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J Clin Invest. 1956;**35**(9):1025-1032. <https://doi.org/10.1172/JCI103348>.

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THE METABOLISM OF CHYLOMICRA. I. THE REMOVAL OF PALMITIC ACID-1-C¹⁴ LABELED CHYLOMICRA FROM DOG PLASMA

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(Submitted for publication April 23, 1956; accepted May 24, 1956)

It is now generally accepted that the intestinal lymphatics constitute the exclusive pathway for the absorption of long chain fatty acids, whether ingested unesterified or as glycerides. These fatty acids appear in the thoracic duct lymph as chylomicra. The subsequent fate of the absorbed fat has been explored very little, and the role of plasma lipoproteins in chylomicron transport and the sites of removal of the absorbed fat remain unknown.

Approximately 95 per cent of long chain fatty acids appear in the lymph as triglycerides and 5 per cent as phospholipids (1). Bergström, Borgström, and Rottenberg (2) fed labeled stearic acid and tristearin to fasting rats. Over a period of 3 to 4 hours, approximately 1 mg. per minute of fatty acids was delivered to and removed from the total plasma, which contained only about 5 mg. of preformed triglycerides. The maximum specific activities of plasma triglyceride fatty acids were only 4 and 15 per cent of the specific activities of the fed stearic acid and tristearin, respectively. The authors suggested that the absorbed triglycerides were not mixing appreciably with the existing plasma triglycerides. Their data did not exclude the possibility that the plasma triglycerides were exchanging rapidly with a large triglyceride pool.

Jones, Gofman, Lindgren, Lyon, Graham, Strisower, and Nichols (3) studied the low-density lipoproteins following fat feeding in humans and found a transient elevation of lipoproteins having flotation rates greater than S_f 60, followed by a stepwise degradation to higher density molecules with flotation rates down to, but not below, S_f 30. They suggested that the low-density lipoproteins constitute a transport system for absorbed fatty acids.

In most studies of exogenous fat metabolism, interpretation of removal rates and mechanisms has been hampered by the variable and uncontrollable factor of fat absorption. In a few studies, chyle has been injected intravenously in order to obviate this difficulty. Marble, Field, Drinker, and Smith (4) found that intravenously administered chyle was rapidly removed from dog plasma. Morris (5) recently reported similar findings in cats. In a previous study in this laboratory, it was found that protamine decreases and heparin increases the rate of removal of intravenously injected chylomicron emulsions from rat plasma (6). It was suggested that the heparin-activated lipolytic enzyme, then termed lipemia clearing factor, which catalyzes the hydrolysis of the triglyceride moiety of chylomicra and low-density lipoproteins, might be involved in fat transport.² The purpose of the present study has been to delineate further the mechanisms by which chylomicra are removed from the plasma. To this end, chylomicra, isolated from thoracic duct lymph of dogs fed sodium palmitate-1-C¹⁴, have been transfused into recipient dogs. The possible participation of plasma unesterified fatty acids and of various lipoprotein fractions in their transport has been examined. The results obtained strengthen the view that lipoprotein lipase is involved in chylomicron removal and cast doubt on the essentiality of the other plasma lipoproteins in this process in the dog.

METHODS

Mongrel dogs of both sexes fed a stock diet were used. Thoracic duct fistulae were prepared in post-absorptive animals according to the technique of Markowitz (8)

² This view has been strengthened by Korn's demonstration of the same enzyme in certain tissues of animals which have not received heparin (7). Because of its specificity, he has termed the enzyme "lipoprotein lipase."

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under Sodium Pentothal® anesthesia. All tributaries of the left external jugular vein except the thoracic duct were ligated and the vein ligated and divided high in the neck. The proximal end of the vein was exteriorized and a large polyethylene catheter inserted. When the animal had recovered from the anesthesia he was placed upright in a sling and the clear lymph collected dropwise in a large centrifuge tube. When a lymph flow of 10 to 20 ml. per hour had been established, the animal was given a tracer amount of sodium palmitate-1- C^{14} (100–200 μ C.) mixed with 10 to 30 ml. of cream containing 36 gm. fat per 100 ml. by stomach tube. Hydration was maintained with skimmed milk and water. The lymph became turbid within one hour after administration of the cream and cleared again in 10 to 20 hours. After each hour the lymph collected was stored at 3° C. and allowed to clot. The clot was removed by centrifugation and the lymph stored at 3° C. Within 24 hours, the chylomicra were isolated from the lymph as follows. The serum was centrifuged at 80,000 \times g. for 30 minutes in the 30 rotor of the Spinco Model L ultracentrifuge at 15° C. The bulk of the chylomicra were packed in a white, butter-like layer at the top of the centrifuge tube with a small amount still dispersed in the upper half of the tube. The packed material was re-emulsified in 0.15 M sodium chloride solution by repeatedly aspirating it through a No. 24 hypodermic needle. The resulting emulsion contained practically no particles over 1 micron diameter on dark field microscopic examination. Recentrifugation of such emulsions can be carried out with little or no change in chemical composition or ease of emulsification. An aliquot of the emulsion was extracted in ethanol-acetone (1:1) for chemical analysis and counting.

Two adult mongrel female dogs weighing 6 kg. were selected to receive the chylomicron emulsions. One dog was used twice; on the first occasion fasted 20 hours (Exper. 1), and two months later, fasted 6 hours (Exper. 2). The other dog was used only once following a 20-hour fast (Exper. 3). The dogs were given sufficient

sodium pentobarbital intravenously to induce basal anesthesia (15 to 20 mg. per kg.). During the experiment they received sufficient diethyl ether by inhalation to maintain light anesthesia. Physiologic saline infusions were begun in both femoral veins and maintained at a rate of approximately 0.5 ml. per minute. The freshly prepared chylomicron emulsion (22 to 36 ml.), containing 2.0 to 3.6 gm. total lipid, was injected intravenously. Twenty-ml. blood samples, mixed with 1.5 mg. sodium oxalate per ml. and chilled immediately in ice, were withdrawn at intervals until the plasma was visibly clear, and for some time thereafter. An aliquot of each blood sample was taken for hematocrit determination. The remainder was centrifuged at 3° C. and the plasma removed.

Separation of chylomicra from plasma

Aliquots of the plasma were centrifuged immediately at 105,000 \times g. for 30 minutes at 3° C. The chylomicra were then packed in a butter-like layer at the top of the tube. The perfectly clear, infranatant plasma was carefully removed by suction through a long needle and centrifuged again under the same conditions. Only a trace of turbid supernatant material was visible after this second centrifugation. The clear, infranatant plasma was removed as before. Prior experiments demonstrated that this chylomicron-free plasma contained essentially all the plasma lipoproteins normally present in post-absorptive dog plasma. The plasma unesterified fatty acids (UFA) were immediately extracted from a two-ml. aliquot of the chylomicron-free plasma (*vide infra*). The impure chylomicra contained in the butter-like layer from the first centrifugation were re-emulsified in physiologic saline and centrifuged for 15 hours at 114,000 \times g. in the 40.3 ultracentrifuge rotor. The chylomicra were concentrated at the top of the tube and the other serum protein constituents concentrated at the bottom. The tube was sliced in a tube slicer and the upper layer emulsified and transferred quantitatively to a 5-ml. volumetric flask (Fraction I).

Lipoprotein fractionation

The remainder of the chylomicron-free plasma was used for the quantitative ultracentrifugal separation of four lipoprotein fractions (9). The densities and nature of the resulting fractions are given in Table I.

Chemical analyses

The plasma and its fractions were extracted in ethanol-acetone (1:1) and analyzed for total cholesterol (10) and lipid phosphorus (11). Phospholipid was estimated by multiplying lipid phosphorus by the factor 25. The plasma and Fraction I were also analyzed for total lipid (12) and free cholesterol (10); and triglyceride was calculated by difference (12). Protein content of the ethanol-acetone insoluble material was determined in the chylomicron samples by the biuret method (13). UFA's were extracted in diethyl ether by the method of Davis (14), in which the plasma is partitioned between the ether and an aqueous phase saturated with so-

TABLE I
Ultracentrifugally separated lipoprotein fractions

Fraction	Nomenclature	Density	Centrifugal conditions
I	Chylomicra	<1.006	105,000 \times g.—30 min.
II	Low-density lipoproteins (Sf >10)	<1.019	114,000 \times g.—15 hr.
III	Beta lipoproteins (Sf 0–10)	1.019–1.063	114,000 \times g.—15 hr.
IV	Alpha lipoproteins (high density)	1.063–1.21	114,000 \times g.—22 hr.
V	Residual serum proteins and UFA	>1.21	114,000 \times g.—22 hr.

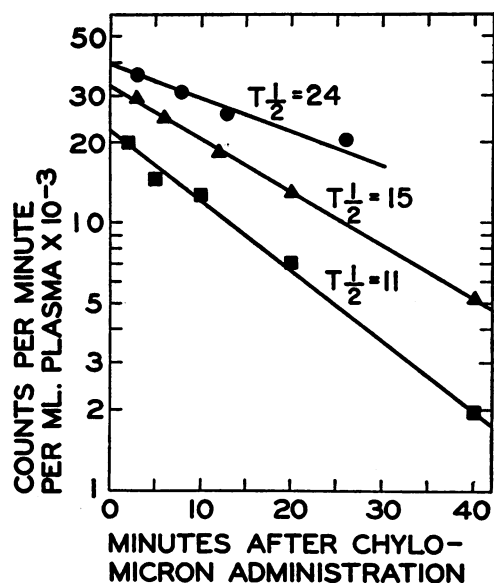


FIG. 1. DISAPPEARANCE OF RADIOACTIVITY FROM DOG PLASMA AFTER INTRAVENOUS ADMINISTRATION OF LABELED CHYLOMICRA

●, ▲, and ■ = Experiments 1, 2, and 3 respectively.

dium sulfate and containing sodium dodecyl sulfate. The ether phase contains all the UFA's, practically all the cholesterol and triglycerides, but almost none of the phospholipids. The ether phase was evaporated to dryness under nitrogen at room temperature, the lipids dissolved in *n*-heptane, and the UFA's separated from the neutral lipids by the method of Borgström (15). The fatty acids were determined by electrometric titration between pH 6.0 and 10.0.⁸ Phospholipids were separated from other lipids of the chylomicra on a silicic acid column according to Borgström (16).

Radioactivity determinations

Samples were prepared in duplicate for assay of radioactivity by mixing suitable aliquots of the lipid extracts with sufficient olive oil to give a total weight approximating 10 mg. These aliquots were evaporated on lens paper in aluminum planchets. Radioactivity was assayed with a Robinson flow gas counter and the results corrected to an absorption weight of 6.2 mg. per cm².

All experimental data for the various fractions and the plasma, with the exception of specific activities, have been corrected to milligrams or counts per minute per ml. of native plasma.

RESULTS

1. *Chylomicron composition.* The chemical composition of the three isolated chylomicron preparations is summarized in Table II. The

⁸ We are indebted to Miss Amelia Cherkes for performing these titrations.

average molecular weight of the fatty acids in preparation No. 1 was estimated to be 260. This figure was determined by dividing the mg. of fatty acid as determined chemically by the concentration in milliequivalents as determined titrimetrically after saponification. The phospholipid fraction contained only 1.5 per cent of the total radioactivity in preparation No. 1. It has been assumed, therefore, in subsequent calculations that all the radioactivity in the chylomicra was contained in triglyceride fatty acids.

2. *In vitro transfer of chylomicron radioactivity.* In order to determine the extent to which transfer of chylomicron radioactivity occurred in the absence of the clearing process, dog chylomicra were incubated *in vitro* with normal dog plasma for 90 minutes at 37° C. Lipoprotein fractions were then separated and total radioactivity in each fraction determined. There was 2.0 per cent of the activity in Fraction II, 0.4 per cent in Fraction III, 0.4 per cent in Fraction IV, and 0.2 per cent in Fraction V.

3. *Removal of chylomicra from the plasma.* The rates of disappearance of radioactivity from the whole plasma are shown in Figure 1. Chylomicron (Fraction I) radioactivity disappeared at the same rate. The removal appeared to follow a first order reaction. The deviations from linearity on the graph are probably due in part to variations in plasma volume secondary to the repeated bleedings, since the hematocrit fell from an average of 45 to 37 during the clearing process. Similar straight lines were obtained when the disappearance of triglycerides and phospholipids from the chylomicra isolated from each sample were plotted on a semilogarithmic scale. The triglyceride and phospholipid half-lives in the three experiments

TABLE II
Chemical composition of dog lymph chylomicra

Substance	Per cent by weight (range)
Cholesterol esters	1.5-2.0
Free cholesterol	0.7-1.0
Phospholipids	8.2-8.9
Triglycerides	86.3-86.5
*Unesterified fatty acids	0.2
Protein	2.2-2.5

* Based on per cent radioactivity in isolated unesterified fatty acids in one experiment.

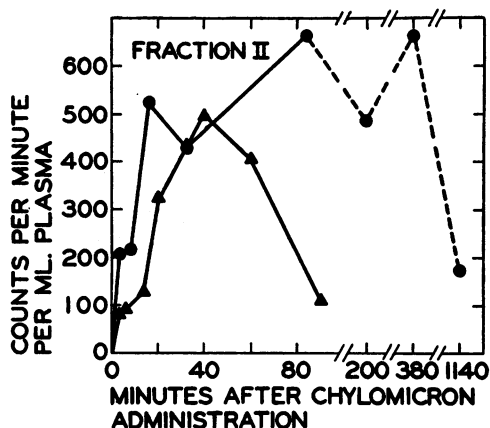


FIG. 2. RADIOACTIVITY IN FRACTION II LIPOPROTEINS FOLLOWING INTRAVENOUS ADMINISTRATION OF LABELED CHYLOMICRA

● and ▲ = Experiments 1 and 2, respectively.

were 28 and 28, 18 and 17, and 11 and 12 minutes, respectively. There was insufficient cholesterol and protein in these preparations to permit accurate determination of half periods. The volume of distribution of the radioactivity at time zero was 50 to 73 ml. per kg., corresponding to accepted values for plasma volume in the dog (17).

4. *Analysis of other lipoprotein fractions.* The appearance of labeled fat in the other fractions during clearing was measured after two separate chylomicron infusions (Figure 2). Radioactivity in Fraction II rose during the early phase of clearing in both experiments. However, the maximum total radioactivity in this fraction never exceeded 2 per cent of the total plasma radioactivity at time zero and there was no detectable change in the total lipid or lipid phosphorus concentrations in this fraction during the clearing process. The pertinent data regarding the S_t 0-10 (Fraction III) and alpha (Fraction IV) lipoproteins are shown for Exper. 1 in Figure 3. The data for Exper. 2 were comparable. It is apparent that only small amounts of radioactivity appeared, and cholesterol and phospholipid concentrations varied little in these fractions during clearing. On the other hand, radioactivity and phospholipid concentrations did rise strikingly in both of these fractions several hours after clearing was complete. When aliquots of the alpha lipoproteins from four serial blood samples (Exper. 2) were extracted by the Davis procedure, it was found that

about 90 per cent of the radioactivity was present in the phospholipid fraction.

5. *Unesterified fatty acids.* The most striking change in all experiments was a significant and progressive rise in the plasma UFA specific activity, the maximal rise occurring when the chylomicra had largely disappeared from the plasma (Figure 4-B). In Exper. 1 the radioactivity contained in Fraction V was measured. The radioactivity varied from 80 to 110 per cent of that found in the UFA's of whole plasma. The serial UFA concentrations in the three experiments are presented in Figure 4-A. There was no significant change in UFA concentration during the clearing process, except for a small rise in Exper. 3. In order to assess the significance of the rise in UFA specific activity during clearing, the following experiment was performed. The non-protein solvent density of a 15-ml. sample of fresh dog serum was brought to 1.21 by addition of potassium bromide. The lipoproteins were removed after centrifuging at $114,000 \times g$ for 22 hours, and the infranatant solution thoroughly dialyzed against physiologic saline. Three mg. of sodium palmitate-1- C^{14} (2.3×10^6 c.p.m.) were dissolved in 10 ml. of physiologic saline with warming to give

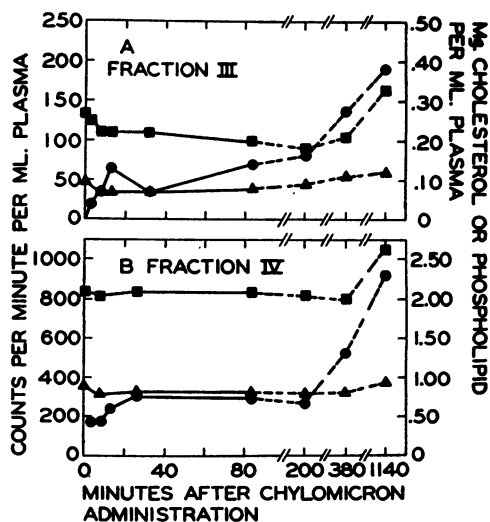


FIG. 3. RADIOACTIVITY AND LIPID CONCENTRATIONS IN FRACTION III AND IV LIPOPROTEINS FOLLOWING INTRAVENOUS ADMINISTRATION OF LABELED CHYLOMICRA IN EXPERIMENT 1

● = radioactivity, ▲ = total cholesterol, and ■ = phospholipids.

an opalescent solution. This solution was mixed with the dialyzed, lipoprotein-free protein solution. A clear solution resulted, with no evidence of turbidity or precipitate, even in the cold. It was previously established by ultracentrifugal fractionation that sodium palmitate added in this way is completely bound to the serum proteins. After removal of aliquots for assay of radioactivity, the remainder of the solution was injected intravenously in a 10-kg. dog fasted for 20 hours. Anesthesia was given as described previously. Five-ml. serial blood samples were withdrawn at close intervals for 11 minutes, and the plasma removed. Plasma aliquots were extracted by the Davis method (14) and the UFA's separated in selected samples (15). The half-life of UFA radioactivity was found to be extremely short, about two minutes (Figure 5). In the samples taken at 1 and 11 minutes, practically all the radioactivity present was in the form of unesterified fatty acids.

6. *Radioactivity in red blood cells.* In Experiment 2, aliquots of the cells remaining after removal of the plasma were washed three times with physiologic saline, lyzed in distilled water, and plated directly on aluminum planchets for assay of their radioactivity. No significant radioactivity was found.

7. *Intravascular lipolysis during clearing.* In Experiments 2 and 3, several aliquots of lipemic plasma, taken following the chylomicron infusion, were incubated at 37° C. for two hours and serial measurements of optical density at 500 m μ made in a Coleman Jr. spectrophotometer. In no instance was there a fall in optical density greater than 0.02 unit during the period of incubation. These measurements were completed within four hours after taking the blood sample, and the plasmas were kept at 3° C. until the time of incubation. These *in vitro* studies suggest a lack of intravascular lipolysis *per se* during the clearing process.

DISCUSSION

Dog chylomicra are composed chiefly of triglycerides. Their composition is similar to that of human chylomicrons.⁴ In our experiments, ap-

⁴ Bragdon, J. H., Havel, R. J., and Boyle, E., data to be published. It should be noted that in the present experiments no attempt was made to remove traces of plasma proteins from the preparations. Therefore, the protein content is probably lower than that reported.

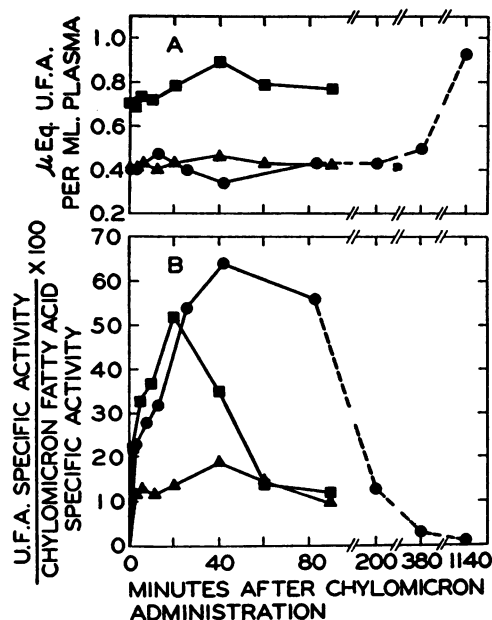


FIG. 4. A. PLASMA UNESTERIFIED FATTY ACID CONCENTRATIONS AFTER INTRAVENOUS ADMINISTRATION OF LABELED CHYLOMICRA

B. SPECIFIC ACTIVITY OF UNESTERIFIED FATTY ACIDS AFTER INTRAVENOUS ADMINISTRATION OF LABELED CHYLOMICRA EXPRESSED AS PER CENT OF THE SPECIFIC ACTIVITY OF THE FATTY ACIDS IN THE ADMINISTERED CHYLOMICRON TRIGLYCERIDES

●, ▲, and ■ = Experiments 1, 2, and 3 respectively.

proximately 97 per cent of the chylomicron radioactivity following feeding of sodium palmitate-1- C^{14} was contained in triglycerides. In calculating the triglyceride fatty acid specific activity, the assumption that all the radioactivity was contained in the triglycerides therefore introduces no significant error. Our finding of 1.5 per cent of the radioactivity in the phospholipid moiety of the chylomicra is in good agreement with that of Bloom, Chaikoff, Reinhardt, and Dauben (1), who fed sodium palmitate-1- C^{14} to rats and found not more than 4 per cent of the label in the phospholipid fatty acids of thoracic duct lymph. While not measured, cholesterol esters could not have accounted theoretically for more than 1 per cent of the radioactivity found in dog chylomicra. The paucity of UFA's in the chylomicra confirms Borgström's finding in rats (15). The small quantity we found could have resulted from *in vitro* lipolysis.

The rapid turnover of chylomicra in dog plasma

confirms the findings of Marble, Field, Drinker, and Smith (4) and of Morris (5). In spite of this rapid turnover, little radioactivity appeared in the other plasma lipoproteins. The maximum radioactivity present in the $S_r > 10$ (Fraction II) lipoproteins was of the order of magnitude of that found in this fraction after simple *in vitro* incubation of dog chylomicra with dog plasma. If this fraction is involved in chylomicron transport as an acceptor of chylomicron lipid, its turnover rate must be very rapid, since lipid failed to accumulate in this fraction during clearing. In the major lipoprotein fractions (III and IV) only very small amounts of radioactivity were found during the clearing process. In Experiment 1, Fraction III contained not more than 0.2 per cent and Fraction IV 0.8 per cent of the radioactivity present in the plasma at time zero. Since the total quantity of triglyceride present in Fractions III and IV was low, the measurement of specific activities was impossible. However, the radioactivity found in these fractions during clearing was similar to that found after *in vitro* incubation of chylomicra with normal dog plasma. The radioactivity found, both *in vitro* and *in vivo*, may have been due to exchange of phospholipids between the chylomicra and the other lipoproteins, since, in Fraction IV, the bulk of the radioactivity was shown to be associated with the phospholipids. The late rise in radioactivity and phospholipid concentrations in Fractions III and IV must represent new lipoprotein formation, perhaps in response to the appreciable blood loss that occurred. The data presented do not support the concept that other lipoproteins are involved in the removal of chylomicra from the plasma. It should be pointed out that the present experiments do not bear upon their possible participation in the *formation* of the chylomicron (18).

Of great interest was the rise in specific activity of the unesterified fatty acids during clearing. This finding suggests that there is an intimate relationship between removal of chylomicra and the hydrolysis of their constituent triglycerides. It is possible that this rise resulted from intravascular lipolysis, but we were unable to observe evidence of continued lipolysis *in vitro* in these experiments. Robinson, Jeffries, and French have reported that *in vitro* lipolysis occurs in the plasma

of one strain of rats after fat feeding, and have calculated that the extent of this lipolysis is sufficient to account for the rate of disappearance of lipemia in these animals (19). We have been unable to reproduce this phenomenon in two other strains of rats.

It is evident from the high specific activity of the plasma UFA's during chylomicron removal that at least part of the UFA's newly released by hydrolysis is returned rapidly to the blood stream. They are apparently transported in the usual manner, namely, complexed with serum albumin, for essentially all the plasma UFA radioactivity was found in the albumin-containing 1.21 density infranates. Although the half-life of the plasma UFA's, and thus the proportion of the cleared triglyceride which was retransported as fatty acid could not be determined during the experiments involving chylomicron clearance, the constancy of the UFA concentration and the high UFA specific activity indicate that UFA transport during the clearing interval is probably extremely active. The magnitude of the UFA turnover possible during clearing may be appreciated by considering the results in Experiment 1. Approximately 2,500 mg. of triglyceride fatty acid disappeared from the plasma in 80 minutes, an average of 30 mg. per minute. If the plasma volume is assumed to be 300 ml., the quantity of plasma UFA's present at any time was about 30 mg., since their concentration remained constant at about 0.1 mg. per ml. plasma during clearing. Thus the plasma UFA's would have to be turned over at least every minute, were all the cleared triglyceride re-transported as fatty acid in this interval. When actually determined in a separate, anesthetized, fasting dog, the plasma UFA half-life was found to be two minutes. This suggests the presence of a mechanism capable of accomplishing the rapid re-transport of much of the chylomicron triglyceride hydrolyzed during clearing. The extreme lability of plasma UFA's in response to various metabolic alterations demonstrated by Gordon and Cherkas (20) also indicates that their turnover can be very rapid under several conditions.

While our findings indicate that the lipolysis associated with the removal of chylomicra from the plasma did not occur in the circulating blood, the actual site of chylomicron removal remains to be

determined. The reticulo-endothelial cells present in the vascular wall of certain organs, or the capillary cells themselves, are suggested as possible sites of removal. Friedman, Byers, and Rosenman (21) have presented evidence that newly absorbed cholesterol is removed by the Kupffer cells of the liver in the rat. The problem of the sites of chylomicron removal is the subject of current investigation in our laboratory.

The close association observed between chylomicron clearance and lipolysis supports the hypothesis that the heparin-activated enzyme, lipoprotein lipase, is involved in the removal of exogenous lipid from the plasma. The observation that protamine sulfate inhibits the *in vitro* activity of lipoprotein lipase (22) can thus explain its retarding effect on chylomicron removal. This retardation of *in vivo* clearing by protamine indicates that lipolysis must be a rate-limiting step in this process.

SUMMARY

Chylomicra have been isolated from the thoracic duct lymph of dogs fed sodium palmitate-1- C^{14} . Their chemical composition was similar to that reported for humans, with approximately 86 per cent triglyceride by weight. After intravenous injection in recipient dogs, the chylomicra disappeared exponentially, with a half-life of 15 to 24 minutes. The triglyceride and phospholipid moieties of the chylomicra disappeared from the plasma at the same rate. Small quantities of radioactivity were found in three ultracentrifugally separated lipoprotein fractions during clearing, but the physiologic significance of this finding in relation to the removal of chylomicra from dog plasma is questioned. In fasted dogs, the specific activity of the plasma unesterified fatty acids rose progressively during clearing and reached a level exceeding 50 per cent of the specific activity of the transfused triglyceride fatty acids. This close association between the removal of chylomicra and the release of unesterified fatty acids into the plasma supports previous work suggesting that the lipolytic enzyme, lipoprotein lipase, is involved in the removal of chylomicra from the plasma. Plasma unesterified fatty acids had a half-life of about two minutes in dogs under the conditions of these experiments. This is interpreted to indicate that an appreciable portion of the cleared chylomicron

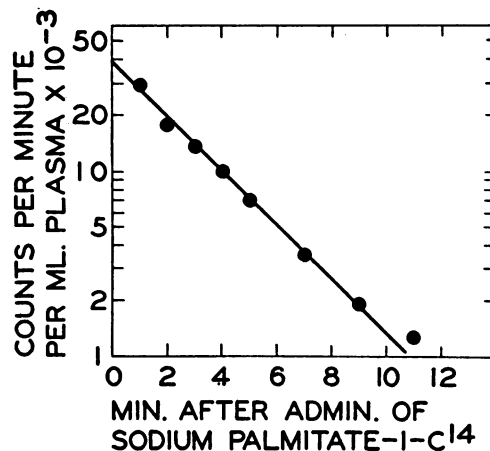


FIG. 5. DISAPPEARANCE OF RADIOACTIVITY FROM DOG PLASMA FOLLOWING INTRAVENOUS ADMINISTRATION OF SODIUM PALMITATE-1- C^{14} (23.5×10^6 COUNTS PER MINUTE) COMPLEXED WITH LIPOPROTEIN-FREE DOG SERUM PROTEINS

triglyceride may be re-transported through the plasma as unesterified fatty acid during the clearing process.

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