

Uptake and metabolism of triglycerides by the rat liver*

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SUMMARY

Triglycerides were prepared with C^{14} in the glycerol moiety and tritium in the fatty acid part as well as with over-all tritium labeling. Tracer amounts of these triglycerides were incorporated into blood plasma lipoproteins and the solutions were injected intravenously into rats. Large amounts of the radioactive material were recovered in the liver 15 minutes after the injection and the triglycerides were located predominantly in the liver mitochondria and microsomes, with relatively little in the fat droplets. Five minutes after the injection of doubly labeled triglycerides, the triglycerides recovered in the liver had practically the same H^3 - to C^{14} -ratio as the injected material, indicating that intravenous lipolysis is not necessary for the uptake of neutral fat by the liver. After longer intervals the H^3 - to C^{14} -ratio in the triglycerides increased rapidly, indicating intrahepatic lipolysis with the loss of free glycerol and re-esterification of the fatty acid with endogenous unlabeled glycerol precursors. Part of the tritiated fatty acids was also recovered in the phospholipid fraction, while very little of the C^{14} -glycerol was used in phospholipid synthesis.

In a previous publication (1) it was shown that free fatty acids (FFR), injected intravenously in tracer amounts, are rapidly removed by the liver and can be recovered to a large extent as triglycerides, located at the sites of their synthesis, i.e., the microsomes and the mitochondria (2). It was shown by perfusion experiments (1) that the triglycerides are gradually removed from these particles and transferred to the "fat droplet fraction" of the liver or released into the blood. After some time, part of the triglyceride fatty acids was also found in the liver phospholipids. Similar results were also reported by Laurell (3), who showed that blood plasma triglycerides found after injection of radioactive palmitic acid are derived from liver triglycerides.

To complete our knowledge of the transport of the fatty acids, it was therefore of importance to follow the fate of plasma triglycerides. These compounds are introduced into the blood from the lymphatic ducts with the chylomicrons and from the liver as lipoproteins. Other sources are possible but have not yet been satisfactorily demonstrated.

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The metabolism of chylomicrons obtained from the lymph of animals fed with labeled fatty acid has been studied in several laboratories (4, 5, 6). The recent literature on this topic, as well as on work in which artificial fat emulsions were used, has been reviewed by Fredrickson and Gordon (7).

Much less information is available on the fate of the endogenous triglycerides which circulate in the blood as a constituent of lipoproteins. They have been labeled by the injection of precursors, like acetic acid or higher fatty acid (3, 8 to 11) and their turnover has been measured and found to exceed that of the plasma phospholipids.

In the present work labeled triglycerides incorporated into plasma lipoproteins were injected intravenously and their disposition was studied. It soon became apparent that with this material, as was previously found with FFA (1) or with lymph chylomicrons (6), a high percentage of the radioactivity was recovered in the liver. The paper presented here therefore deals solely with the uptake and metabolism of the triglycerides by the liver.

The following points have been investigated: What cytoplasmic particles are the acceptors of plasma triglycerides? Is cleavage to FFA a necessary pre-

liminary reaction to their penetration into the tissue? Does cleavage occur in the organ after penetration of the triglycerides?

In order to test these points it was necessary to inject into the blood stream triglycerides in a form representative of their natural state in the plasma, i.e., as lipoproteins or chylomicrons. By the use of tritiated fatty acids of high specific activity, it was possible to incorporate very small amounts of triglycerides into the plasma lipids without any marked change in their quantity or quality. By high speed centrifugation, the labeled plasma lipids could also be separated arbitrarily into fractions with finer and coarser particles; and possible differences in behavior, due to the physical state of the compounds, could be tested. Finally, since one of the aims of this work was to determine the extent of intravascular lipolysis ("clearing") accompanying the uptake of the triglycerides, these substances were synthesized from C^{14} -glycerol and H^3 -fatty acid. With these doubly labeled compounds a change in the H^3 - to C^{14} -ratio would be expected in the case of lipolysis. Partial lipolysis and replacement of the H^3 -fatty acids with unlabeled endogenous ones would bring about a drop in this ratio. Complete cleavage, on the other hand, would cause a rise in this ratio, as free glycerol is converted to a large extent into carbohydrate metabolites and the fatty acids would be re-esterified with endogenous glycerol precursors.

MATERIAL AND METHODS

Preparation of Labeled Triglycerides. Three types of labeled triglycerides were prepared and used in this investigation with similar results. Tritiated triolein was prepared by tritiating 30 to 40 mg. triolein according to Wilzbach (12). In view of the findings of Nystrom *et al.* (13), it may be assumed that tritiation resulted in partial saturation of the double bond and that a mixture of oleate and stearate was obtained. The triolein, spread in a thin layer, was exposed to 10 c. of tritium gas for 10 days. After removing the bulk of the gas, the material was dissolved in ethanol, allowed to stand for several hours to equilibrate the exchangeable tritium, and the ethanol was then evaporated. Solution in ethanol and evaporation were repeated several times. The product was then taken up in ether and the solution washed twice with a 1 per cent aqueous solution of sodium carbonate to remove water-soluble breakdown products and fatty acids. The residue in the ether solution was chromatographed on a silicic acid column according to Borgström (14), and

the triglyceride fraction eluted by benzene was used for the experiments reported.

Doubly labeled triglyceride (glyceryl-1- C^{14} trioleate- H^3) was prepared by esterifying glycerol-1- C^{14} with the H^3 -fatty acid obtained by saponification of the tritiated triolein. Esterification was carried out by interacting the tritiated fatty acid with glycerol-1- C^{14} .¹ To 30 μ moles glycerol with 100 μ c. C^{14} the tritiated fatty acid with carrier was added to make a three- to fourfold excess over the theoretically required amount. The mixture was heated in a closed vessel to 180°C for 12 hours in the presence of small amounts of $SnCl_2$ as catalyst and concentrated sulfuric acid in a side arm to remove the water produced. This was preferred to flushing through with dry CO_2 , generally recommended for this esterification, since it was found that with the small amounts of glycerol used, the loss of glycerol by evaporation was considerable. The reaction mixture was extracted with petroleum ether and the extract was washed repeatedly with water. The petroleum ether was evaporated and the residue was dissolved in anhydrous acetone and passed through a column of MgO and Celite (1:1) to remove the unesterified oleic acid. Several μ moles of fatty acid carrier were added and the acetone solution was passed again through a MgO-Celite column. The acetone eluate was evaporated and the residue dissolved in petroleum ether. This solution was then chromatographed on a silicic acid column according to Borgström (14) to separate the triglycerides from the lower glycerides and other contaminants.

Glycerol-1- C^{14} tripalmitate-9,10- H^3 was prepared as described for the previous preparation, using palmitic acid-9,10- H^3 .²

Labeled Lipoproteins. Incorporation of the glycerides into plasma lipoproteins was achieved by adding 0.05 ml. of an alcoholic solution of the labeled triglyceride (containing not more than 0.2 μ moles) to 13 ml. fresh rat serum and incubating for 30 to 60 minutes at 37°C with shaking. The lipoproteins were then fractionated by chilling the serum in ice and centrifuging at 100,000 $\times g$ for 60 minutes. The bottom of the plastic centrifuge tube was punctured and the lowest 3 ml. collected (fraction 1). Two more fractions of 3 ml. were obtained from the middle of the tube (fractions 2 and 3) and finally the upper layer (fraction 4) was collected. The last fraction was usually turbid and contained the serum chylomicrons.

¹ Obtained from the Radiochemical Centre, Amersham, Buckinghamshire, England.

² See footnote 1.

For technical reasons the method of centrifugal fractionation used was an arbitrary one, and the fractions obtained did not coincide with the generally accepted division of lipoprotein fractions. The aim of this fractionation was solely to establish whether any gross difference appears with lipoproteins of different degrees of dispersion.

Paper electrophoresis of these serum fractions showed that the bulk of the radioactivity migrated with the β lipoprotein and the rest stuck to the point of origin. No detectable quantities migrated with the serum albumin, indicating that no significant lipolysis occurred during the incubation of triglycerides in the sera.

The experimental procedure consisted of injecting 0.5 to 1.0 ml. of the serum fractions into the tail vein of male rats weighing 100 to 120 g., fed on an ordinary stock diet. At the indicated time intervals the animals were bled under light ether anesthesia from the abdominal aorta. The liver was chilled immediately in crushed ice and homogenized in KCl-Tris buffer and fractionated by centrifugation into debris (comprising intact cells, nuclei, and trapped cytoplasmic particles), mitochondria, supernatant (containing the microsomes), and floating fat, as described in a previous publication (2). Extraction and fractionation of the lipids were also performed as described in that paper (2).

Assay of radioactivity was performed with a Packard Tri-Carb liquid scintillation counter in which simultaneous counts of H^3 and C^{14} were determined. The degree of quenching was examined in the triglyceride and in phospholipid fractions by introducing internal standards. With the former this was negligible, and with the latter it was minimized by using low concentrations.

RESULTS

When emulsions of triglycerides were injected intravenously, a considerable portion of the injected material was found in the liver 15 minutes after the injection. This was the case when the highly dispersed lipoprotein fraction (fraction 1) was used, as well as when the coarser serum chylomicron fraction (fraction 4) was injected (Table 1). Similar results were obtained by Bragdon and Gordon (6) when lymph chylomicrons were injected. It has been observed that this deposition in the liver is the rule whenever well-dispersed fatty compounds are injected. When coarser, artificial fat emulsions are used, considerable portions can be found in the lungs. This has been found to be

an indicator for the adequacy of emulsification in intravenous lipid applications. Furthermore, the results presented show that the triglycerides penetrating the liver cell are deposited primarily in the metabolically active cytoplasmic particles of the liver, as was previously shown for the glycerides formed from injected FFA (1). The fat droplets of the liver contained

TABLE 1. DISTRIBUTION OF TRITIATED TRIOLEIN IN RAT LIVER 15 MINUTES AFTER INJECTION

Serum Fraction Injected*	Per Cent of Injected Dose			
	Whole Liver	Mitochondria	Supernatant†	Fat‡
1	24	5	7.5	0.4
2	27	5	10	0.3
3	26	7	10	0.3
4	60	10	15	4.0

* For preparation of fractions see Materials and Methods in text.

† Supernatant at $20,000 \times g$ for 30 minutes.

‡ Combined fatty layers separated after each centrifugation.

relatively little radioactivity when examined 15 minutes after the injection. As with the triglycerides derived from FFA, here too the distribution of the triglycerides is gradually shifted; and larger amounts can be found in the free fat as time progresses. There was no gross difference in the behavior of the various serum fractions. Fraction 4, which contained most of the serum chylomicrons, was taken up by the liver at a more rapid rate and larger portions were found in the droplets with these lipid emulsions.

The figures given in Table 1 for distribution in the various particles are not absolute and indicate only the relative distribution in the various fractions. They do not add up to the total amount found in the liver, since considerable amounts are present in the debris, obtained after low speed centrifugation, i.e., in the fraction containing intact cells, cell nuclei, and also trapped cytoplasmic particles. It should further be remarked that the glycerides found in the supernatant, obtained after centrifugation at $20,000 \times g$, are mainly bound to the microsome fraction and can be precipitated by prolonged centrifugation in the Spinco centrifuge at $100,000 \times g$. This was done only in a

few experiments and the results are presented as "supernatant."

In Table 2 the results of experiments are given in which doubly labeled triglycerides were injected intravenously. There was no significant difference between triglycerides labeled with palmitic acid-9,10-H³ or with "tritiated oleic acid."

It is evident that the triglycerides, isolated from the liver 5 minutes after the injection, have H³- to C¹⁴-ratios, which are practically identical with those of the substance injected. This ratio increases rapidly

ratio at least five times that of the original triglyceride. This finding indicates that the phospholipids are formed predominantly from the fatty acids derived from triglycerides, and only very little from the glycerol.

DISCUSSION

The results presented show that triglycerides injected intravenously in the form of serum chylomicrons, or incorporated into lipoproteins, are rapidly removed and that a considerable portion is found in the liver. The localization of these triglycerides in the liver was found to be similar to that found after injection of FFA (1) and indicates that the liver contains at least two compartments for triglycerides: one with a rapid turnover and metabolism situated on the active particles, and a second, slower one in the fat droplets. This compartmentalization may explain the finding of Waddell *et al.* (15) with artificial fat emulsions, and of French and Morris (5) with chylomicron fatty acids, that the clearance of plasma triglyceride decreases rapidly with increasing doses. It is likely that with small doses all the injected material is cleared by the particles. However, when these sites become saturated by increasing loads, the clearance becomes dependent upon the removal of the glycerides from the particles, which is a much slower process.

The experiments with the doubly labeled triglycerides showed that for the penetration of triglycerides into the liver cells, intravascular lipolysis is not re-

TABLE 2. H³/C¹⁴ IN RAT LIVER NEUTRAL GLYCERIDES FOLLOWING INJECTION OF LABELED SERUM*

Minutes After Injection	Glyceryl-1-C ¹⁴ tripalmitate-9,10-H ³	Glyceryl-1-C ¹⁴ trioleate-H ³
5	1.18 ± 0.02†	1.05 ± 0.02
15		1.25 ± 0.04
30	1.43 ± 0.12	1.56 ± 0.25
60	1.92 ± 0.08	2.07 ± 0.06
120		2.58 ± 0.08
720		6.0

* Relative to the ratio of the injected material, which is taken as 1.0.

† Standard deviation of the mean, obtained in each case from 3 to 4 experiments, except for the figure for 720 minutes, which is single.

and 15 minutes after the injection is already significantly higher than the original one. These results indicate that there was no marked intravascular lipolysis and that the triglycerides were deposited in the liver intact. In this organ, however, a rapid dissociation between the glycerol moiety and the fatty acids takes place, as indicated by the rapid rise in the H³- to C¹⁴-ratio in the glycerides. With this technique no accumulation of lower glycerides was observed, i.e., no temporary drop in the ratio was found.

Concomitant with the increase in the isotope ratio and the decrease in total triglyceride activity there is a gradual rise in the radioactivity of the phospholipid fraction (Table 3). The phospholipids formed from the injected triglycerides have a H³- to C¹⁴-

TABLE 3. LIVER PHOSPHOLIPIDS AND NEUTRAL GLYCERIDES FOLLOWING INJECTION OF MIXED LABELED TRIGLYCERIDES

Minutes After Injection	Per Cent of Injected Fatty Acids		Per Cent of Injected Glycerol	
	Phospholipids	Glycerides	Phospholipids	Glycerides
5	3.0 ± 0.6*	37 ± 5.8	1.85 ± 0.5	35 ± 6
15		44 ± 4.2		35.5 ± 3.4
30	16 ± 2.8	21 ± 3.6	2.75 ± 0.7	13.5 ± 2.5
60	23 ± 3.6	23 ± 2.4	3.85 ± 0.5	11 ± 1.4
120	15.5 ± 2.4	16 ± 0.8	1.45 ± 0.3	6.2 ± 0.3
720	10	5.5	0.80	0.80

* Standard deviation of the mean, obtained in each case from 3 to 4 experiments, except for the longest period (720 minutes), where only single values are available.

quired since the ratio between labeled glycerol-C¹⁴ to fatty acid-H³ in the liver glycerides, shortly after the injection, was the same as that in the injected triglyceride.

It may be claimed that the lipoproteins employed in this study have been altered by the incubation process. The procedure used was, however, extremely mild. After incubation of serum with minute amounts of triglycerides it was demonstrated by paper electrophoresis and high speed centrifugation that the triglyceride was strongly attached to the proteins. Lacking any absolute criteria for establishing the nativity of the lipoproteins, one still has to keep in mind that the material obtained in this way may differ somewhat from the natural one. This reservation does not invalidate our main conclusion, that well-dispersed triglycerides penetrate the liver cells without prior cleavage. Bragdon and Gordon (6) arrived at a similar conclusion on the basis of differences in the tissue distribution of chylomicron triglycerides and FFA. The capability of the liver to ingest intact triglycerides is in agreement with the findings of Courtice and Morris (16), that chylomicrons are transferred freely from the plasma to hepatic lymph.

The present demonstration of a rapid uptake of serum triglycerides by the liver, together with the previous results (1) which showed release of liver triglycerides into the blood, point to a circular process involving the liver and blood serum. Liver and serum triglycerides may therefore be looked upon as constituents of a common pool which equilibrates rapidly. The results of Laurell (3) lend themselves to similar conclusions.

It is known that material is constantly being added to this triglyceride pool by the mobilization of FFA from adipose tissue and their esterification to triglycerides in the liver (1, 3). Several processes are known for the disposal of this pool: Combustion of triglyceride fatty acid to CO₂ has been demonstrated by Bragdon (17) and Morris (18); assimilation of triglycerides by adipose tissue *in vitro* has been demonstrated in our laboratory (19); and Bragdon and Gordon (6) have presented evidence that chylomicron triglycerides, especially after carbohydrate feeding, are taken up by the depots without intravascular hydrolysis. Triglyceride fatty acids are transferred to phospholipids as shown in the present paper, in agreement with Harper *et al.* (9) and with our previous report (1). The comparatively high degree of labeling of the phospholipids makes it unlikely that the triglycerides have to be split into FFA for subsequent reincorporation into phospholipids. In such a pathway the

fatty acids would have been diluted to very low activity by the plasma FFA, which were previously shown to be practically devoid of radioactivity, and to mix very rapidly with the liver particles' fatty acid pool (1). The results seem to indicate some mechanism of transesterification, not involving FFA formation.

The glycerol moiety of the liver triglycerides undergoes a much more rapid turnover than the fatty acid part, as is indicated by the rapid increase in the H³- to C¹⁴-ratio in the glyceride fraction. This increase indicates complete cleavage of the triglycerides into free glycerol and re-esterification of the fatty acid moiety with endogenous glycerol precursors. The radioactive glycerol is probably incorporated into the metabolism of carbohydrates. If di- and monoglycerides had accumulated during triglyceride cleavage, a decrease in the H³- to C¹⁴-ratio would have resulted. The fact that at no time could such a drop be detected indicates that the triglycerides which undergo degradation are cleaved predominantly by three successive stages without accumulation of intermediates. This conclusion is corroborated by the finding that the incorporation of the glycerol moiety of the triglycerides into phospholipids is much lower than that of the fatty acid moiety, whereas it would have been expected, according to the scheme of phospholipid synthesis of Kennedy (20), that if diglycerides were formed, they would serve as efficient precursors of phospholipids.

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