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Disappearance of the cholesterol moiety of an injected chylomicron-containing fraction of chyle from the circulation of the rat*

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SUMMARY

A chylomicron-containing, low-density lipoprotein fraction of thoracic duct chyle (S_f classes 20 and higher) obtained from rats fed either cholesterol-4-C^{14} or palmitic acid-1-C^{14} was injected intravenously into rats. Blood samples were obtained every 5 to 10 min for the first 1.5 hr, and thereafter at intervals of 30 min or longer up to 2.5 or 6.5 hr. The disappearance curves for the labeled cholesterol differed strikingly from those for the labeled triglyceride. The latter first declined rapidly and then more slowly. The former consisted of three phases: (1) rapid decline, (2) rising, and (3) leveling off. During the first phase, lipid-C^{14} disappeared from the circulation less rapidly after the labeled cholesterol injection than after injection of the labeled triglyceride. The percentage of lipid-C^{14} in the circulation increased about threefold during the rising phase of the cholesterol curves, and almost all of the terminal plasma-C^{14} was present in the higher-density lipoproteins (S_f classes less than 20). Disappearance curves derived for rats injected with cholesterol-C^{14} in the form of *in vitro*-prepared, higher-density plasma lipoproteins (S_f classes less than 20) showed a gradual initial decline followed by a leveling off. The following conclusions were drawn from these and other available data: The first phase of the curves for the cholesterol-labeled, chylomicron-containing chyle fraction results from an initial rapid removal of cholesterol-C^{14} of chylomicrons accompanied by a slow removal of cholesterol-C^{14} that has been transferred intravascularly to higher-density lipoproteins. This phase, during which most of the chylomicron-cholesterol-C^{14} is removed from the circulation, is followed by a period during which labeled free sterol is rapidly recirculated from the liver as higher-density lipoproteins. The greater proportion of the labeled sterol in the plasma lipoproteins during the terminal phase is derived from the sterol that has been recirculated.

The rate of disappearance, from whole blood or plasma, of injected, isotopically-labeled, very low-density chyle lipoproteins has been studied only insofar as their triglyceride and phospholipid moieties are concerned (1–4). The disappearance curve for the triglyceride moiety declined rapidly at first and more slowly during its second phase. The disappearance rate of the phospholipid moiety from the circulation was much slower than that of the triglyceride. Recent observations from this laboratory (5) indicated that the disappearance curves of the cholesterol moiety of a chylomicron-containing fraction of chyle might be more complex than the curves for triglycerides and phospholipids. Consequently, we have compared the disappearance of labeled triglyceride and cholesterol moieties of a low-density fraction of chyle from the blood stream of rats. It is shown here that the disappearance curves for the isotopic cholesterol are indeed unique. To assist in the interpretation of these curves, the disappearance from the circulation of a cholesterol-labeled lipoprotein fraction of plasma (S_f classes of less than 20), prepared *in vitro*, was also studied.

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EXPERIMENTAL METHODS

Materials

Labeled Compounds. Palmitic acid-1-C^{14} (Research Specialties Co., Richmond, Calif.) and cholesterol-4-

C^{14} (Nuclear Chicago Corp., Chicago, Ill.) were purified on silicic acid columns by the method of Barron and Hanahan (6).

Preparation of Chyle Lipoproteins Labeled with Palmitic Acid-1-C^{14} or Cholesterol-4-C^{14}. The thoracic ducts of donor rats were cannulated as previously described (7). The cannulated rats were placed in restraining cages and allowed access to saline for drinking purposes. Provided a steady flow of chyle continued for 12 hr, such rats were used for the preparation of the labeled chyle lipoproteins. Palmitic acid-1-C^{14} or cholesterol-4-C^{14}, dissolved in 0.5 ml of olive oil, was fed by stomach tube, and the chyle from the thoracic duct was collected in ice-cold tubes for the next 12 hr. The fibrin clot was removed with a wooden applicator. The defibrinated chyle was diluted ninefold with 1.1% NaCl (D = 1.006 g per ml) and centrifuged at 79,420 × *g* for 16 hr at 4° in a model L Spinco ultracentrifuge. The chylomicron-containing surface layer was removed by means of mild suction and used within 24 hr.

As determined by silicic acid chromatography (6), the cholesterol-labeled chyle preparations contained 71.1% of the sterol-C^{14} in the esterified form for rats 1 and 2 and 75.6% for rats 3-6.

Preparation of Cholesterol-4-C^{14}-Labeled Plasma Lipoproteins. Rat serum was incubated, as described by Avigan (8), with free or esterified cholesterol-4-C^{14}, which had been dispersed on celite, and the mixture was filtered through a fine-sintered glass funnel. The filtrate was diluted with a NaCl solution of density 1.006 (2 ml serum + 7 ml salt solution) and centrifuged at 79,420 × *g* for 16 hr at 4° in the model L Spinco ultracentrifuge. The upper layer was removed and discarded, and the lower layer (the bottom 1.5 cm) was saved. All of the plasma proteins other than those of S_f classes 20 and higher were thus collected, but as shown by Avigan (8) the labeled cholesterol of this preparation is associated almost exclusively with the lipoproteins.

Two lipoprotein preparations labeled by incubation with free cholesterol-4-C^{14} were used. In one, 97.1% of the sterol-C^{14} was in the free form; in the other, 96.8% was free. Two rats were injected with each preparation.

To prepare the lipoproteins labeled with cholesterol esters, the esters were first synthesized by incubating free cholesterol-4-C^{14} with serum by the method of Avigan but at about $^1/_{50}$ the concentration used in that procedure. In this manner, 40-60% of the cholesterol-4-C^{14} is esterified. After extraction of the lipids from the incubation mixture, the labeled cholesterol esters were isolated by silicic acid chromatography. They were then incorporated into lipoproteins by incubation with serum. This preparation contained 98.8% of the sterol-C^{14} in the esterified form. It was injected into two rats.

Treatment of Animals

Male rats of the Long-Evans strain, weighing from 220 to 240 g, were used. Food was withheld from all rats for 16 hr before the start of the experiment. The rats designated *fed* received 7 ml of a 35% glucose solution by stomach tube one hour before injection of the labeled lipoprotein. The rats were lightly anesthetized with ether, and 1 ml of the C^{14} preparations was injected into either the leg or tail vein over a period of about 45 sec.

The rats were placed in restraining cages immediately after the injections. Serial blood samples were taken from the tail vein (which was warmed each time to facilitate bleeding) every 5 min for the first 20 min, every 10 min during the next hour and 10 min, and thereafter at intervals of 30 min or longer, up to 2.5 or 6.5 hr. With the exception of the rats injected with the plasma lipoprotein preparation containing esterified C^{14}-cholesterol, two 50-λ blood samples were taken at each bleeding. They were immediately hemolyzed with 0.5 ml of distilled water, and, to the solution thus obtained, 50 ml of a ethanol–ether 3:1 solution was added. The total amount of blood removed did not exceed 7% of a rat's blood volume. A terminal blood sample (8–10 ml) was obtained by heart puncture from the rats injected with the cholesterol-labeled chyle lipoproteins and used for ultracentrifugal separation of plasma lipoprotein fractions.

The *in vitro* incorporation of the labeled esterified cholesterol into lipoproteins was much lower than that of the free cholesterol so that the plasma lipoprotein preparation containing the esterified C^{14}-cholesterol had only about $^1/_{10}$ to $^1/_{20}$ as much C^{14} per ml as did the other preparations. For this reason, single 200-λ samples were taken at each bleeding, from rats injected with the cholesterol-ester-labeled preparation, and at half as many time intervals as in all other rats. In this way, the same volume of blood was removed from all rats.

Analytical Procedures

Extraction of Lipids. Total lipids of each blood sample were extracted overnight with an ethanol–ether 3:1 solution, at room temperature, followed by two further extractions (each for about 2 hr) with the ethanol-ether mixture and with ethyl ether. The solvents of the combined extracts were removed, under reduced pressure, in a Rinco rotary evaporator, and the residue was dissolved in hexane.

TABLE 1. DISTRIBUTION OF LIPID-C^{14} AMONG ULTRACENTRIFUGALLY SEPARATED PLASMA FRACTIONS AFTER INTRAVENOUS INJECTION OF A CHOLESTEROL-4-C^{14}-LABELED, CHYLOMICRON-CONTAINING FRACTION OF THORACIC DUCT CHYLE

1.0 ml injected intravenously. The preparation injected into rats 1 and 2 contained 76.1% of the sterol-C^{14} in esterified form and 23.9% in free form. The preparation used for rats 3–6 contained 75.6% of the C^{14} in the ester and 24.4% as free sterol.

Rat No.	Weight	Fed* or Fasted†	Minutes after Injection	% Lipid-C^{14} Recovered in Fraction			
				Supernatant	Infranatant A	Infranatant B	Pellet
	g						
1	235	Fasted	150	2‡	1‡	86‡	11‡
2	226	Fasted	150	3	4	81	12
3	240	Fasted	407	3	2	83	12
4	244	Fasted	390	3	2	84	11
5	220	Fed	425	1	2	87	10
6	240	Fed	390	4	5	74	17

* Food withdrawn 16 hr before injections. These rats received 7.0 ml of a 35% glucose solution 1 hr prior to injection of chylomicrons.

† Food withdrawn 16 hr before injection.

‡ Each value in these columns refers to a percentage of the sum of the values for the four fractions. Supernatant refers to the top layer containing S_f classes of 20 and higher. Infranatant B refers to the bottom layer containing S_f classes of less than 20. Infranatant A is the layer between the above two.

Ultracentrifugal Fractionation of Terminal Plasma Samples. Two ml of the terminal plasma samples were diluted to 9 ml with 1.1% NaCl (D = 1.006 g per ml) and centrifuged at 79,420 × *g* for 16 hr at 4° in a model L Spinco ultracentrifuge. The tubes were sliced, and the plasma fractions listed in Table 1 were obtained as previously described (5). The supernatant was the chylomicron-containing layer, and the bottom layer, infranatant B, contained the bulk of the α and β lipoproteins. Infranatant fraction A, the layer between the other two fractions, was slightly contaminated by them. The pellet, containing the remaining plasma proteins, was washed with saline, and the washings were added to infranatant B. This washing undoubtedly did not remove all of the α-lipoproteins from the pellet.

The lipids in all four fractions were extracted by the same method as that used for extraction of lipids from the blood samples.

C^{14}-Assays. The dried lipid extracts were dissolved in 15 ml of toluene containing 45 mg of 2,5-diphenyloxazole and 1.5 mg of 1,4-bis-2-(5-phenyloxazoyl)-benzene and assayed for C^{14} in a Packard tricarb liquid scintillation spectrometer. Lipid extracts from whole blood were invariably slightly colored. An internal C^{14} standard was used to correct the activity in each sample for color quenching. No sample contained less than 18 times the radioactivity of the background in the experiments with the chyle preparations and 7 times the background in the plasma lipoprotein experiments.

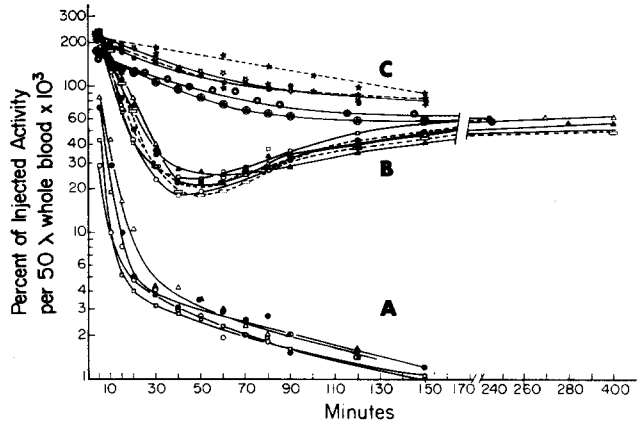

FIG. 1. Disappearance of lipid-C^{14} from circulation of rats injected intravenously with (A) a chylomicron-containing chyle fraction (S_f classes 20 and higher) labeled with tripalmitin-1-C^{14}, (B) the same chyle fraction labeled with cholesterol-4-C^{14}, and (C) a cholesterol-4-C^{14}-labeled plasma lipoprotein fraction (S_f classes less than 20) prepared *in vitro*. In A and C, all rats fasted. In B, solid lines: fasted rats; broken lines: glucose-fed. In C, solid lines: experiments with preparation labeled predominantly with free cholesterol; broken lines: preparation labeled predominantly with esterified cholesterol.

RESULTS

1. Experiments with Chylomicron-Containing Chyle Preparations

The disappearance curves for 6 rats (4 fasted, 2 fed) injected with the cholesterol-labeled chyle preparation and for 4 rats (all fasted) injected with the triglyceride-labeled chyle preparation are shown in Figure 1. The percentages of the injected C^{14} recovered in the extracted lipids of 50 λ of blood × 10^3 are plotted semi-logarithmically against time.

The curves for the chylomicron-containing fraction of chyle labeled with cholesterol-4-C^{14} differed strikingly from those for the chylomicron-containing fraction of chyle labeled with the C^{14} triglyceride. The latter consisted of two phases, both declining. The former showed three phases: an initial decline, followed by a rise, and terminating in a leveling off. The slopes of the initial phase of the cholesterol curves were not so steep as those of the triglyceride curves.

An average of 8.3% of the injected C^{14} was present in total blood volume of the rats at the end of the first phase in the experiments in which the cholesterol-

labeled chyle preparation was injected (calculated on the assumption that blood represents 8% of the body weight); 2.3% (average) of the injected C^{14} was so recovered at the end of the first phase after injection of the labeled triglyceride. At the time the rats injected with the cholesterol-labeled chyle preparation were killed (2.5 or 6.5 hr after the injection), the injected C^{14} recovered as lipids in whole blood had risen to an average of 22%. In the two rats killed 2.5 hr after injection of the cholesterol-labeled chyle preparation, plasma lipids accounted for about 50% of whole blood lipid-C^{14}.

Table 1 shows the distribution of lipid-C^{14} among the ultracentrifugally separated fractions prepared from terminal plasma samples of the rats injected with the cholesterol-labeled chyle preparation. At 2.5 as well as at 6.5 hr after the injection, only a small percentage of the plasma lipid-C^{14} remained in the chylomicron-containing (supernatant) fraction. Most of the lipid-C^{14} was found in the fraction containing the bulk of the higher-density α- and β-lipoproteins (infranatant fraction B).

In the experiments with cholesterol-labeled chyle preparations, no differences between fed and fasted rats were observed in the disappearance curves or in the terminal plasma analysis.

2. Experiments with Cholesterol-Labeled Plasma Lipoproteins Prepared In Vitro

The disappearance curves for the rats injected with the cholesterol-labeled lipoproteins of plasma (S$_f$ classes less than 20) did not show a sharply declining phase. This was true for both the predominantly free and the predominantly esterified cholesterol-C^{14}-labeled plasma lipoproteins. The slope of one of the curves derived for the plasma lipoproteins labeled with cholesterol esters was not as steep initially as were those of the other curves. For this reason, and also because the radioactivity of the lipoproteins labeled with the ester was too low to permit us to take as many blood samples from the rats injected with that preparation as were taken from rats injected with free cholesterol, we cannot state with assurance that the rates of removal of the injected lipoproteins labeled in the free and esterified forms are identical. Our curves do provide, however, a clear-cut distinction between the disappearance from the circulation of cholesterol injected in the form of low-density chyle lipoproteins and cholesterol injected as higher-density plasma lipoproteins.

The cells of the terminal blood samples (2.5 hr after injection) of the rats injected with the labeled cholesterol esters contained a smaller proportion of lipid-C^{14} than did the cells of the rats injected with free cholesterol. The cells of the former contained about 15% of the total blood-C^{14}; those of the latter contained about 50%. In both cases, practically all of the C^{14} in the cells was recovered as free sterol. If, in the rats injected with the cholesterol esters, the labeled sterol entered blood cells predominantly in the free form, one source of this free sterol might be labeled free sterol recirculated from the liver after uptake and hydrolysis of the esters.

DISCUSSION

Our disappearance curves for the triglyceride moiety of palmitic acid-1-C^{14}-labeled chyle preparations closely resemble those reported for rats by French and Morris (2). They are also similar to those observed in dogs (1, 3). These curves, which consist of two phases, both declining, have been adequately discussed elsewhere (1–3, 9).

A unique feature of the curves for the cholesterol-labeled, chylomicron-containing chyle fraction was a rising phase, which came after an initial, rapidly declining phase. This means that the initial period, during which the injected cholesterol-C^{14} is rapidly removed from the circulation, is followed by one in which C^{14} is rapidly returned to the circulation.

The first-phase slopes of the curves for the cholesterol- and triglyceride-labeled chyle lipoproteins also differed. The slopes of the cholesterol curves were not so steep. This difference might be accounted for by removal of the two moieties of chylomicrons from the circulation at different rates. It could also be accounted for by removal of the two moieties at the same rate, accompanied by either (*a*) recirculation of cholesterol, but not of triglycerides, into higher-density lipoproteins or (*b*) intravascular transfer of a part of the chylomicron cholesterol, but not triglycerides, into higher-density lipoproteins, and removal of the cholesterol-labeled, higher-density lipoproteins more slowly than the labeled chylomicrons. Transfer of labeled cholesterol to blood cells would also reduce the rate of removal of the cholesterol from the circulation. Intravascular transfer of cholesterol to higher-density lipoproteins and removal of the cholesterol from the circulation more slowly in this form than as chylomicrons appears to be the explanation best supported by the available evidence:

1. Rapid transfer of labeled cholesterol from injected low-density chyle lipoproteins to higher-density plasma lipoprotein occurs in the intact rat and dog (5, 10). In the rat, 10 min after injection of a cholesterol-labeled chyle preparation, about one-fourth of the plasma-C^{14} is present in higher-density plasma lipo-

proteins (5). It has been shown in dogs, on the other hand, that only slight amounts of C^{14} can be recovered in higher-density plasma lipoproteins at a time when injected triglyceride-labeled, low-density lipoproteins are being rapidly removed from plasma (1).

2. It seems probable that the transfer of labeled cholesterol from low-density chyle lipoproteins to higher-density plasma lipoproteins *in vivo* occurs intravascularly. After injection of a cholesterol-C^{14}-labeled, chylomicron-containing fraction of chyle into totally hepatectomized-eviscerated rats (in which it would be expected that recirculation of labeled cholesterol would be entirely or almost entirely eliminated), about 25% of the plasma lipid-C^{14} was recovered in higher-density lipoproteins.[1]

3. Our disappearance curves for injected plasma lipoproteins indicate also (provided that *in vivo*-labeled lipoproteins disappear at a rate similar to *in vitro*-labeled lipoproteins) that labeled cholesterol that is transferred to higher-density plasma lipoproteins disappears much more slowly from the circulation than does the labeled cholesterol of the low-density chyle lipoproteins during the initial phase of the disappearance curves.

The transfer of cholesterol-C^{14} of the injected low-density chyle lipoproteins to blood cells does not appear to occur as rapidly as the transfer to higher density lipoproteins, at least during the first 10 min. Ten minutes after the injection of our cholesterol-labeled chyle preparation, only about 5% of the blood lipid-C^{14} was found in the cells (5).

It therefore seems probable that, within minutes after the intravenous injection of a cholesterol-labeled, chylomicron-containing fraction of chyle, a considerable proportion of the labeled sterol is transferred intravascularly to higher-density lipoproteins and that the first phase of the curves derived for this preparation results from a *rapid* disappearance of cholesterol-C^{14} of the low-density chyle lipoproteins (S_f classes 20 and higher) accompanied by a *slow* disappearance of cholesterol-C^{14} that had been transferred to the higher-density plasma lipoproteins (S_f classes less than 20).

After this early, almost complete removal of chylomicron-C^{14} from the circulation, labeled sterol is rapidly recirculated. In an earlier communication (5), we concluded that the liver, after removing the bulk of labeled cholesterol of our injected chyle preparation, hydrolyzed the esters and recirculated the labeled free cholesterol most rapidly some time between 10 min and 2 hr — a period when the rising phase of the curves derived for the cholesterol-labeled chyle preparations in the present study was observed. Recirculation of the labeled sterol from the liver could result from a rapid exchange of free cholesterol between liver and plasma (11) or from the release of lipoproteins newly synthesized by the liver.

Only 2-3% of the terminal plasma lipid-C^{14} (obtained at 2.5 or 6.5 hr after injection of the cholesterol-labeled chyle preparation) was recovered in the chylomicron-containing (S_f classes 20 and higher) fraction, whereas over 80% was in the fraction containing the bulk of the α- and β-lipoproteins. The labeled sterol in the latter fraction was derived presumably both from intravascular transfer and recirculation from the liver. However, since it was found that, during the rising phase of the curves, the percentage of lipid-C^{14} in the circulation increased about threefold (from 8–22%), it would seem that the greater proportion of the C^{14} circulating in plasma lipoproteins during the terminal phase was derived from recirculated rather than intravascularly transferred labeled sterol.

At 2.5 hr after injection of the cholesterol-labeled chyle preparation, the plasma lipid-C^{14} (labeled chiefly in the higher-density plasma lipoproteins) accounted for about half of the blood lipid-C^{14}. Thus, the terminal (leveling off) phase of the chyle lipoprotein curves represents, almost exclusively, turnover of the labeled sterol in the blood cells as well as in the higher-density plasma lipoproteins.

[1] Lossow, W. J., N. Brot, and I. L. Chaikoff. Unpublished observations.

REFERENCES

1. Havel, R. J., and D. S. Fredrickson, *J. Clin. Invest.* **35**: 1025, 1956.
2. French, J. E., and B. Morris, *J. Physiol.* **138**: 326, 1957.
3. Fredrickson, D. S., D. L. McCollester, and K. Ono. *J. Clin. Invest.* **37**: 1333, 1958.
4. McCandless, E. L., and D. B. Zilversmit. *Am. J. Physiol.* **193**: 294, 1958.
5. Lossow, W. J., N. Brot, and I. L. Chaikoff. *J. Lipid Research* **3**: 207, 1962.
6. Barron, E. J., and D. J. Hanahan. *J. Biol. Chem.* **231**: 493, 1958.
7. Bloom, B., I. L. Chaikoff, W. O. Reinhardt, C. Entenman, and W. G. Dauben. *J. Biol. Chem.* **184**: 1, 1950.
8. Avigan, J. *J. Biol. Chem.* **234**: 787, 1959.
9. Olivecrona, T., E. P. George, and B. Borgström. *Federation Proc.* **20**: 928, 1961.
10. Fredrickson, D. S., D. L. McCollister, R. J. Havel, and K. Ono. In *Chemistry of Lipids as Related to Atherosclerosis*, edited by I. H. Page, Springfield, Illinois, Charles C Thomas, 1958, p. 205.
11. Eckles, N. E., C. B. Taylor, D. J. Campbell, and R. G. Gould. *J. Lab. Clin. Med.* **46**: 359, 1955.