Metabolism of doubly-labeled chylomicron cholesteryl esters in the rat

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ABSTRACT Chylomicrons labeled in vitro with doubly-labeled cholesteryl esters were injected intravenously into fasted rats, and the tissue distribution and chemical form of each isotope were observed for 24 hr. The use of doubly-labeled cholesteryl esters provided information about the metabolism of both the sterol and the fatty acid moieties.

Similar results were obtained with doubly-labeled cholesteryl palmitate, oleate, and linoleate. In each instance, most (80–90%) of the chylomicron cholesteryl ester was removed from the plasma by the liver; small amounts were also taken up by all other tissues examined. There was no hydrolysis during uptake. In the liver the newly absorbed cholesteryl esters underwent slow hydrolysis (60% after 1 hr and 85–90% after 3.5 hr); the rate of reesterification of the liberated cholesterol was still slower. After 24 hr only 20–28% of the labeled cholesterol present in the animal was found in the liver.

Labeled fatty acid disappeared from the liver, and was redistributed among other tissues, much more rapidly than the labeled cholesterol. Most of the labeled fatty acid apparently underwent oxidation, since only 15–20% of the injected labeled fatty acid was present in the animal after 24 hr. At this time the three fatty acids were differently distributed between and within the tissues. These differences reflected some known differences of fatty acid concentration and lipid composition in the various tissues.

KEY WORDS cholesteryl esters · doubly-labeled · in chylomicrons · rat · plasma clearance · hepatic uptake · hydrolysis · metabolism · reesterification

DURING THE INTESTINAL ABSORPTION of cholesterol, the cholesterol is largely esterified with long-chain fatty acids, and is then transported via the intestinal lymphatics as part of lymph chylomicrons (1–5). The chylomicron cholesteryl ester then enters the vascular compartment, from which it is cleared mainly by the liver (6–8). The extent to which the liver predominates in the initial metabolism of chylomicron cholesteryl ester has been demonstrated after the intravenous injection of chylomicrons containing newly absorbed, labeled cholesterol into intact rats or dogs (6–8). As much as 90% of the injected labeled cholesterol was found in the liver of both rats (6) and dogs (8) after appropriate short time intervals. Although small amounts of chylomicron cholesterol can be taken up by extrahepatic tissues (6, 7, 9), the liver is the only organ which normally plays a quantitatively significant role in the initial uptake of chylomicron cholesterol from the blood.

It was previously reported that chylomicron cholesteryl ester is apparently taken up by the liver without hydrolysis (6). This conclusion was suggested by the observation that after short time intervals the percentage of labeled cholesterol present in the liver as free cholesterol was unchanged from that in the injected chylomicrons. The possibility that uptake by the liver involved hydrolysis and reesterification could not, however, be ruled out (6). After uptake by the liver a slow net hydrolysis of the labeled cholesteryl ester occurred, with a rise in the liver free cholesterol-^{14}C from 13% to 40% in 1 hr, and to 80% in 3.5 hr. Here, too, the possibility could not be excluded that during this time interval a substantial amount of reesterification, and hence a much larger amount of total hydrolysis, had occurred. Concomitant with net ester hydrolysis there was a slow, progressive loss of labeled cholesterol from the liver as equilibration occurred between the cholesterol pools of liver, blood, and peripheral tissues. After 24 hr only 20% of the labeled cholesterol was found in the liver, with considerable amounts present in the peripheral tissues.

The experiments reported here were designed to examine more definitively the events occurring during the

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uptake and subsequent metabolism of chylomicron cholesteryl esters by the liver. In these experiments, doubly-labeled cholesteryl esters were incorporated into chylomicrons in vitro, and the metabolism of both the sterol and of the fatty acid components in intact rats was subsequently examined. The use of doubly-labeled cholesteryl esters also permitted a detailed comparison of the metabolism of different, individual cholesteryl esters.

EXPERIMENTAL PROCEDURES

Doubly-labeled cholesteryl palmitate, oleate, and linoleate, each labeled with ^{3}H in the 7α position of the sterol ring and with ^{14}C in the carboxyl group of the fatty acid moiety, were prepared by mixing appropriate amounts of the corresponding ^{3}H- and ^{14}C-labeled cholesteryl esters. The labeled esters were either obtained commercially (Nuclear-Chicago Corporation, Des Plaines, Ill. or New England Nuclear Corp., Boston, Mass.) or synthesized as described previously (10). Each labeled ester was purified by chromatography on a small column of alumina (10), and then assayed for purity by thin-layer chromatography on Silica Gel G impregnated with silver nitrate (11). When necessary, the latter procedure was employed on a preparative scale in order to ensure that all of the label resided in the desired ester. The specific radioactivities of the ^{3}H-labeled esters were all approximately 50 $\mu c/\mu mole$, and of the ^{14}C-labeled esters 10 $\mu c/\mu mole$.

Chylomicrons were obtained by feeding a mixture of corn oil and olive oil (1:1, v/v) to rats with cannulae implanted in their thoracic ducts. The chylomicrons were isolated and washed by flotation as described elsewhere (12), and then diluted to a concentration of 10 mg of total lipid per ml. The chylomicrons were labeled in vitro with one of the doubly-labeled cholesteryl esters, by the method described elsewhere (13). With this procedure, 25μg of doubly-labeled cholesteryl ester was incorporated into chylomicrons, per ml of chylomicron suspension. The labeled chylomicrons were used for experiments within 24 hr.

In one study (with cholesteryl linoleate) chylomicrons of different size were separated by sucrose density gradient centrifugation (12, 14). Suspensions of large and small particles were each labeled in vitro in the same manner as that employed with the whole chylomicron suspension.

The animals used in all studies were male Sprague-Dawley rats weighing 150–200 g and fasted for 12–16 hr prior to injection. The rats were kept fasting throughout the experiment. Each rat received 1.5–2 ml of a suspension of labeled chylomicrons by injection into a tail vein. At specified time intervals (20 min, 1 hr, 3.5 hr, 24 hr) the animals were anesthetized with ether, and blood (5–7 ml) was withdrawn from the abdominal aorta into heparinized syringes. As rapidly as possible, the liver, epididymal fat bodies, kidneys, and adrenal glands were removed, rinsed, blotted dry, weighed, and extracted with 20–40 vol (v/w) ethanol–acetone 1:1 (6, 15). The rest of the animal, including the carcass and all remaining viscera, was ground and extracted with 20 vol of ethanol–acetone. The blood was chilled and centrifuged. The plasma was removed, the red cells were washed twice with isotonic saline, and plasma and red cell samples were extracted with chloroform–methanol 2:1.

Portions of each tissue and "carcass" extract were assayed for radioactivity, and the recovery and distribution of each isotope in each rat was determined. Other portions of the extracts were chromatographed on columns of silicic acid (Unisil, 100–200 mesh, Clarkson Chemical Company, Inc., Williamsport, Pa.), with a load of 75 mg or less of total lipid per 5 g of silicic acid. Serial elutions were carried out with (per 5 g of silicic acid): 95 ml 21% benzene in hexane; 50 ml of benzene then 100 ml of chloroform; and 100 ml of methanol. The three fractions contained, respectively, cholesteryl esters, triglycerides plus free cholesterol (and free fatty acids), and phospholipids. As reported previously (16), this method gives quantitative recovery of cholesteryl esters uncontaminated by triglycerides. The column fractions were evaporated and assayed for radioactivity.

Radioassay was carried out in a Packard Tri-Carb liquid scintillation counter with 0.5% diphenyloxazole in toluene as scintillation solvent. After the initial radioassay, the extent of quenching was determined for each isotope by adding first a ^{14}C and then a ^{3}H internal standard to each vial, and redetermining radioactivity after each addition. All radioassay results were automatically punched on data processing tape. The tape was analyzed by a computer which was programmed to make the appropriate corrections for quenching, and to determine the amount (dpm) of each isotope, present in each sample, according to the equations of Okita, Kabara, Richardson, and LeRoy (17).

The distribution of each isotope in the organs and tissues was calculated from the observed amounts of ^{14}C and ^{3}H in the measured samples used for analysis. Depot fat was taken as 7.08% (18), and plasma volume as 3.5% of the body weight. The red cell volume was determined from the measured hematocrit. The extent of net hydrolysis of injected cholesteryl ester, in each sample, was determined from the relative amounts of ^{3}H found in the free cholesterol and in the cholesteryl ester fractions after silicic acid column chromatography. The extent to which the labeled cholesteryl ester found in each sample represented labeled cholesterol that had been liberated by hydrolysis of the injected ester and then reesterified, was

determined from the isotope ratio in each cholesteryl ester fraction, as follows: % reesterified cholesterol = 100 × (observed minus injected ratio of ^{3}H:^{14}C)/(observed ratio of ^{3}H:^{14}C). This calculation assumes that the labeled fatty acids liberated from hydrolyzed injected cholesteryl ester were not used to reesterify the liberated labeled free sterol, and hence provides a minimum estimate of the extent of reesterification. As described in the tables, most of the recovery and tissue distribution data, as well as of the chromatographic results, represent average values obtained with a pair of identically treated animals.

RESULTS

The amount of chylomicron lipid injected into each rat is listed in Table 1. These values all lie within a range which has been shown to have approximately physiological clearance characteristics when expressed in terms of the fractional disappearance rate from blood of injected chylomicron lipid (19).

The total recovery of each isotope, as lipid-soluble radioactivity, in each entire animal is shown in Table 1. The recovery of both isotopes was nearly quantitative after 20 min in all three studies (with doubly labeled cholesteryl palmitate, oleate, or linoleate). During the first 3.5 hr there was no significant loss of ^{3}H-labeled sterol from the body, although by 24 hr the recovery of ^{3}H had decreased to some extent. In contrast, the recovery of ^{14}C-labeled fatty acid decreased rapidly in all three studies. Only about 30% of the injected ^{14}C was recovered after 3.5 hr, and only 15–20% after 24 hr.

TABLE 1 RECOVERY OF LIPID-SOLUBLE RADIOACTIVITY AFTER INJECTION OF CHYLOMICRONS CONTAINING DOUBLY-LABELED CHOLESTERYL ESTERS (^{3}H-CHOLESTEROL–FATTY ACID-^{14}C)

Time	Total Lipid Injected	Label Injected		Label Recovered in Whole Animal*	
		^{3}H	^{14}C	^{3}H	^{14}C
	mg	*μc*		%	
Cholesteryl palmitate					
20 min	23.1	0.54	0.14	89	102
60 min	23.1	0.54	0.14	100	77
3.48 hr	23.1	0.54	0.14	99	33
24.0 hr	23.1	0.54	0.14	58	20
Cholesteryl oleate					
21 min	18.0	1.52	0.27	93	97
59 min	15.0	1.26	0.23	93	62
3.50 hr	18.0	1.52	0.27	97	30
24.2 hr	18.0	1.52	0.27	77	14
Cholesteryl linoleate					
20 min	20.0	0.74	0.10	88	86
60 min	22.2	0.81	0.11	82	50
3.50 hr	22.2	0.81	0.11	83	28
24.0 hr	22.2	0.81	0.11	69	19

* All values listed represent the average results for a pair of rats, except for the 60 min and 24 hr values for cholesteryl palmitate, which represent single animals.

The tissue distribution of each isotope in each rat is presented in Table 2. The liver was responsible for most of the uptake of the doubly-labeled cholesteryl esters in all of the animals: after 20 min about 70% of both isotopes was found in the liver, and 15–20% remained in the plasma. The radioactivity found in the plasma at this time presumably represented chylomicron cholesteryl ester still undergoing clearance by the liver. After 60 min, 87% of the ^{3}H-cholesterol and 73–78% of the ^{14}C-fatty acid was found in the liver, in the studies with cholesteryl palmitate and linoleate. For cholesteryl oleate, a comparable amount (83%) of ^{3}H-sterol, but a somewhat lesser amount (57%) of ^{14}C-fatty acid was found in the liver at this time. In all three studies the amount of ^{3}H-cholesterol in the liver slowly declined after 60 min, so that 66–81% of the recovered ^{3}H was found in the liver after 3.5 hr, and 20–28% after 24 hr. Along with the loss of ^{3}H-cholesterol from the liver a rise occurred in the ^{3}H content of the other tissues examined (except plasma). By 24 hr most of the labeled cholesterol in the animal's body was found in those parts of the animal ("rest of animal") that were not separately examined. Most of this labeled sterol was presumably in the muscle and carcass (6).

The disappearance of ^{14}C-labeled fatty acid from the liver and the redistribution of ^{14}C among other tissues occurred more rapidly than the redistribution of the labeled cholesterol (Table 2). In all three studies, by 24 hr a large fraction (40–64%) of the ^{14}C-fatty acid remaining in the animal was found in the adipose tissue. Relatively more ^{14}C-labeled palmitate and oleate than linoleate was found in the body fat at this time. On the other hand, much more ^{14}C-labeled linoleate than either oleate or palmitate was found in the muscle plus carcass ("rest of animal") after 24 hr.

The percentage of ^{3}H-cholesterol present as esterified sterol in each tissue at each time interval is recorded in Table 3. The extent to which each cholesteryl ester sample represented reesterified labeled cholesterol is also shown. In all three studies, after 20 min most of the ^{3}H-sterol present in the liver was found in the form of intact, unhydrolyzed cholesteryl ester, with approximately the same isotope ratio as that present in the injected material. In the liver, a steady hydrolysis occurred, so that the percentage of labeled cholesterol present as esterified sterol declined to 31–43% after 60 min, and to 8–16% after 3.5 hr. Relatively little reesterification of the liberated free cholesterol took place during the early phases of this hydrolysis. Thus, after 60 min virtually all of the labeled cholesteryl ester present in the liver was still found as intact, unhydrolyzed doubly labeled ester,

TABLE 2 TISSUE DISTRIBUTION OF RECOVERED RADIOACTIVITY

	Distribution of Recovered Radioactivity*							
	20 Min		60 Min		3.5 Hr		24 Hr	
	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C
	%		%		%		%	
Cholesteryl palmitate								
Liver	69.8	71.9	87.3	78.2	81.0	62.9	28.0	29.3
Plasma	21.4	19.8	4.3	5.5	4.8	2.2	2.9	1.3
Red cells	0.3	0.3	0.6	0.5	4.9	0.5	6.4	1.4
Fat	6.4	6.3	3.8	6.8	1.6	11.1	5.6	64.3
Kidneys	0.2	0.3	0.1	0.7	0.3	1.4	1.2	1.1
Adrenals	0.04	0.04	0.05	0.06	0.3	0.2	2.2	0.4
Rest of animal	1.9	1.4	3.9	8.3	7.1	21.7	53.7	2.2
Cholesteryl oleate								
Liver	69.7	67.8	82.5	57.4	66.0	32.9	24.4	17.0
Plasma	17.6	17.7	5.5	9.5	4.8	2.1	6.3	2.9
Red cells	0.2	0.3	0.3	0.4	6.4	2.1	10.3	3.9
Fat	6.4	8.9	4.2	12.7	2.7	14.1	7.2	56.2
Kidneys	0.1	0.1	0.1	0.7	0.4	1.4	1.3	1.9
Adrenals	0.03	0.04	0.08	0.12	0.2	0.3	1.2	1.2
Rest of animal	5.9	5.1	7.3	19.2	20.1	47.1	49.3	16.9
Cholesteryl linoleate								
Liver	70.3	61.9	87.5	72.5	66.2	42.2	19.8	15.2
Plasma	16.4	19.4	2.9	5.4	5.4	2.9	3.4	2.3
Red cells	0.4	0.8	0.3	0.7	3.4	0.8	7.8	3.5
Fat	2.7	8.8	0.7	8.5	2.8	22.9	8.9	39.7
Kidneys	0.2	1.2	0.1	0.6	0.4	0.8	1.1	1.1
Adrenals	0.3	0.4	0.1	0.15	0.2	0.3	1.1	1.2
Rest of animal	9.7	7.5	8.4	12.1	21.6	30.1	57.9	37.0

* See footnote to Table 1.

despite the fact that more than half of the injected cholesteryl ester had already undergone hydrolysis. Even after 3.5 hr, in two of the studies (cholesteryl oleate and linoleate) more than half of the small amount of esterified labeled cholesterol found in the liver was still present as intact, unhydrolyzed ester. In the study with cholesteryl oleate, in fact, almost all of the labeled cholesteryl ester present after 3.5 hr represented unhydrolyzed ester,[1] although more than 90% of the injected cholesteryl ester had undergone hydrolysis by this time. After 24 hr, only 8–12% of the ^{3}H-cholesterol in the liver was present as esterified cholesterol, and most (80–92%) of this represented reesterified, labeled cholesterol.

After 20 min, all of the labeled cholesteryl ester in plasma was found as intact, unhydrolyzed ester, which is consistent with the above interpretation that this radioactivity represented chylomicron cholesteryl ester undergoing hepatic clearance. After 60 min most of the labeled cholesterol in plasma was still intact, unhydrolyzed ester, but by 3.5 hr 90% of it represented reesterified labeled cholesterol. Reesterified ^{3}H-cholesterol comprised all (99%) of the labeled plasma cholesteryl ester after 24 hr.

In all of the other tissues examined (except red cells), at 20 and 60 min a large fraction of the ^{3}H present was found as esterified sterol, and most of the labeled cholesteryl ester was intact and unhydrolyzed. In all of these tissues except the adrenals the percentage of ^{3}H present as esterified sterol declined progressively with time so that labeled free sterol represented 80% or more of the tissue ^{3}H at 24 hr. Despite this decline, significant amounts of intact, unhydrolyzed cholesteryl esters were found in the fat and "rest of rat" samples after 24 hr. This was particularly so for fat, which contained hardly any reesterified cholesterol ester even after 24 hr. In the adrenals the percentage of ^{3}H present as esterified sterol declined between 60 min and 3.5 hr, but then rose to a level of 76–81% at 24 hr. As in the plasma, reesterified ^{3}H-cholesterol comprised almost all of the labeled adrenal cholesteryl ester after 24 hr.

The distribution of ^{14}C-fatty acid among different lipids of each tissue and at each time interval is presented in Table 4. In the liver, in all three studies, after 20 min the ^{14}C-fatty acid was predominantly in cholesteryl esters. At subsequent times progressively less ^{14}C was found in cholesteryl esters; at 24 hr, in fact, less than

[1] The value listed in Table 3 (4% reesterified ester after 3.5 hr) may be falsely low because of the preferential formation of cholesteryl oleate in rat liver (20); some of the liberated ^{14}C-oleate may hence have been used for reesterification (see also Table 4 and text).

TABLE 3 DISTRIBUTION OF LABELED CHOLESTEROL BETWEEN FREE AND ESTERIFIED CHOLESTEROL AND THE EXTENT TO WHICH THE ESTERIFIED STEROL REPRESENTED REESTERIFIED LABELED CHOLESTEROL

Tissue*	Time	Ester Injected: Cholesteryl Palmitate % of ^3H as ester	Cholesteryl Palmitate Ratio† $^3H/^{14}C$ in ester	Cholesteryl Palmitate % reesterified ester‡	Cholesteryl Oleate % of ^3H as ester	Cholesteryl Oleate Ratio† $^3H/^{14}C$ in ester	Cholesteryl Oleate % reesterified ester‡	Cholesteryl Linoleate % of ^3H as ester	Cholesteryl Linoleate Ratio† $^3H/^{14}C$ in ester	Cholesteryl Linoleate % reesterified ester‡
Liver										
	20 min	73	0.85	0	85	0.87	0	83	1.03	3
	60 min	42	1.09	8	31	0.91	0	43	1.10	9
	3.5 hr	16	3.71	73	8	1.04	4	14	1.96	49
	24 hr	8	8.5	88	11	5.4	80	12	12.5	92
Plasma§										
	20 min	91	0.98	0	82	0.87	0	92	1.00	0
	60 min	99	1.02	2	80	0.86	0	69	1.15	13
	3.5 hr	47	8.2	88	32	9.2	89	50	10.7	90
	24 hr	90	84.	99	52	107.	99	63	65.	99
Fat										
	20 min	97	1.02	2	—	—	—"	95	0.94	0
	60 min	45	0.96	0	86	<1	0	10	—	—
	3.5 hr	2	—	—	—	—	—	4	0.88	0
	24 hr	14	—	—	10	<1	0	20	1.15	13
Kidneys										
	60 min	33	1.17	15	73	0.87	0	30	1.15	13
	3.5 hr	12	4.9	79	13	2.47	59	16	3.52	71
	24 hr	—	—	—	4	4.54	78	8	3.84	74
Adrenals										
	60 min	—	—	—	64	0.8	0	45	1.18	15
	3.5 hr	55	50	99	37	2.6	62	32	3.27	69
	24 hr	81	35	98	76	17.5	95	78	152.	99
"Rest of rat"										
	20 min	90	1.03	3	89	0.8	0	80	1.10	10
	60 min	—	—	—	73	0.81	0	22	1.04	4
	3.5 hr	18	1.15	13	14	0.91	0	2	3.4	70
	24 hr	8	1.94	49	6	1.64	39	2	4.2	77

* Analyses were carried out on all samples of liver and plasma. All values for liver and plasma hence represent the average results for a pair of rats, except for the 60 min and 24 hr values for cholesteryl palmitate, which represent single animals. Analyses for all the other tissues listed were carried out on only one member of each duplicate pair of samples, and the results are hence less reliable and more subject to individual variation.

† The ratios listed are the ratio of $^3H/^{14}C$ dpm in the cholesteryl esters, normalized to the corresponding ratio of $^3H/^{14}C$ dpm in the injected chylomicrons (which was assigned the value of 1.00 in each study).

‡ These values represent the % of labeled esterified sterol that had been formed by hydrolysis of the injected doubly-labeled cholesteryl ester, followed by reesterification of the liberated free cholesterol with unlabeled fatty acid (see text).

§ Analysis of some of the red cell samples in each study revealed that in all three studies more than 98% of the labeled cholesterol was present as free sterol at 3.5 and 24 hr. A small amount of esterified labeled cholesterol (ca. 10% of the labeled red cell cholesterol) was present at 60 min; the amount of labeled cholesteryl ester was, however, too small to permit an accurate determination of the extent to which the ester represented unhydrolyzed injected ester.

" The absence of listed values indicates that analyses were not carried out on those samples, or that the assays were technically unreliable. Analyses were not carried out on the 20 min samples of kidneys or adrenals, because of the relatively large amount of "contaminating" plasma radioactivity in these samples.

20% of the hepatic ^{14}C-fatty acid was found in cholesteryl esters. Relatively more ^{14}C-labeled oleate than linoleate or palmitate was found in liver cholesteryl esters at 24 hr. This finding is consistent with the known preferential formation of cholesteryl oleate in rat liver (20). At 24 hr, other significant differences in the hepatic distribution of the three fatty acids were also apparent: ^{14}C-labeled palmitate and linoleate were found mainly in liver phospholipids, whereas almost half of the ^{14}C-oleate was found in triglycerides.

The ^{14}C-fatty acid distribution in plasma was similar to that in liver. After 24 hr ^{14}C-labeled palmitate and linoleate were found mainly in plasma phospholipids, whereas most of the plasma ^{14}C-oleate was found in the triglyceride fraction. In the adipose tissue, each of the three labeled fatty acids was found mainly in triglycerides after 24 hr. Relatively more ^{14}C-labeled linoleate than oleate or palmitate was found in adipose tissue phospholipids at this time. In the kidneys, all three labeled fatty acids were found predominantly in phospholipids at the

TABLE 4 DISTRIBUTION OF LABELED FATTY ACID AMONG DIFFERENT TISSUE LIPIDS

Tissue*	Time	Distribution of Recovered ^{14}C†								
		Cholesteryl Palmitate			Cholesteryl Oleate			Cholesteryl Linoleate		
		CE	TG	PL	CE	TG	PL	CE	TG	PL
			%			%			%	
Liver										
	20 min	82	5	13	90	7	3	89	7	4
	60 min	56	15	29	66	22	12	65	14	21
	3.5 hr	14	14	72	44	21	35	32	21	47
	24 hr	3	5	92	18	46	36	6	12	83
Plasma										
	20 min	91	8	1	81	15	4	92	7	2
	60 min	85	15	1	74	22	4	62	28	10
	3.5 hr	33	34	33	40	44	16	36	19	45
	24 hr	20	9	71	29	51	20	9	38	53
Fat										
	20 min	95	3	2	—	—	—‡	68	11	21
	60 min	48	51	1	48	38	14	4	31	65
	3.5 hr	1	99	1	—	—	—	—	—	—
	24 hr	1	98	1	14	83	3	15	65	20
Kidneys										
	60 min	12	12	75	21	29	50	8	17	75
	3.5 hr	3	15	82	5	22	73	10	30	60
	24 hr	—	—	—	5	14	81	10	6	84
Adrenals										
	60 min	—	—	—	58	34	8	18	23	58
	3.5 hr	—	—	—	56	36	8	26	5	69
	24 hr	—	—	—	28	67	5	—	—	—
"Rest of rat"										
	20 min	91	8	1	88	10	2	78	12	10
	60 min	—	—	—	49	28	23	53	40	7
	3.5 hr	17	42	40	25	38	37	3	42	55
	24 hr	14	46	40	18	46	36	6	35	59

*,‡ See corresponding footnotes * and ‖ for Table 3.

† The values listed represent the distribution of recovered ^{14}C amongst 3 column fractions: CE, cholesteryl esters; TG, triglycerides + partial glycerides + free fatty acids; PL, phospholipids.

later time intervals. In the carcass ("rest of rat") samples, a large fraction of each of the three ^{14}C-fatty acids was found in both the triglyceride and phospholipid fractions at the later time intervals.

At the same time as the study with doubly labeled cholesteryl linoleate, we examined the effect of chylomicron size on the metabolism of chylomicron cholesteryl ester. Small and large chylomicrons were separately labeled with doubly labeled cholesteryl linoleate and injected intravenously into intact rats. The results presented in Table 5 demonstrate that there were no major differences in the metabolism of cholesteryl esters in large vs. small chylomicrons, except that plasma clearance of the labeled cholesteryl ester in large chlyomicrons occurred more rapidly, as previously demonstrated (12, 13). The small differences observed (Table 5) in the rate of hydrolysis and in tissue distribution were all compatible with the expected more rapid hepatic uptake and hydrolysis when cholesteryl esters are injected in the form of large chylomicrons.

DISCUSSION

The use of chylomicrons labeled in vitro with doubly-labeled cholesteryl esters permitted a detailed examination of some of the events occurring during the hepatic uptake and subsequent metabolism of the cholesteryl esters. The physiological validity of the data is, however, dependent on the assumption that the labeled cholesteryl ester added in vitro was metabolized in the same manner as normal chylomicron cholesteryl ester. This assumption seems justified, since we have previously shown (13) that the physical and metabolic properties of labeled cholesteryl ester added in vitro were the same as those of cholesteryl esters incorporated into chylomicrons in vivo, except that the added ester was relatively more abundant in the larger chylomicrons. This difference in the distribution of cholesteryl esters added in vitro, with respect to chylomicron size, did not appear to affect the metabolism of the doubly labeled cholesteryl esters significantly, since there were no major differences in the metabolism of labeled cholesteryl esters in small vs. large chylo-

TABLE 5 METABOLISM OF ^{3}H-CHOLESTERYL LINOLEATE-^{14}C IN LARGE AND SMALL CHYLOMICRONS*

Total Recovery of Radioactivity in Whole Animal

Chylomicron size	Time	Total Lipid Injected	Label Injected		Label Recovered in Whole Rat	
			^{3}H	^{14}C	^{3}H	^{14}C
		mg	μc		%	%
Small	60 min	26.7	1.0	0.14	83	64
Large	60 min	15.0	0.91	0.13	89	54
Small	3.5 hr	26.7	1.0	0.14	82	32
Large	3.5 hr	15.0	0.91	0.13	86	28
Small	24 hr	26.7	1.0	0.14	64	15
Large	24 hr	15.0	0.91	0.13	67	14

Tissue Distribution of Recovered Radioactivity

Tissue	Distribution of Recovered Radioactivity					
	60 min		3.5 hr		24 hr	
	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C
	%		%		%	
Small chylomicrons						
Liver	69	53	60	39	24	19
Plasma	14	18	5	3	4	4
Red cell	0.3	0.4	4	1	8	3
Rest of animal†	17	29	31	57	64	74
Large chylomicrons						
Liver	89	67	74	40	24	16
Plasma	2	5	3	3	5	2
Red cells	0.2	0.6	2	2	10	4
Rest of animal†	9	27	21	55	61	78

Distribution of ^{3}H-Cholesterol in Liver and Plasma‡

Tissue	Time	Small Chylomicrons			Large Chylomicrons		
		% of Cholesterol (^{3}H) as Ester	Ratio $^{3}H/^{14}C$ in Ester	% Re-esterified Ester	% of Cholesterol (^{3}H) as Ester	Ratio $^{3}H/^{14}C$ in Ester	% Re-esterified Ester
Liver							
	60 min	52	1.09	8	38	1.20	17
	3.5 hr	19	1.72	42	23	7.3	86
	24 hr	9	4.2	76	16	17.6	94
Plasma							
	60 min	94	1.06	6	74	1.07	7
	3.5 hr	37	9.3	89	29	4.4	77
	24 hr	48	141.	99	54	35.	97

Distribution of ^{14}C-Linoleate in Liver and Plasma‡

Tissue	Time	Distribution of Recovered ^{14}C					
		Small Chylomicrons			Large Chylomicrons		
		CE	TG	PL	CE	TG	PL
			%			%	
Liver							
	60 min	69	15	17	61	22	17
	3.5 hr	41	18	41	16	31	53
	24 hr	3	14	84	8	17	75
Plasma							
	60 min	81	16	3	52	39	9
	3.5 hr	30	22	48	29	17	55
	24 hr	9	38	53	22	35	44

* All values listed in this Table represent results obtained with a single rat for each time interval.
† In this Table, the "rest of animal" values include all organs and tissues other than liver and blood.
‡ See Tables 3 and 4, for definitions of column heading.

microns (Table 5). Delay in removal of smaller particles from the circulation was the only significant difference, but this did not influence the subsequent pattern of metabolism of the cholesteryl ester in these particles. In addition, the over-all metabolism of the cholesterol moiety of the doubly labeled cholesteryl esters was generally similar to the metabolism of labeled cholesterol incorporated into chylomicrons in vivo, as previously reported (6).

A similar over-all pattern of cholesteryl ester metabolism was observed in each of the three studies carried out with different doubly labeled cholesteryl esters. Upon entry into the vascular compartment, 80–90% of the cholesteryl esters was removed by the liver. Hepatic uptake was achieved without hydrolysis of the cholesteryl ester. This was established by the finding that, at the early time intervals studied, the ratio of the two isotopes in liver cholesteryl esters was identical with the ratio in the injected chylomicrons. In the liver the newly absorbed cholesteryl esters underwent a slow, steady hydrolysis, to the extent of about 60% after 60 min and about 85–90% after 3.5 hr. Despite this steady hydrolysis, however, the isotope ratio of hepatic cholesteryl esters did not change for more than 1 hr, and in one instance (with cholesteryl oleate) for more than 3.5 hr. This indicates that some newly absorbed cholesteryl ester remained in the liver without hydrolysis for a substantial period of time, and that the rate of reesterification of the liberated cholesterol was slow, relative to the rate of hydrolysis of the newly absorbed cholesteryl esters. The total rate of hydrolysis under these conditions was hence similar to the net rate of hydrolysis, as determined by the percentage of labeled cholesterol found as free cholesterol. As previously reported (6), the net rate of hydrolysis in vivo is also similar to the maximal rate of enzymatic hydrolysis observed in vitro (10).

These results demonstrate that the initial metabolism of chylomicron cholesteryl ester differs significantly from the metabolism of chylomicron triglyceride. Recent studies by a number of investigators (21–24) have indicated that the liver is responsible for the direct clearance of only about one-third of injected chylomicron triglyceride. Most of the chylomicron triglyceride fatty acids are cleared from the circulation by extrahepatic, peripheral tissues. In contrast, 80–90% of chylomicron cholesteryl ester is cleared by, and can be recovered in, the liver. The chylomicron triglyceride is initially taken up from the circulation by the liver without hydrolysis of the triglyceride ester bonds [demonstrated by experiments with chylomicrons doubly labeled in both the glycerol and fatty acid parts of the triglycerides (22, 23, 25, 26)]. The liver therefore takes up both chylomicron triglyceride and cholesteryl ester without ester bond hydrolysis. Chylomicron triglycerides taken up by the liver are metabolized much more rapidly than chylomicron cholesteryl esters, with triglyceride hydrolysis occurring rapidly in the interval 10–20 min after chylomicron injection (19, 22, 23). In contrast, about 80% of the labeled cholesteryl esters are still intact after 20 min, and 40% after 60 min.

Recent studies by Felts (27, 28), by Belfrage (26), and by Schotz, Arnesjö, and Olivecrona (29) have suggested that intact chylomicron triglyceride molecules taken up by the liver do not actually traverse the hepatic cell membrane without first undergoing hydrolysis. These studies have demonstrated that much of the radioactivity recovered in the liver soon after the administration of labeled chylomicron triglyceride represents intact triglyceride "entrapped" in the extracellular spaces of the liver. This triglyceride can be washed out of the liver to a large extent by perfusing the liver with saline. It has accordingly been suggested that intact chylomicron triglyceride taken up by the liver initially remains in the spaces of Disse, or attached to the outer surface of the cell membrane, and requires hydrolysis prior to entry into the cell. Felts has suggested that this hydrolysis takes place mainly in peripheral tissues (27, 28), whereas Belfrage (26) and Schotz et al. (29) have presented evidence that it occurs to a large extent in the liver. Uptake and hydrolysis of chylomicron triglyceride at the surface of isolated liver cells has been reported by Green and Webb (30).

The results presented in this paper do not provide definite information as to whether or not intact, unhydrolyzed cholesteryl esters taken up by the liver remain in the extracellular compartment (outside the hepatic cell membrane) prior to hydrolysis. On the one hand, the evidence described above—that intact chylomicron triglycerides remain extracellular prior to hydrolysis—suggests that chylomicron cholesteryl esters may well be metabolized in a similar fashion. On the other hand, there are enough differences in the metabolism of chylomicron triglycerides and cholesteryl esters to raise the possibility that these two kinds of molecules are metabolized differently in the liver. It is, for example, known that most of the liver's capacity for hydrolysis of cholesteryl esters is attributable to a soluble enzyme (10), presumably present in the cytoplasmic portion of the hepatic cell. Intact cholesteryl esters would probably have to traverse the cell membrane in order to come in contact with this hydrolytic enzyme. It should also be noted that although substantial amounts of unhydrolyzed cholesteryl ester remained in the liver for relatively long periods of time (1–3 hr), there appeared to be little, if any, loss of intact ester from the liver during this period. Future studies are planned which will focus on the question of whether or not chylomicron cholesteryl esters can be taken up intact into the liver cell.

In addition to hepatic uptake, small amounts of the labeled chylomicron cholesteryl esters were also taken up by all of the other tissues examined (adipose tissue, kidneys, adrenals, "carcass"). Extrahepatic uptake has previously been demonstrated by the finding of small amounts of labeled cholesterol in all tissues examined at short time intervals after the injection of labeled chylomicrons (6, 7), and by the slow but definite removal of labeled cholesterol, initially in chylomicrons, from the blood of a functionally hepatectomized dog (8). In the present studies the cholesteryl esters were taken up intact, without hydrolysis, by the extrahepatic tissues, and subsequently underwent hydrolysis in situ. These findings are consistent with the results of studies in vitro (9) that have demonstrated the uptake and hydrolysis of small amounts of chylomicron cholesteryl esters by a number of extrahepatic tissues. In contrast, it seems well established that chylomicron triglyceride is not taken up intact by peripheral tissues (specifically adipose tissue and muscle), but rather is hydrolyzed in the tissue capillaries (presumably by lipoprotein lipase) prior to or during tissue uptake (22, 23, 25).

As previously reported (6), during and after hydrolysis there was a slow, progressive loss of labeled cholesterol from the liver as equilibration occurred between the cholesterol pools of liver, blood, and peripheral tissues. These equilibrations continued throughout the experimental period, and were reflected in a rise in the ^{3}H content of the peripheral tissues. During this time there was also a moderate (25–40%) loss of ^{3}H from the body, presumably due to fecal excretion of ^{3}H-labeled sterols and bile acids during the experimental period. By 24 hr most of the labeled cholesterol in the animal's body was found in the peripheral tissues (mainly muscle and carcass). Most of this labeled cholesterol was present as free cholesterol; this observation is consistent with the hypothesis that transfer of label to peripheral tissues occurred largely by equilibration of the various pools of free cholesterol (5, 6). This equilibration probably involved mainly the poorly understood mechanism of isotopic exchange (5, 6). It has been pointed out (31) that recirculation of labeled sterol from the liver could occur by exchange of free cholesterol between liver and plasma, or by release of lipoproteins newly synthesized by the liver. The observed rates suggest that exchange was the major process involved. It should be noted that most (80–90%) of the total cholesterol of most tissues consists of free sterol; prominent exceptions are red blood cells and the nervous system, which contain almost entirely free sterol, and plasma and adrenals, which contain predominantly sterol ester. In the present experiments, by 24 hr only plasma and adrenal glands contained more esterified than free labeled cholesterol. This indicated that considerable equilibration had occurred between the free and esterified cholesterol pools in these tissues. At 24 hr almost all of the labeled cholesteryl ester in both plasma and adrenals consisted of reesterified labeled cholesterol. It is likely that, subsequent to the initial uptake (and later hydrolysis) of a small amount of intact cholesteryl ester by the adrenals, most of the labeled cholesterol accumulated in the adrenals as free cholesterol and was then esterified in situ.

The results obtained with the plasma samples raise the possibility that small amounts of cholesteryl ester might be taken up by the liver and subsequently released in the form of plasma lipoproteins, without ever having undergone hydrolysis. This possibility is suggested by our observation that after 60 min the plasma cholesteryl esters still had the same isotope ratio as the injected chylomicron cholesteryl esters. Another explanation for this finding is possibly that a small amount of the injected doubly labeled cholesteryl ester became associated with higher density plasma lipoproteins by exchange during the plasma clearance of the injected chylomicrons. Additional experiments, of a different type, will be needed in order to answer this question.

After hydrolysis of cholesteryl esters within the liver, the liberated ^{14}C-labeled fatty acids presumably entered into the same metabolic pathways as the fatty acids released by hepatic hydrolysis of chylomicron triglycerides. Since chylomicron triglycerides are hydrolyzed much more rapidly (21–23), the metabolism of the ^{14}C-fatty acids probably lagged behind that of most of the triglyceride fatty acids. As with the metabolism of the ^{3}H-cholesterol, a similar over-all pattern of ^{14}C-fatty acid metabolism was observed in all three studies. In all the studies labeled fatty acid disappeared relatively rapidly from the liver, leaving only 10–20% of the injected ^{14}C still in the liver after 3.5 hr. Disappearance of ^{14}C from the liver apparently involved both oxidation of the labeled fatty acids to CO_2 and redistribution of labeled fatty acids to peripheral tissues. Most of the ^{14}C-fatty acid presumably underwent oxidation, since only 30% of the injected ^{14}C was recovered in the entire animal after 3.5 hr, and only 15–20% after 24 hr. Significant amounts of ^{14}C-fatty acid were also transferred to peripheral tissue, however, and by 24 hr only 15–30% of the ^{14}C remaining in the animal was still in the liver.

At later time intervals, differences were observed in the distribution of the different fatty acids between and within given tissues. Thus, although in all three studies after 24 hr a large fraction of the total remaining ^{14}C was found in body fat, relatively more ^{14}C-labeled palmitate and oleate than linoleate was found in adipose tissue at this time. On the other hand, much more ^{14}C-labeled linoleate than either oleate or palmitate was found in the muscle plus carcass ("rest of rat") after 24 hr. In adipose tissue, although each of the ^{14}C-fatty acids was found

mainly in triglycerides after 24 hr, relatively more ^{14}C-labeled linoleate than oleate or palmitate was found in phospholipids. In both the liver and plasma, ^{14}C-labeled palmitate and linoleate were found mainly in phospholipids, whereas ^{14}C-oleate was found predominantly in the triglyceride fraction. These differences presumably reflected to some extent the concentration and the fatty acid composition of different lipids within the various tissues.

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