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THE REMOVAL OF ^{14}C-LABELLED CHYLOMICRON FAT FROM THE CIRCULATION IN RATS

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During the absorption of a fatty meal the triglycerides of the long chain fatty acids are carried to the blood stream by the lymph in the form of emulsified droplets, the chylomicrons. It has been shown experimentally, by injection of homologous chyle into the circulation, that this chylomicron fat leaves the blood stream rapidly (Marble, Field, Drinker & Smith, 1934; Little, Harrison & Blalock, 1942; Morris, 1954; Havel & Fredrickson, 1956). The mechanisms concerned in this process are poorly understood and, in particular, it is uncertain whether the chylomicrons are removed as intact particles, or are first broken down in the blood stream with the release of the fat in a more readily diffusible form.

When the plasma of animals given an intravenous injection of heparin is incubated with added chyle *in vitro*, the chylomicron fat is rapidly hydrolysed (Robinson, Jeffries & French, 1954). There is evidence that during fat absorption a lipase, analogous to this heparin-clearing factor, is present in the blood and this has led to the suggestion that chylomicron fat is hydrolysed before being removed from the circulation (Jeffries, 1954; Robinson *et al.* 1954). It has not been established, however, that hydrolysis is an essential step in the removal of the chylomicron fat from the blood. The appearance of chylomicrons in increased numbers in the lymph from the leg, head and neck, and liver following the injection of chyle intravenously has suggested, on the other hand, that at least a proportion of the chylomicrons can pass unchanged through vessel walls at some sites in the body (Courtice & Morris, 1955; Morris & Courtice, 1956).

One of the difficulties in assessing these possibilities is that there is little quantitative information available about the rate of removal of chylomicron triglyceride from the blood. Several investigations of disappearance rates have been carried out using artificial emulsions (Meng & Freeman, 1948;

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Lerner, Chaikoff, Entenman & Dauben, 1949; Rutenberg, Seligman & Fine, 1949; Goldman, Chaikoff, Reinhardt, Entenman & Dauben, 1950*a*; Meng, 1952; Becker, Rall & Grossman, 1955) or heterologous lipoproteins (Bragdon & Havel, 1954). It seems doubtful, however, whether these results can be related directly to the behaviour of the chylomicrons since, as shown by Waddell, Geyer, Saslaw & Stare (1953), the behaviour of emulsified particles in the circulation is largely determined by the nature of the surface active agent used to stabilize them.

In the experiments described in this paper, the removal of chylomicron fat from the circulation has been studied by injecting ^{14}C-labelled homologous chyle intravenously into rats and following its rate of disappearance from the blood. The effects of intravenous injections of heparin and of inhibitors of the heparin clearing reaction on the rate of disappearance have also been studied and an attempt has been made to measure the exchange of chylomicron fat between the plasma and the lymph.

METHODS

Albino rats of both sexes with body weights between 180 and 190 g were used for all the experiments. The animals were starved for 18 hr before use.

Collection of ^{14}C-labelled chyle. Thoracic duct fistulae were established in rats by the technique of Bollman, Cain & Grindlay (1948). At operation a gastrostomy tube was inserted so that fluids and radioactive fat could be administered readily. On the day following the operation 30 μc of glyceryl tri(palmitate-1-^{14}C), specific activity 1–2 mc/m-mole, dissolved in 1 ml. of olive oil, were given through the gastrostomy tube. The lymph from the thoracic duct was collected into 3·8% sodium citrate solution throughout the subsequent period of fat absorption. The lymph, containing one volume of citrate in ten, was stored at 0° C before use.

Intravenous injections and blood sampling. The rats were anaesthetized with ether and injections were made into an exposed femoral vein. The radioactive chyle was injected in volumes varying from 0·2 to 2·0 ml. over periods from 10 to 60 sec. Heparin (Pularin, Evans Medical Supplies) was injected in doses of 200 u./kg body wt.; protamine in doses of 5 mg/kg body wt. and Triton WR-1339 (Rohm and Haas Co.) in doses of 250 mg/kg body wt. Blood samples for radioactive assay were taken from the cut end of the tail. An infra-red lamp was used to keep the animals warm throughout the experiments and to ensure a free blood flow from the tail. Less than 5% of the estimated blood volume of the animals was removed by the sampling procedure during the course of the experiments.

Radioactive assay. Samples of whole blood or lymph (0·03 ml.) were pipetted on to aluminium planchets, and thoroughly mixed with 0·1 ml. of a solution of Teepol (Shell Chemicals; Liq. Sulphestolis, B.P.C. 1949) diluted 1:1000 in water. The mixture was spread over the surface of the planchet and a disk of lens tissue was placed on top. The sample was evaporated to dryness and counted to an error of 5% with a thin mica end-window GM tube. The results from replicate samples of blood or lymph prepared in this way agreed within 1%. The activities in blood samples are expressed as percentages of the initial activities in the circulation. These latter values were obtained by extrapolation to zero time of the curves calculated from the experimental data.

The radioactivity in lipid extracts was measured by mounting 0·1 ml. portions directly on the planchets and covering them with lens paper, as described by Entenman, Lerner, Chaikoff & Dauben (1949).

Chemical analyses. Total esterified fatty acids in the samples of lymph were estimated by the colorimetric method of Sterne & Shapiro (1953). The partition of the lymph into phospholipid

and triglyceride fractions was carried out by the method of Goldman, Chaikoff, Reinhardt, Entenman & Dauben (1950*b*).

Centrifugation. Chylomicrons were separated qualitatively from samples of lymph by centrifugation at 20,000 *g* for 30 min in a Spinco preparative ultracentrifuge. The creamy surface layer was pipetted off the clear lymph beneath.

Electrophoresis. The protein and lipid components of the lymph were separated by zone electrophoresis in barbiturate buffer (pH 8·6) at an ionic strength of 0·06 M. Duplicate paper strips were stained with bromphenol blue or with Sudan black. The distribution of protein and fat in these strips was determined by eluting 1 cm segments and measuring the optical densities of the eluates in a Beckman spectrophotometer at a wave-length of 590 mμ (Swahn, 1953). When the optical densities of the Sudan black eluates had been measured the samples were assayed for their ^{14}C content.

RESULTS

Analysis of the ^{14}C-labelled chyle

To determine the form in which the label was present in the radioactive chyle, 0·1 ml. samples were extracted into 10 ml. of boiling 3:1 alcohol-ether mixture, and the mixture reduced to a small volume by careful evaporation. The residue was extracted with petroleum ether and samples of the petroleum-ether extract assayed for radioactivity. More than 95% of the total activity present in the samples was recovered in this lipid extract. Estimation of radioactivity in the phospolipid fraction of this material showed that 2–3% of the ^{14}C activity was present as phospholipid fatty acid. This is in agreement with more detailed investigations by other workers (Bloom, Chaikoff, Reinhardt, Entenman & Dauben, 1950; Borgström, 1952) showing that when labelled triglyceride is fed to rats it appears in the chyle almost entirely as triglyceride.

To determine the distribution of ^{14}C activity between visible chylomicrons and other components, the chyle samples were separated into turbid and clear fractions by centrifugation and the ^{14}C content of the two fractions analysed. The radioactivity per unit volume in the turbid fraction was 30–40 times that in the clear fraction. Additional evidence that the radioactivity was present mainly in the chylomicrons was obtained by electrophoretic analysis. The ^{14}C activity followed the distribution of the Sudan staining and was localized near the point at which the samples were applied (cf. Swahn, 1953).

The disappearance of ^{14}C-labelled chylomicron fat from the circulation

To establish the rate at which chylomicron fat was removed from the circulation, serial blood samples were taken from ten rats which had each been injected intravenously with 1 ml. of labelled chyle containing 47·5 mg of total fatty acids. From the results obtained, the mean circulating half-life of the fat was calculated as 11·3 min $\pm$ S.E. 0·62.

The mean disappearance curve for two rats, studied throughout a period of 3 hr following the injection of 2 ml. of labelled chyle, is shown in Fig. 1. The

percentages of the initial ^{14}C activity in the blood plotted against time on semilogarithmic co-ordinates give a composite curve which can be represented by two exponential components and hence by the general expression

$$P_t = P_{1_0} \ln^{-k_1 t} + P_{2_0} \ln^{-k_2 t},$$

where P_t is the percentage of the initial radioactivity present in the plasma at any time t, P_0 is the percentage present at the time t_0, and k is a proportionality factor representing the fractional decrease in percentage P with time. In the experiment represented in Fig. 1 the percentage remaining at the time t is given by the equation

$$P_t = 94{\cdot}2 \ln^{-0{\cdot}0774t} + 5{\cdot}8 \ln^{-0{\cdot}0104t},$$

in which the constants (k_1 and k_2) represent half-lives of 8·9 and 66·5 min respectively.

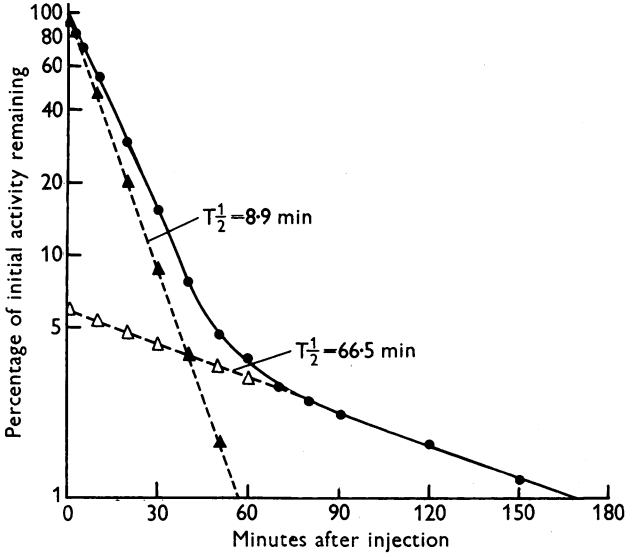

Fig. 1. The disappearance of ^{14}C-labelled chylomicron fat from the circulation. Semilogarithmic plot of the mean results for two rats injected intravenously with 2 ml. of labelled chyle. $T\frac{1}{2}$ = half-life.

The composite nature of the disappearance curves suggested that there might be components in the injected chyle which were being removed at different rates. As already stated, some activity remained in the lymph of the lower layer after removing the chylomicrons by centrifugation. An experiment was therefore carried out to determine the effect of this component in the disappearance curves. A sample of labelled chyle was spun at 15,000 *g* for 30 min and the chylomicron layer separated from the clear lower layer. The chylomicron fraction was resuspended in 0·9% saline and thoroughly emulsified. The turbid and clear fractions were then injected separately into rats

and the rates of disappearance from the blood measured. Fig. 2 shows the mean disappearance curves for two pairs of rats injected with these fractions of chyle. It can be seen that in both cases the disappearance curves could be resolved into two exponential components as in the case of whole chyle. However, with the clear fraction the slower component of the curves accounted for the removal of a much higher proportion of the total activity than was observed either with the chylomicron fraction or with whole chyle.

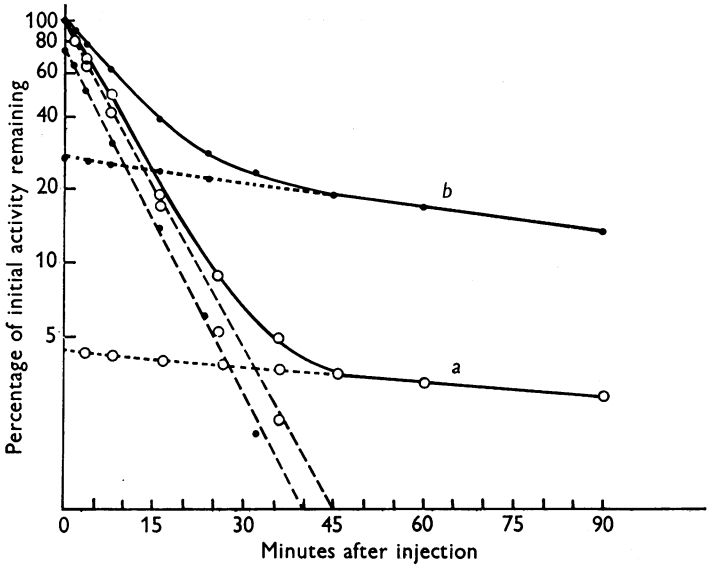

Fig. 2. The disappearance of ^{14}C-labelled fat from the circulation following the injection of the chylomicron layer and the clear lower layer of chyle. Semilogarithmic plot. Curve (*a*): mean results from two rats injected with 0·5 ml. of the chylomicron fraction containing 14·3 mg of total fatty acids. The percentage remaining at $t = 95{\cdot}6 \ln^{-0{\cdot}1042t} + 4{\cdot}4 \ln^{-0{\cdot}0048t}$. Curve (*b*): mean results from two rats injected with 1·5 ml. of the clear lower layer containing 5·0 mg of total fatty acid. The percentage remaining at $t = 73{\cdot}0 \ln^{-0{\cdot}1072t} + 27{\cdot}0 \ln^{-0{\cdot}0076t}$.

In the experiments with whole chyle the dominant exponential accounted for the removal of approximately 90% of the injected fat in the first 30 min. In the subsequent experiments, when the disappearance of labelled fat was followed for 30 min only, the composite nature of the curve was disregarded and the logarithmic transformation of the experimental points was represented as a single regression. Comparisons of removal rates were made between the means of the calculated regression coefficients for each group by use of the *t* test.

The effect of the amount of fat injected on the removal rate

The exponential nature of the disappearance curve showed that the rate at which chylomicron fat was removed was related to its concentration in the

blood. When varying amounts of chyle were injected the individual disappearance curves each represented an exponential function, but it was found that the slopes of the curves were not parallel. This effect of initial fat concentration on the removal rate is shown in Fig. 3, where it can be seen that the removal rate varied inversely with the amount injected. For example, in this group of experiments, when 9·7 mg of total fatty acid was injected the plasma half-life of the chylomicron fat was 6·2 min compared to 14·1 min when 77·6 mg of total fatty acid was injected.

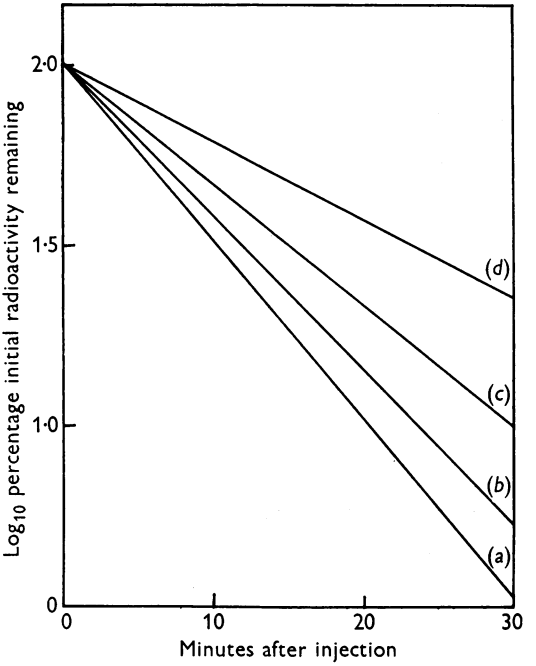

Fig. 3. The change in slope of the disappearance curve of ^{14}C-labelled chylomicron fat which occurs when increasing amounts of fat are injected intravenously. The amount of fatty acids injected and the regression equations are given:

(*a*) 9·7 mg total fatty acids, $Y = 1{\cdot}37 - 0{\cdot}0484(x - 13{\cdot}1)$;
(*b*) 19·4 mg total fatty acids, $Y = 1{\cdot}45 - 0{\cdot}0408(x - 13{\cdot}1)$;
(*c*) 38·3 mg total fatty acids, $Y = 1{\cdot}57 - 0{\cdot}0324(x - 13{\cdot}1)$;
(*d*) 77·6 mg total fatty acids, $Y = 1{\cdot}72 - 0{\cdot}0213(x - 13{\cdot}1)$;

where $Y = \log_{10}$ percentage of initial activity remaining and $x =$ minutes after injection.

When a second amount of chyle was injected before the first had been removed completely from the blood there was a change in the slope of the disappearance curve. Fig. 4 illustrates an experiment in which 1 ml. of unlabelled chyle was injected 10 min after the injection of 1 ml. of the labelled chyle. The second injection changed the half-life of the labelled fat from 12 to 21 min.

The effect of injection of heparin, protamine or Triton WR-1339 on the removal of chylomicron fat from the blood stream

In the experiments described in this section the test and control groups of rats were injected with the same amounts of chylomicron fat.

The effect of heparin on the removal rate was examined by giving rats heparin intravenously 1 min before the injection of the labelled chyle. This injection of heparin caused a significant acceleration in the rate of removal (Table 1).

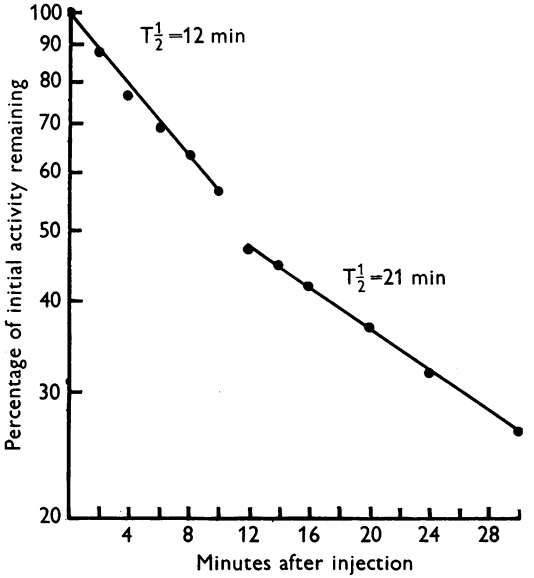

Fig. 4. The effect of a second injection of chyle on the rate of removal of ^{14}C-labelled chylomicron fat from the circulation. An initial intravenous injection of 57·5 mg of ^{14}C-labelled chylomicron fat was given and was followed after 10 min by an injection of 48·6 mg of unlabelled fat. Semilogarithmic plot. $T\frac{1}{2}$ = half-life.

Experiments were carried out in groups of rats given injections of protamine sulphate and Triton WR-1339. It is known that these substances inhibit the heparin clearing reaction *in vitro* (Robinson *et al.* 1954). Protamine sulphate and Triton WR-1339, given 1 and 5 min respectively before the injection of labelled chyle, caused a significant reduction in the rate at which the fat left the blood stream (Table 1). Nevertheless, the chylomicron fat was still removed from the circulation. In view of this, tests were carried out to determine whether the doses used in these experiments were adequate to cause complete inhibition of the clearing reaction. Injections of the same doses of protamine or Triton WR-1339 were given to rats 15 min before the injection of heparin. The animals were bled after a further 5 min and their plasma was tested by following the change in optical density which occurred when fatty

chyle was added (French, Robinson & Florey, 1953). The plasma from these rats showed no clearing activity in response to heparin injection, whereas the plasma from rats given heparin alone produced complete clearing of the added chyle in 30 min.

TABLE 1. The effect of intravenous injection of heparin, protamine or Triton WR-1339 on the removal of chylomicron fat from the circulation

Treatment	Rat no.	Regression coefficient	Circulating half-life (min)	Significance of difference from normal
Normal	1	−0·0268	11·2	—
	2	−0·0305	9·9	—
	3	−0·0323	9·3	—
Heparin, 200 u./kg	1	−0·0553	5·4	<0·001
	2	−0·0553	5·4	
	3	−0·0831	3·6	
Protamine sulphate, 5 mg/kg	1	−0·0106	28·4	<0·001
	2	−0·0098	30·4	
	3	−0·0115	26·2	
Triton WR-1339, 250 mg/kg	1	−0·0102	29·5	<0·001
	2	−0·0158	19·1	
	3	−0·0103	29·4	

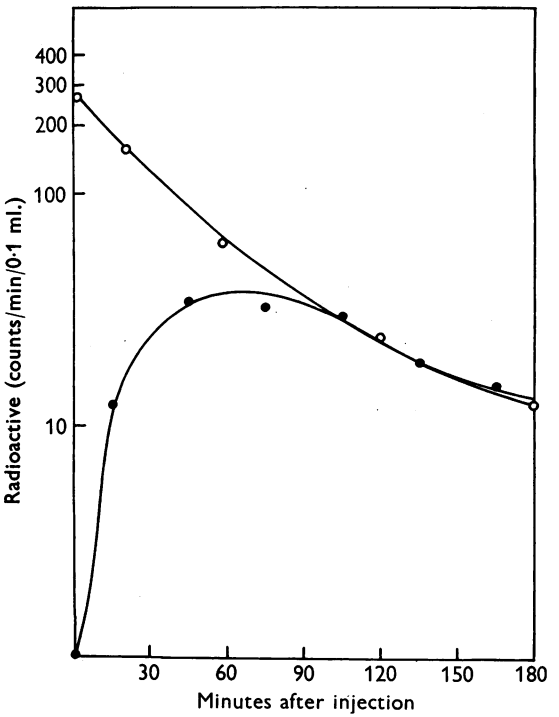

Fig. 5. The appearance of ^{14}C activity in the thoracic duct lymph of a rat injected intravenously with 2 ml. of ^{14}C-labelled fatty chyle. Semilogarithmic plot. ○, Blood; ●, Thoracic duct lymph.

The recirculation of fat in the lymph following intravenous injection of chyle

Labelled chyle was injected intravenously into six rats with established thoracic duct fistulae, and the ^{14}C activity in samples of blood and lymph was estimated during the 3 hr period following the injection (Fig. 5). Radioactivity was present in the lymph collected in the first half hour and the concentration rose to a maximum after about 2 hr. In some experiments the ^{14}C activity in the lymph reached higher levels than in blood samples collected at the same time. The mean total amount of ^{14}C activity recovered in the lymph was 0·9% ± S.E. 0·1 of the amount injected.

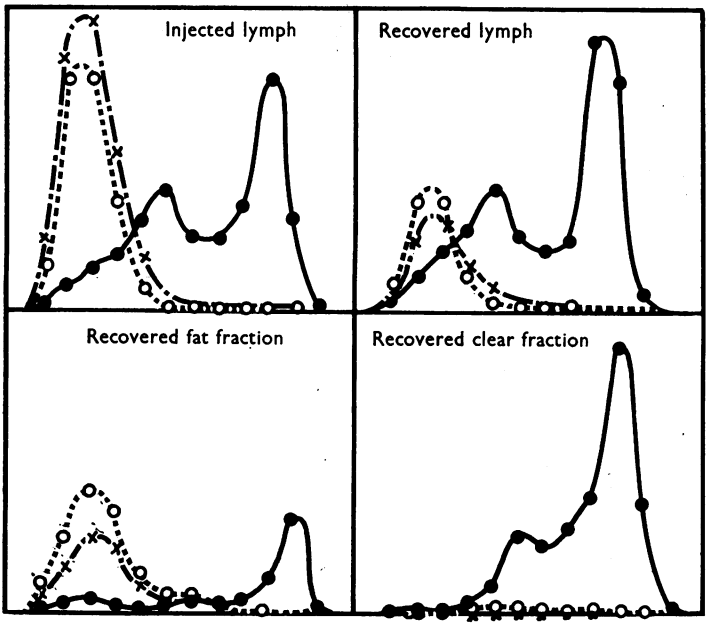

Fig. 6. The electrophoretic elution diagrams of ^{14}C-labelled lymph injected intravenously into and recovered from a rat with a thoracic duct fistula. The recovered lymph was separated into fatty and clear fractions by high-speed centrifugation. ●——●, protein distribution; O---O, ^{14}C distribution; ×-·-×, fat distribution: the direction of migration of the samples is from left to right.

Similar experiments were carried out in two additional groups of six rats to determine whether injections of protamine or heparin produced any change in the amount of fat returned by the lymph. In both these groups of experiments measurable amounts of radioactivity appeared in the lymph during the first half hour, but again the maximum activity occurred after about 2 hr. In the heparin-treated group, a mean of 1·1% ± S.E. 0·1 of the injected fat was recovered; in the protamine group the mean recovery was 0·6% ± S.E. 0·1.

In order to determine the form in which the radioactive material was present

in the recovered lymph, samples were analysed by the methods already described for the chyle. About 90% of the total activity in the lymph was present in the rectified alcohol-ether extract, and of this 3–4% was present as phospholipid fatty acid. On centrifugation, the radioactivity per unit volume in the chylomicron fraction was 5–6 times that in the clear fraction. Electrophoresis of the recovered lymph showed that the radioactivity was concentrated in stainable fat at the point of application of the sample (Fig. 6).

In the samples of lymph obtained from rats receiving heparin or protamine before the chyle injection this distribution was essentially the same. It appeared, therefore, that in each group of animals a proportion of the ^{14}C label was present in chylomicron fat in the recovered lymph.

DISCUSSION

The rate of removal of chylomicron fat from the blood in rats is of the same order as that reported for dogs by Havel & Fredrickson (1956). It appears to be higher than has been reported in experiments in which various artificial emulsions have been used, but direct comparisons are difficult to make as the amounts of fat injected in other experiments have been much larger than those used here. During fat absorption the chylomicron concentration, even at the height of lipaemia, is less than the initial concentration which followed the injection of 1 ml. of chyle. The rate of removal observed following the injection of 0·2 ml. is probably a closer measure therefore of the circulating half-life of the chylomicrons under physiological conditions.

The disappearance curves represent the removal of chylomicron fat from the blood but do not discriminate between removal as chylomicrons or removal as the products of hydrolysis. In general, the curves appear to favour the first of these possibilities and are similar to those obtained after the injection of a variety of particulate substances into the blood stream.

A composite disappearance curve has been described following the intravenous injection of labelled colloids, and it has been shown that this is due to variation in particle size with the smaller particles accounting for the slowly removed 'tail' (Dobson, Gofman, Jones, Kelly & Walker, 1949). In the case of injected chyle, it appears that variation in particle size could account at least in part for the composite nature of the disappearance curves. The label was probably distributed over a range of particles of varying size which were separated roughly into a large particle and a small particle fraction by the centrifugation procedure. From the experiment in which these fractions were injected separately, it appeared that the fat in the larger visible particles disappeared the most rapidly from the blood stream and that the slower phase of the curves was partly accounted for by the presence of colloidal fat particles which were too small to scatter light. This is an incomplete explanation, however. Some of the activity in the later parts of the curves would be

accounted for by labelled fat which was returned to the blood in the lymph or remobilized from the tissues, and by metabolic intermediaries formed by the oxidation of the chylomicron fat.

The observation that the disappearance of chylomicron fat from the blood followed an exponential function but that the rate was inversely proportional to the amount injected is similar to results obtained with other colloidal particles (Frimmer, 1953; Biozzi, Benaceraf & Halpern, 1953; Neveu, Biozzi, Benaceraf, Stiffel & Halpern, 1956). With such colloids it has been shown that a second intravenous dose will change the slope of the disappearance curves (Biozzi *et al.* 1953). In the case of these materials which are known to be taken up by reticulo-endothelial cells, this form of disappearance has been attributed to a saturation effect whereby the capacity of the cells to take up material from the blood is progressively reduced. It is possible that during the disappearance of chylomicron fat there is a saturation of the removal mechanism which could explain the change in slope of the disappearance curves when increasing amounts of fat are injected.

Direct evidence that some of the chylomicron fat left the blood stream as particles was obtained in the experiments in which labelled fat was identified in the recovered lymph as chylomicrons. The recovery of about 1% of the injected chylomicron fat in the lymph agrees closely with the results obtained in cats by Morris & Courtice (1956). Some of the activity in the recovered lymph was not present as fat. Freely diffusible oxidation products, such as glucose and bicarbonate, probably accounted for the final approximation of the levels of radioactivity which occurred in blood and lymph.

If the chylomicron fat does leave the blood stream as particles it is unknown whether the particles pass through the capillary membrane or are in some way engulfed by the endothelial cells. It has been suggested that chylomicrons are taken up by the reticulo-endothelial cells. Following the injection of artificial emulsions fat accumulates in these cells, particularly in the Kupffer cells of the liver (Gilbert & Jomier, 1908; Jaffé & Berman, 1928). There is also evidence that exogenous cholesterol, reaching the blood stream in chylomicrons, is concentrated in reticulo-endothelial cells (Friedman, Byers & Rosenman, 1954). It has never been shown conclusively that chylomicron triglyceride is taken up in this way (Murray & Freeman, 1951). As already pointed out, the form of the disappearance curves for chylomicron fat is similar to that observed for materials taken up by reticulo-endothelial cells. There is no reason to suppose, however, that this type of disappearance is a characteristic solely of reticulo-endothelial activity.

The possibility that the fat leaves the blood stream in a form which is more readily diffusible than the chylomicrons has also to be considered. If hydrolysis of the fat occurs before removal, there is evidence from studies *in vitro* that a series of complexes could be formed by association of fatty acids and

residual unhydrolysed fat with the plasma proteins (cf. Robinson & French, 1957). In view of the rapid disappearance of chylomicron fat from the blood, however, it is unlikely that removal can depend on the formation and transfer of lipoprotein complexes through aqueous diffusion channels. It is known that lipoproteins exchange between plasma and lymph in a similar way to the plasma proteins which have circulating half-lives of several hours (Wasserman & Mayerson, 1951; Courtice & Morris, 1955). The demonstration by Havel & Fredrickson (1956) that unesterified fatty acids leave the circulation at a rapid rate suggests that these substances can be removed independently of protein.

In the present experiments it has been shown that heparin, which is known to induce intravascular hydrolysis, causes an acceleration in the rate of removal of chylomicron fat from the circulation and that inhibitors of the heparin clearing reaction, protamine and Triton WR-1339, slow down the rate of removal. This is consistent with the view that a hydrolytic reaction, analogous to the heparin clearing reaction, is concerned in the removal of chylomicron fat. On the other hand, no evidence was obtained that intravascular hydrolysis was *essential* for removal. In the presence of concentrations of protamine or Triton WR-1339, sufficient to inhibit hydrolysis induced by heparin, fat still left the circulation. Further, the slower rate of removal of chylomicron fat in the presence of protamine or Triton WR-1339, while possibly due to inhibition of intravascular hydrolysis, could also be due to changes in the surface properties of the chylomicrons which modify in some other way their removal from the blood.

SUMMARY

1. The removal of chylomicron fat from the circulation in rats has been studied by observing the rate of disappearance of ^{14}C-labelled tripalmitin injected intravenously in the form of homologous chyle.

2. The disappearance of the injected fat followed an exponential function but at a rate which was inversely proportional to the amount of fat injected.

3. The chyle contained labelled components which were removed from the circulation at different rates. Fat in the form of visible chylomicrons was removed more rapidly than the fat present in invisible particles.

4. The rate of removal of chylomicron fat was increased by the intravenous injection of heparin, and slowed, but not stopped, by injection of protamine sulphate or Triton WR-1339.

5. Approximately 1% of the labelled fat was recovered in thoracic duct lymph in the 3 hr following intravenous injection. Some of the labelled fat recovered in the lymph was present in chylomicron form.

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