtained and chylomicrons were isolated by centrifugation using the same procedure as for lymph chylomicrons (14). Triglyceride concentration and oxidation were then determined in chylomicrons isolated from the postprandial serum. Oxidation was assessed by measuring conjugated dienes (7, 10).

Tissue distribution of control and oxidized chylomicrons

Control and oxidized [^{14}C]linoleic acid-labeled lymph chylomicrons (10 mg triglyceride) were injected via tail vein into unanesthetized male rats and the recovery of [^{14}C]linoleic acid label was determined in livers 10, 20, and 30 min after the injection (14). At the indicated time, livers were removed and perfused with ice-cold saline to remove blood, weighed, and duplicate 1-g portions of liver were homogenized in 1 ml of 0.15 M NaCl and extracted with 40 ml of chloroform–methanol 2:1. Duplicate 5-ml aliquots of the extracts were evaporated to dryness, redissolved in 1 ml heptane and 10 ml Bio-Safe II counting cocktail for scintillation counting.

To determine the distribution of nonoxidized and oxidized [^{14}C]linoleic acid from control and oxidized chylomicrons in extra hepatic tissues, 30 min after the

TABLE 1. VLDL output by the liver

	[^{14}C]Linoleic	Triglyceride
	% administered dose	*mg*
Control perfusate (n=8)	0.86 ± 0.07	1.72 ± 0.09
Oxidized perfusate (n=8)	0.70 ± 0.09	1.40 ± 0.29
	NS	NS

VLDL were isolated by ultracentrifugation from liver perfusates after 2 h of perfusion. The output of control and oxidized [^{14}C]linoleic acid and triglycerides was calculated for each liver. Values are given as mean ± SE; NS, not significant.

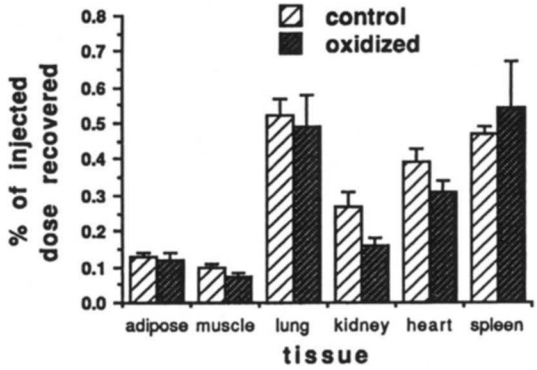

Fig. 3. Recovery of [^{14}C]linoleic acid label in tissues at 30 min after the intravenous injection of either nonoxidized [^{14}C]linoleic acid-labeled control or oxidized [^{14}C]linoleic acid-labeled oxidized chylomicrons (10 mg triglyceride). The values are expressed per g tissue (adipose tissue, skeletal muscle, lung) and per whole organ (kidney, heart, spleen). There were 4 rats in each group. All data are expressed as means ± SEM.

administration of the radiolabeled chylomicrons, whole adrenals, 0.5 g epididymal adipose tissue, 0.5 g gluteus muscle, 0.5 g lung, whole kidney, heart, and spleen were removed. The tissues were washed in ice-cold saline and blotted between several sheets of filter paper to remove excess liquid. Tissues were then homogenized in 20 ml of chloroform–methanol 2:1 and 5 ml of the filtered homogenate was evaporated and counted. The counts due to the residual radioactivity in blood were assessed as described by Koeltz et al. (15); however, at 30 min, the radioactivity remaining in blood was minimal and negligible corrections were required.

Liver perfusion

The apparatus, solutions, and methods were similar to those described by Brissot et al. (16). Control and oxidized chylomicrons (50 mg triglyceride) were injected into male Sprague-Dawley rats (250 g) and livers were isolated 50 min after the injection. Livers were surgically removed by a modification of the procedure of Hems et al. (17) and placed on a nylon mesh to facilitate handling and rinsed with saline solution maintained at 37°C. After temporary obstruction of the outflow catheter, the portal vein catheter was connected to the perfusion system containing 50 ml perfusion solution preequilibrated with 95% O_2, 5% CO_2 in the recirculating mode at a rate of 5 ml/min. The liver was lowered onto the perfusion platform and the position of the inflow and outflow catheters was adjusted. Then the flow rate was gradually increased to 20–25 ml/min. The pressure was monitored for evidence of any obstruction. Initially, livers were perfused for 10 min, then the perfusing solution was removed and perfusion was continued with a new perfusing solution for 2 h for VLDL collection. Bile was collected throughout the 2-h perfusion period.

[^{14}C]linoleic acid recovery in VLDL isolated from the perfusate

VLDL secreted by perfused livers were isolated from 50 ml perfusate by ultracentrifugation. Initially, BHT (final concentration 1 μg/ml) and EDTA were added to the perfusate to 0.5 μM and the perfusate was centrifuged in a Beckman 30 rotor at 30,000 rpm for 18 h. The isolated VLDL was concentrated further by centrifugation in a Beckman 50.3 Ti rotor at 35,000 rpm for 18 h at 5°C. VLDL was removed with a tube cutter and used for the determinations of [^{14}C]linoleic acid incorporation, triglyceride concentration, chemical composition, [^{14}C]linoleic acid label distribution, and elution pattern in gel filtration column. Control VLDL was obtained from livers after the injection of control chylomicrons and oxidized VLDL was obtained after the injection of oxidized chylomicrons. VLDL from rat serum was prepared for comparison purposes by centrifugation the serum at d < 1.006 (18).

To measure [^{14}C]linoleic acid incorporation into VLDL, VLDL was extracted as described by Folch, Lees, and Sloane Stanley (19), solvent was evaporated and redissolved in 1 ml heptane and counted.

Sepharose CL 2B gel filtration of VLDL isolated from the perfusate

VLDL, derived from oxidized chylomicrons and containing oxidized [^{14}C]linoleic acid, was isolated from

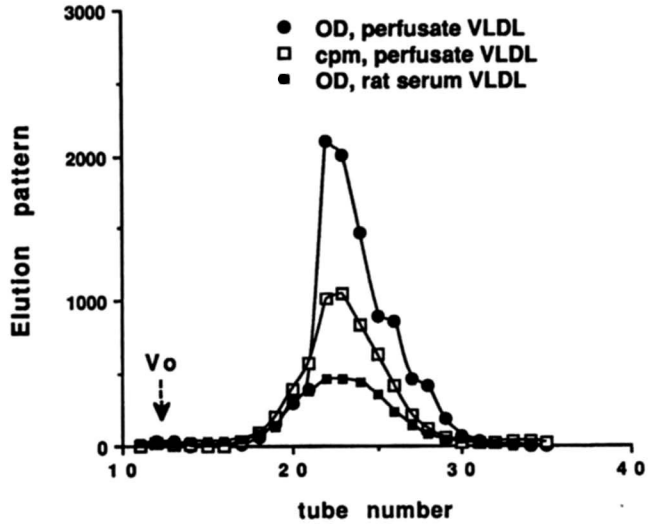

Fig. 4. Elution pattern of the secreted VLDL from an Sepharose 2B column (0.9 × 90 cm) determined by optical density (OD × 1000) and counts per min (cpm) per fraction. The elution rate was 9.0 ml/h and 1.8-ml fractions were collected. The elution of rat serum VLDL is shown for comparison purposes. Chylomicrons and chylomicron remnants elute at void volume indicated by the arrow (V_0).

TABLE 2. Chemical composition of VLDL

Source	Triglycerides	Cholesteryl Esters	Free Cholesterol	Phospholipids	Proteins
		% by wt			
Plasma VLDL	60.2	7.5	2.7	19.1	10.6
Control perfusate (n=3)	67.75 ± .077	3.45 ± 0.57	3.43 ± 0.50	14.77 ± 0.99	11.80 ± 0.16
Oxidized perfusate (n=3)	62.53 ± 1.64	4.60 ± 0.06	4.07 ± 0.56	15.77 ± 1.67	13.03 ± 1.04

VLDL were isolated by ultracentrifugation from liver perfusates after 2 h of perfusion and chemical analyses were determined as described in Methods. Values are given as mean ± SE.

combined perfusates from two livers and applied to a Sepharose 2B gel filtration column (95 cm × 0.9 cm) using an elution buffer consisting of 0.15 M NaCl, 0.04% EDTA, and 0.02 M Tris-HCl, pH 7.0. The column was eluted at 9 ml/h and 1.8-ml fractions were collected. Each fraction was analyzed for radioactivity (cpm) and optical density (OD) at 280 nm. Chylomicrons, isolated from rat mesenteric lymph, chylomicron remnants, and VLDL, isolated from rat serum, were used as elution markers. Chylomicron remnants were prepared in vivo from a functionally eviscerated rat (20) and tested in a liver perfusion system. At 10 min, > 90% of remnants were taken up by the liver.

Identification of oxidized linoleic acid species in oxidized VLDL secreted by the liver

During lipid peroxidation, 9- and 13-hydroperoxides are formed from linoleic acid. These unstable hydroperoxides, when reduced, form stable 9- and 13-hydroxy octadecanoic acids (OH-18). To confirm that oxidized VLDL contains oxidized linoleic acids, we measured OH-18 that are derived from hydroperoxy linoleic acid (oxidized linoleic acid) in the oxidized VLDL radioactive fraction. The following procedure was used. Five mg linoleic acid carrier and 0.10 mCi [^{14}C]linoleic acid were oxidized using the procedure of Peers and Coxton (21). Briefly, linoleic acid was dissolved in 10 ml petroleum ether in a 500 ml round flask and evaporated in a rotary evaporator. The flask was flushed with O_2 and placed in a 37°C oven for 20 h. For chylomicron labeling, hydroperoxides were separated from the starting material on an open silica column as described by Wang and Powell (22). The column was eluted with a heptane ethyl acetate mixture. Any remaining radioactive nonoxidized linoleic acid was eluted with 3% ethyl acetate in heptane and oxidized linoleic acid was eluted with 30% ethyl acetate. The solvent in the 30% ethyl acetate fraction was evaporated; the material was redissolved in a corn oil lipid emulsion and administered to rats as described above for oxidized chylomicron preparation. For oxidized linoleic acid identification, gas chromatographic (GLC) analysis was carried out using the exact procedure developed by Nikkari et al. (23). Briefly, to the oxidized VLDL isolated from the liver perfusate, 50 μg internal standard triheptadecanoin was added and lipids were extracted using the procedure of Folch, Lees, and Sloane Stanley (19). The key steps in this procedure are reduction of the oxidized linoleic acid to OH-18 and gas chromatography of OH-18 methyl esters. The VLDL chloroform extract was evaporated to dryness, redissolved in methanol, reduced by catalytic hydrogenation (23), saponified, methylated, and finally extracted in petroleum ether. The hydroxylinoleic acid products were purified on an open silica column as described above (22). Nonoxidized linoleic acid methyl esters were eluted with 3% ethyl acetate in heptane and hydroxy octadecanoic acid methyl ester was eluted with 30% ethyl acetate. A portion of this fraction was used for radioactivity determinations and the remainder was used for OH-18 measurements by GLC. A column, 30 m × 0.5 mm i.d. fused silica capillary column coated with DB1 (J&W Scientific, Inc., Folsom, CA), in a Hewlett-Packard 5890 Series II gas chromatograph was utilized. The temperature was programmed 25°/min from 50°C to 170°C and 4°/min from 170°C to 300°C. The injection and the detector temperatures were 250°C and 300°C, respectively. The flame detector response was monitored with a Hewlett-Packard HP 3396 ChemStation program connected to a printer. Each sample was chromatographed both before and after derivitization of the hydroxy groups by acetylation as acetylation changes retention times that are specific to OH-18 (23). Commercially available HODE standards were carried through the same procedure and were used for concentration determinations and the elution time standardization. This procedure was also used to identify OH-18 in oxidized linoleic acid used for oxidized chylomicron preparation and in oxidized lymph chylomicrons. Control chylomicrons and control VLDL were also analyzed for control purposes.

Analytic methods

Triglycerides and protein were determined as described previously (6). The total and free cholesterol and free fatty acid composition in chylomicrons were determined by GLC (Hewlett-Packard, Palo Alto, CA) (24).

Phospholipid was measured following the procedure of Bartlett (25). For the determination of the distribution of [^{14}C]linoleic acid label among lipid classes, thin-layer chromatography on silica gel plates was used (26). Bands corresponding to triglyceride, phospholipid, and cholesteryl ester were extracted and the radioactivity was determined. Assays for ^{14}C and ^{3}H were performed in a Tri-Carb liquid scintillation spectrometer (model B2450; Packard Instrument Co., Inc., Downers Grove, IL). Vitamin E in oils was determined by HPLC (Rainin) as described (27). Student's *t* test was used to test for the significance between means. Significance was expressed as $P < 0.05$.

RESULTS

Oxidized chylomicrons in postprandial rat serum

Previously we have shown that after intragastric administration of oxidized lipids to rats, chylomicrons isolated from mesenteric lymph contained oxidized lipids and the levels of oxidized lipids in lymph chylomicrons were directly related to the quantity of oxidized lipid administered (4, 6). In the present study, we examined whether dietary oxidized lipids were also present in the chylomicron fraction isolated from postprandial serum. Two h after the administration of control and oxidized lipid, we measured conjugated dienes in postprandial chylomicrons and found that the levels of oxidized lipids were increased more than 100% in the animals that had been administered oxidized lipid (**Fig. 1**). Significant differences were also found when the data in Fig. 1 were expressed as nmol conjugated dienes/mg triglyceride (8.34 ± 0.75 vs. 16.31 ± 1.41, $P < 0.001$). Thus, oxidized lipids in the diet result in an increase in the quantity of oxidized lipids in the chylomicron fraction isolated from postprandial serum.

Distribution of [^{14}C]linoleic acid label from control and oxidized chylomicrons

To determine whether dietary oxidized lipids are delivered to the liver, we next compared the hepatic uptake of oxidized [^{14}C]linoleic acid in oxidized chylomicrons and nonoxidized [^{14}C]linoleic acid in control chylomicrons (10 mg triglyceride administered). As shown in **Fig. 2,** 10, 20, and 30 min after the administration of control or oxidized chylomicrons, the quantity of nonoxidized and oxidized [^{14}C]linoleic acid was similar (approximately 15% of the administered label). Thus, chylomicrons deliver both nonoxidized and oxidized dietary linoleic acid to the liver where it could be reutilized for VLDL synthesis.

As only a small percentage of chylomicron fatty acids is taken up by the liver, we next examined the extrahepatic distribution of nonoxidized and oxidized [^{14}C]linoleic acid. Thirty min after labeled chylomicron administration, the quantity of nonoxidized and oxidized linoleic acid label was similar in all tissues studied (**Fig. 3**). These studies demonstrate that the tissue distribution of oxidized [^{14}C]linoleic acid in oxidized chylomicrons is virtually identical to that of nonoxidized [^{14}C]linoleic acid in control chylomicrons.

Accumulation of VLDL in the liver perfusate

We next examined whether both nonoxidized and oxidized linoleic acid would be utilized for VLDL production using an in vitro liver perfusion system after the in vivo administration of labeled control and oxidized chylomicrons to rats. It has been established that during in vitro liver perfusion, hepatic secretion of triglycerides occurs at a constant or increasing rate for at least 6 h (28–30). When we perfused livers isolated from animals that had been injected with nonoxidized [^{14}C]linoleic acid in control chylomicrons and oxidized [^{14}C]linoleic acid in oxidized chylomicrons, we also found that the output of [^{14}C]linoleic acid-labeled VLDL increased linearly during the 2-h time period of study. (1 h nonoxidized 0.40%, 2 h nonoxidized 1.03%; 1 h oxidized 0.29%, 2 h oxidized 0.83%, n = 2 for each group). In a larger group of animals, the secretion of [^{14}C]linoleic acid into VLDL was similar in livers after the uptake of either control or oxidized chylomicrons with 0.86 ± 0.07% and 0.70 ± 0.09% of the nonoxidized and oxidized [^{14}C]linoleic acid secreted, respectively (**Table 1**). In view of the large endogenous fatty acid pool in livers (4.5 mg

TABLE 3. Label distribution in VDVL

Souorce	Triglycerides	Cholesterol Esters	Phospholipids
	% of total		
Control perfusate (n=6)	96.33 ± 0.45	2.69 ± 0.42	0.97 ± 0.10
Oxidized perfusate (n=5)	96.95 ± 0.55	1.56 ± 0.12	1.36 ± 0.19
	NS	$P<0.05$	NS

VLDL were isolated by centrifugation from liver perfusates after 2 h of perfusion. The label distribution among lipid classes was determined by TLC as described in Methods. Values are given as mean ± SE; NS, not significant.

triglyceride/g liver) (31), the low recovery of labeled linoleic acid in nascent VLDL is not unexpected.

The bile output was constant throughout the perfusion period and was similar after administration of either control or oxidized chylomicrons. When the collected bile was examined for the recovery of radioactivity, we found that at 2 h, < 0.002% of the injected dose was recovered after administration of both the control and oxidized chylomicrons.

The quantity of triglycerides in VLDL was also similar after administration of either control or oxidized chylomicron preparations (1.72 ± 0.16 vs. 1.40 ± 0.50 mg) (Table 1). This recovery of triglycerides is similar to those found by other investigators using similar perfusion systems (28, 30, 31). Thus, our results show that oxidized [^{14}C]linoleic acid in oxidized chylomicrons is taken up by the liver and incorporated into VLDL similarly to nonoxidized [^{14}C]linoleic acid in control chylomicrons.

To eliminate the possibility that the radioactive counts in the VLDL fraction are due to residual chylomicrons/chylomicron remnants entrapped in the interstices of the sinusoids or the space of Disse that are then washed out during the perfusion procedure, we labeled chylomicrons with [^{3}H]retinol. Retinol, carried in the chylomicron particles, remains with the chylomicron remnant particle, and therefore has been used to follow chylomicron particle metabolism (32). Using our perfusion system, no [^{3}H]retinol counts were detected in the perfusate, indicating that trapped chylomicrons/chylomicron remnants did not contribute to the labeled VLDL in the perfusate.

Sepharose 2B gel filtration of oxidized VLDL isolated from the perfusate

The association of the oxidized [^{14}C]linoleic acid radioactivity with VLDL isolated from the perfusate was further confirmed by gel filtration column chromatography. When VLDL secreted from livers after the uptake of oxidized [^{14}C]linoleic acid was examined by Sepharose 2B column chromatography (**Fig. 4**), both the radioactivity and VLDL mass coeluted from the column as a single peak at the same elution volume as VLDL isolated from rat serum. No detectable chylomicron/chylomicron remnant peak that elutes at the void volume (V_0) of the column was observed. These results provide additional evidence that dietary oxidized linoleic acid is incorporated into VLDL secreted by the liver.

Chemical composition of secreted VLDL in the perfusate

To verify the identity of VLDL isolated from liver perfusates, the chemical composition was determined after administration of either control or oxidized chylomicrons (**Table 2**). No significant differences in lipid composition were observed between VLDL preparations secreted after the hepatic uptake of either control or oxidized chylomicrons. Furthermore, the composition of VLDL recovered from the perfusion medium

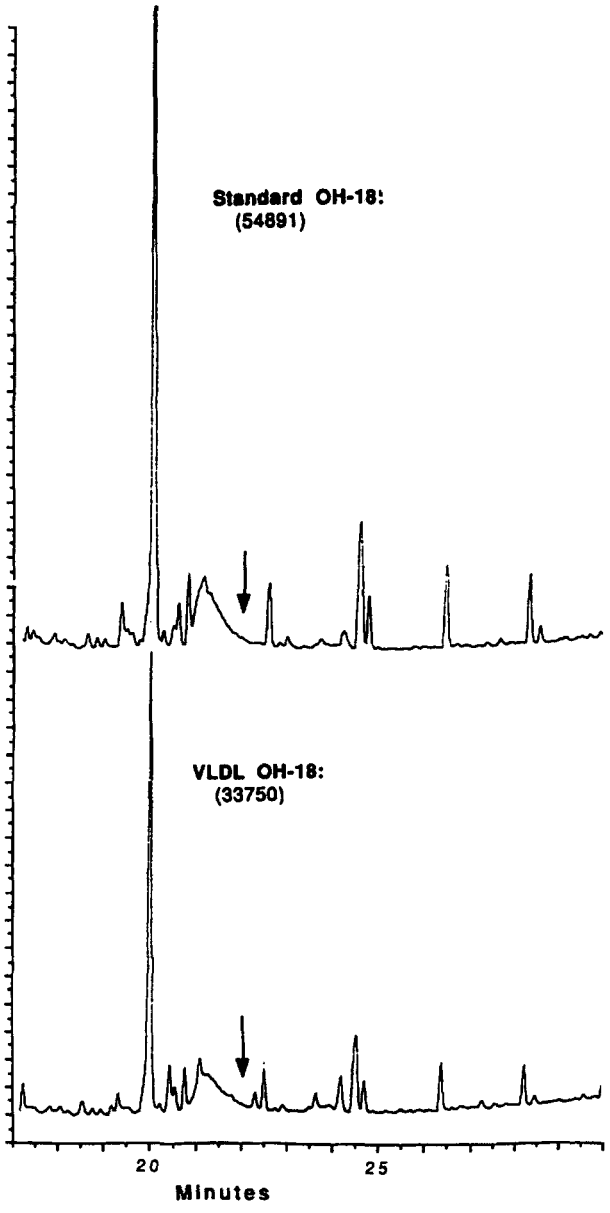

Fig. 5. Gas chromatographic tracings of hydrogenated hydroxy octadecanoic acid (OH-18) methyl esters obtained from (upper panel) 1 μg commercially available standard 13-HODE and (lower panel) OH-18 derived from 10 μg VLDL isolated from liver perfusate. VLDL was delipidated, hydrogenated, saponified, methylated, and then purified on a silica gel column. For quantitation, the areas were compared to internal standards and to the standard of commercially available HODE. Areas of each peak in the original tracing are given in parentheses. The peak positions of these samples, when acetylated, are indicated by arrows.

closely resembled the composition for nascent VLDL isolated from the liver perfusate by other investigators (28, 31, 33) and VLDL isolated from rat serum.

Distribution of [^{14}C]linoleic acid label among lipid classes

It has been shown previously that liver utilizes chylomicron remnant fatty acids to synthesize cholesterol ester and phospholipids that are secreted in VLDL (34–36). When we examined the nonoxidized and oxidized [^{14}C]linoleic acid label distribution in VLDL isolated from the perfusate, we found that most of the label was found in triglycerides (**Table 3**). After administration of control chylomicrons, the amount of label found in cholesterol ester and phospholipid was 2.69 ± 0.42% and 0.97 ± 0.10%, respectively. After administration of oxidized chylomicrons, the amount of the label in cholesteryl ester was 1.56 ± 0.12% and in phospholipid 1.36 ± 0.19%. There were no significant differences in label distribution in phospholipids between nonoxidized and oxidized [^{14}C]linoleic acid, however, less label was incorporated into cholesteryl ester in livers after the uptake of oxidized chylomicrons ($P < 0.05$). It is clear that both cholesteryl ester and phospholipids were synthesized in the liver as the administered chylomicrons contained almost all of the nonoxidized and oxidized [^{14}C]linoleic acid label in triglycerides with only 1.0% present in cholesteryl ester plus phospholipids. This preferential distribution is in agreement with results reported by Leyton, Drury, and Crawford (36) and Wang and Koo (37). They found that there was a preferential incorporation of dietary linoleic into hepatic triglycerides.

Identification of oxidized linoleic acid in oxidized VLDL as hydroxy octadecanoic acid (OH-18)

To confirm that oxidized VLDL contains not only diet-derived radioactivity but also oxidized linoleic acid, we next analyzed the radioactive fraction of oxidized VLDL for the presence of OH-18 that is generated during linoleic acid oxidation when hydroperoxides are reduced to hydroxy fatty acids. **Figure 5** shows the analysis of OH-18 derived from hydrogenated and methylated commercially available standard HODE (upper panel) and from VLDL isolated from the liver perfusate (lower panel). The peak in the upper panel, eluting at 19.87 min, was derived from 13-HODE. After acetylation of hydroxy group, as expected, the elution time increased to 22.0 min (23) (the elution time is indicated by an arrow). One μg of OH-18 standard yielded an area of 54,891 (arbitrary units). The lower panel shows a typical pattern of methylated OH-18 isolated from oxidized VLDL. The elution time before and after acetylation was similar to that of the OH-18 derived from 13-HODE. A peak area of 33,750 was derived from oxidized VLDL containing 10 μg triglycerides. Thus, this oxidized VLDL sample contained approximately 6% oxidized fatty acids. In the VLDL sample, most of the material corresponded to OH-18 derived from 13-HODE; however, a 9-HODE standard-derived OH-18 elution time was almost indistinguishable (19.79 min, data not shown). Moreover, all the radioactive counts in oxidized VLDL were found to be in the oxidized fatty acid fraction. OH-18 was also detected in oxidized linoleic acid added to the diet and in the oxidized chylomicrons administered to rats. Thus, we have demonstrated the presence of both the radioactivity and OH-18 in the oxidized diet administered to rats, in the oxidized chylomicrons, and in the oxidized VLDL isolated from liver perfusates. Control chylomicrons and control VLDL contained no detectable OH-18 when analyzed by this procedure.

DISCUSSION

Oxidized lipids are present in the mesenteric lymph of rodents (4–6) and we have shown a direct relationship between the quantity of oxidized lipid in the diet and the levels of oxidized lipid in lymph chylomicrons (4, 6). Moreover, the present study demonstrates that in rats oxidized lipids are also found in the postprandial serum chylomicron fraction. When we added relatively small quantities of oxidized lipid to the diet, the quantity of oxidized lipid in serum postprandial chylomicrons was twice as great. These results indicate that in rodents oxidized lipids in the diet are absorbed by the small intestine and secreted in chylomicron particles. Furthermore, recent studies in humans have also demonstrated that the quantity of oxidized lipid in the diet directly correlates with the level of oxidized lipid in serum postprandial chylomicrons (7).

We have also shown that in rats the levels of oxidized lipids in serum VLDL plus LDL fraction are related to the quantity of oxidized lipids in the diet (6). Animals fed a diet that did not contain oxidized lipids had very low levels of oxidized lipids in their VLDL plus LDL fraction. Conversely, when rats were fed diets containing large quantities of oxidized lipid, the levels of oxidized lipids in the VLDL plus LDL fraction were high. Thus, dietary oxidized lipids affect not only the level of oxidized lipids in chylomicrons but also the level of oxidized lipids in endogenous lipoprotein particles.

In the present study, we have examined the metabolism of nonoxidized [^{14}C]linoleic acid-labeled chylomicrons and oxidized [^{14}C]linoleic acid-labeled oxidized chylomicrons to determine whether the fate of dietary linoleic acid is altered by oxidation. We found that the hepatic uptake of nonoxidized and oxidized linoleic acid

was similar with approximately 15% of nonoxidized and oxidized linoleic acid delivered to the liver 30 min after chylomicron administration. Additionally, the uptake of nonoxidized and oxidized linoleic acid by extrahepatic tissues was also very similar. These results are consistent with previous studies where we demonstrated that the hepatic uptake of control and oxidized chylomicrons was essentially the same 30 min after the administration of chylomicrons (4). In these earlier studies we labeled chylomicrons with [^{3}H]oleic acid and [^{14}C]cholesterol and therefore could not specifically trace the metabolism of the oxidized lipid moiety (linoleic acid) in chylomicrons. In the present study, we labeled the oxidized chylomicrons with oxidized ^{14}C linoleic acid to definitely determine the metabolism of oxidized dietary lipid. These studies indicate that mild oxidation does not alter the metabolism of chylomicrons and that oxidized lipids carried in oxidized chylomicrons are metabolized similarly to nonoxidized lipid with approximately 15.0% of the fatty acids being transported to the liver. It should be noted that these studies only examined bulk chylomicron metabolism and a small but potentially clinically significant increase in the uptake of oxidized chylomicrons/chylomicron remnants by macrophages, endothelial cells or smooth muscle cells would not be recognized.

Fatty acids delivered to the liver can be utilized for the formation of the lipid components of VLDL (34–37). In the present study we demonstrate that oxidized linoleic acid delivered to the liver in chylomicrons is utilized for the formation of VLDL to a similar extent as nonoxidized linoleic acid. After administration of labeled control and oxidized linoleic acid to intact animals, 0.86 ± 0.07% and 0.70 ± 0.09% of the administered label was found in vitro in the liver perfusate at 2 h, respectively. We assume that the low recovery of the label in VLDL is primarily due to the dilution of the label in the liver by endogenous fatty acids and not due to oxidation. Leyton et al. (36) have shown that at 1 h after the oral administration of [^{14}C]linoleic acid, only 9% is oxidized and at 3 h oxidation increases to only 13%. Moreover, we have no explanation for the preferential incorporation of linoleic acid into triglycerides as compared to phospholipid and cholesterol esters. However, this observation is in agreement with the results obtained by Leyton et al. (36) and Wang et al. (37). They found that distribution of each fatty acid among lipoprotein species is different and that linoleic acid and oleic acid were incorporated mainly into triglycerides.

We have also demonstrated that oxidized VLDL contains hydroxy octadecanoic acid originating from oxidized linoleic acid. The presence of hydroxy octadecenoic acid was traced from the oxidized lipid diet administered to the rats to lymph chylomicrons and finally to VLDL secreted by the liver. Thus, lipids in the diet are delivered to the liver in chylomicrons and these oxidized lipids can be utilized for the formation and secretion of VLDL. This could be one mechanism by which the quantity of oxidized lipids in the diet influence the level of oxidized lipids in endogenous lipoproteins such as VLDL. Other potential pathways by which dietary oxidized lipid could be incorporated into VLDL include the direct transfer of oxidized lipid from chylomicrons to other lipoproteins or the initial storage of oxidized fatty acids in adipose tissue with the later transfer of these oxidized fatty acids to the liver where they could be utilized for VLDL synthesis.

That the label was in newly secreted VLDL rather than in chylomicrons that were adsorbed and released during the perfusion is shown by the following observations. First, when control or oxidized chylomicrons were labeled with [^{3}H]retinol, which is a good marker for the core of chylomicrons/chylomicron remnants, no label was found in the perfusate. Second, the secretion of labeled linoleic acid increased linearly with time. Third, the distribution of labeled fatty acids differed between the chylomicrons administered and the VLDL in the perfusate with greater quantities of labeled linoleic acid in the cholesteryl ester and phospholipid fraction in the VLDL perfusate. Fourth, the chemical composition of the VLDL in the perfusate is very similar to serum VLDL and is not similar to the composition of the administered chylomicrons. Last, on gel filtration, the label was exclusively in the VLDL fraction. Taken together these observations provide strong evidence that the labeled oxidized linoleic acid is taken up by the liver and repackaged into VLDL.

The significance of oxidized VLDL in the atherosclerotic process still needs to be established; however, it is clear that oxidized lipids in the diet can contribute to oxidized serum lipoproteins such as chylomicrons and VLDL. Moreover, many foods consumed in a typical American diet contain substantial quantities of polyunsaturated fatty acids that have been subjected to various degrees of processing and heat treatment, particularly deep fat frying, which is known to lead to oxidation. Frankel et al. (38) have examined the relative percent of oxidation products in frying oils used in fast foods and found that the majority of these foods contain oxidized lipids. For example, French fried potatoes contain up to 8.2% of oxidized material. Alexander (39) has shown that oils used in many restaurants are kept at 180°C for extended time periods which results in the appearance of oxidized lipids. Thompson et al. (40) have also performed oxidized lipid measurements and have shown that oils used for deep-frying in restaurants, institutions, and armed services contained high quantities of oxidized lipids. Yagi et al. (11) measured perox-

ides in 30 kinds of foods and found that the peroxide content could be as high as 600 nmol/g of food. Thus, there is abundant evidence that oxidized lipids are frequently present in the typical American diet. Furthermore, the quantity of oxidized lipids found in a typical American diet is higher than the levels of oxidized lipids used in our experiments.

In summary, our study demonstrates that dietary oxidized lipids are delivered to the liver via chylomicrons where they are utilized for the synthesis of triglycerides, cholesteryl ester, and phospholipids which are secreted in VLDL particles. Thus, dietary oxidized fat could affect the level of oxidation of endogenous lipoprotein particles such as VLDL.

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