THE METABOLISM OF CHYLOMICRON
CHOLESTERYL ESTER IN RAT LIVER
A Combined Radioautographic-Electron
Microscopic and Biochemical Study

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ABSTRACT
Chylomicrons containing labeled cholesterol, mainly (70%) present as cholesteryl ester, were
injected intravenously into intact rats, and samples of liver were obtained 27-210 min later. Most
(58-75%) of the injected label was recovered in the liver after 27-75 min. Hepatic
uptake occurred without hydrolysis of the labeled cholesteryl ester. In separate experiments,
in vitro perfusion of livers of similarly treated rats for 30-35 min washed out only 3-9% of
the labeled sterol. Samples of liver and small intestine were prepared for electron microscopy
with Aquon as the dehydrating agent. Good retention (70% or more) of labeled cholesterol
and satisfactory preservation of ultrastructure were obtained. After 30 min, the radio-
autographic reaction was localized mainly over the region of the cell boundary of the
parenchymal liver cells, with fewer grains being present over intracellular organelles. At
later time intervals, when considerable hydrolysis of the labeled cholesteryl ester had oc-
curred, the radioautographic reaction was more evenly distributed. Phagocytosed labeled
lipid was seen in Kupffer cells after the larger lipid load; phagocytosis by parenchymal cells
was not seen. In other experiments, cholesteryl ester hydrolase activity was found in all
subcellular fractions, the microsome and plasma membrane fractions showing the highest
activity per mg protein. The mechanism of cholesteryl ester transport into the liver cell may
involve: (1) hydrolysis at the cell surface; or (2) slow entry of intact molecules followed by
intracellular hydrolysis of the ester bond.

INTRODUCTION
It is now well established that the liver is the
major organ involved in the initial metabolism
of chylomicron cholesterol. Previous studies with
rats and with dogs demonstrated that most of
the labeled cholesterol is removed from the
circulation by the liver after the intravenous
injection of chylomicrons containing labeled
cholesterol (mainly present as cholesteryl ester)
(1-4). Studies with chylomicrons containing
doubly-labeled cholesteryl esters established that
hepatic uptake takes place without hydrolysis
of the chylomicron cholesteryl esters (4). In the
Liver, the newly absorbed cholesteryl esters undergo a slow hydrolysis. In addition, during and after hydrolysis there occurs a slow progressive loss of labeled cholesterol from the liver, and an accumulation of labeled sterol in peripheral tissues (4).

The experiments reported here were designed to provide information about the anatomic localization at the ultrastructural level of newly absorbed chylomicron cholesterol within rat liver. An electron microscopic-radioautographic study was conducted which examined the ultrastructural distribution of labeled cholesterol at several time intervals after the intravenous injection of chylomicrons containing labeled cholesterol (mainly present as labeled cholesteryl ester) into intact rats. The time intervals were selected so as to provide information representing times before, during, and after cholesteryl ester hydrolysis had occurred. In addition, related biochemical studies were conducted to examine whether newly absorbed intact cholesteryl esters could be washed out of the liver during in vitro perfusion, and whether hepatic cell plasma membranes contained the enzymatic capacity to catalyze the hydrolysis of cholesteryl esters.

**EXPERIMENTAL PROCEDURE**

**Liver Perfusion Study**

Chylomicrons were obtained by feeding cholesterol-7α-3H in oil (olive oil: corn oil = 3:1) to a rat with a cannula implanted in the thoracic duct. The chylomicrons were isolated and washed by flotation as described elsewhere (5). The chylomicron suspension contained 22 mg of total lipid and approximately 840,000 dpm of 3H per ml; 50% of the 3H was present as cholesteryl ester and 50% as free cholesterol.

Male Sprague-Dawley rats weighing approximately 150 g each were fasted overnight, and then each given 0.9-1.0 ml of the labeled chylomicron suspension as an intravenous injection into the tail vein. At given time intervals (see Table III), the rats were anesthetized with diethyl ether, the abdomen and chest were opened, and cannulae were inserted into the portal and hepatic veins. Liver perfusion (6) was begun immediately and was continued for 30-35 min. All of the perfusate was collected after a single passage through the liver, i.e. no perfusate was recirculated. The perfusate was Krebs-Ringer bicarbonate buffer through which 95% O2-5% CO2 was bubbled just prior to passage through the liver. In most instances, the perfusion pressure was 10-15 cm of water, and the flow rate was 8-10 ml per minute. The liver and perfusate were maintained at 37°C during the perfusion.

After the perfusion, the entire liver was weighed and then homogenized with 20 volumes of CHCl3-CH3OH, 2:1, so as to obtain a total lipid extract of the organ. Portions of the liver lipid extract were assayed for 3H; other portions were chromatographed on columns of alumina, grade II (7), so as to separate ester from free cholesterol, and the separated fractions were then radioassayed. Portions of each perfusate sample (the perfusates were collected in serial 5-min samples) were also extracted, chromatographed, and radioassayed. A total of eight rats were injected with labeled chylomicrons, and their livers were perfused. In addition, five rats were injected with the chylomicrons, and their livers were removed and extracted without perfusion.

**Radioautographic Study: Biochemical Aspects**

Cholesterol-7α-3H (New England Nuclear Corp., Boston), 25 mc (specific radioactivity 25 mc/m mole) was dissolved in 400 mg of a monolein-oelie acid mixture (2:3, by weight). The entire sample was fed by stomach tube to a lymph fistula rat, and was washed in with 0.75 ml of freshly collected rat bile. Lymph was collected at room temperature for 20 hr. The lymph contained 37% of the administered 3H. Chylomicrons were isolated and washed by flotation through isotonic saline at 20°C. The chylomicron suspension was analyzed for its total lipid content by the method of Stern and Shapiro (8), and was also radioassayed for its content of 3H. The final preparation contained 25 mg of total lipid and 678 X 106 dpm of 3H per ml. Cholesteryl ester comprised 69.9% of the chylomicron 3H.

27 hr after the cholesterol-3H feeding, the lymph fistula rat was anesthetized with ether and the abdomen was opened. A loop of proximal jejunum was removed, washed with saline, and fixed in 3% glutaraldehyde in 0.1 M phosphate buffer pH 7.4.

The chylomicron injection study was carried out on the morning immediately following the preparation of the labeled chylomicron suspension. Male Sprague-Dawley rats, weighing 140-150 g each, were fasted overnight and injected with either 1 or 2 ml of the labeled chylomicrons into a tail vein. After a desired time interval had elapsed (see Table IV), the rats were anesthetized with ether, the aorta was cut and blood was collected, and the liver was partially washed out by perfusion in situ through the portal vein with 13 ml of warm isotonic saline. Pieces of liver were taken for electron microscopy; other weighed pieces were extracted with CHCl3-CH3OH, 2:1, after homogenization in a Dounce homogenizer. Plasma samples were extracted with 20 volumes of CHCl3-CH3OH, 2:1. The liver and plasma lipid extracts were assayed for 3H, and were chroma-
Fate of Radioactivity in Rat Liver during Specimen Preparation for Electron Microscopy, after Injection of Chylomicrons Containing Labeled Cholesterol, with Use of Epon

TABLE I

<table>
<thead>
<tr>
<th>Time after injection (min):</th>
<th>43</th>
<th>60</th>
<th>107</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample analyzed</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Radioactivity recovered*</td>
<td>%</td>
<td>%</td>
<td>%</td>
</tr>
<tr>
<td>Glutaraldehyde</td>
<td>1.4</td>
<td>4.8</td>
<td>2.8</td>
</tr>
<tr>
<td>Buffer</td>
<td>0.3</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>Ethanol 70%</td>
<td>6.8</td>
<td>7.3</td>
<td>7.2</td>
</tr>
<tr>
<td>Ethanol 95%</td>
<td>21.4</td>
<td>23.2</td>
<td>20.4</td>
</tr>
<tr>
<td>Epon</td>
<td>19.2</td>
<td>29.3</td>
<td>22.7</td>
</tr>
<tr>
<td>Epon mixture</td>
<td>1.8</td>
<td>1.1</td>
<td>1.1</td>
</tr>
<tr>
<td>Tissue</td>
<td>32.1</td>
<td>29.0</td>
<td>23.0</td>
</tr>
<tr>
<td>Total recovery</td>
<td>83.0</td>
<td>95.2</td>
<td>82.2</td>
</tr>
</tbody>
</table>

* Aliquots of liver obtained at various time intervals after injection of chylomicrons containing cholesterol-3H (62% esterified) were homogenized in chloroform-methanol 2:1 (v/v), and the radioactivity recovered per unit weight was taken as 100%. Other aliquots were fixed in glutaraldehyde, washed in buffer, postfixed in osmium tetroxide, and dehydrated as shown above. Negligible amounts of radioactivity were recovered in osmium tetroxide from livers prefixed in glutaraldehyde. The per cent of cholesterol-3H present in the liver as cholesteryl ester was 43, 27, and 20% for the three livers (43-, 60-, and 107-min rats, respectively).

tographed on alumina (7), in order to separate free and esterified cholesterol and hence determine the per cent of cholesterol-3H present as esterified sterol in each sample.

Radioautographic Study: Preparation of Tissue for Electron Microscopy

Fixation of tissue was carried out either in two changes of acetate-veronal buffer containing 2% osmium tetroxide for 2 hr, or in paraformaldehyde-glutaraldehyde fixative (9) followed by postfixation with osmium tetroxide as above. All tissues were dehydrated in Aquon at 4°C with the following schedule: 15 min in 20%, 10 min in 50%, 30 min in 70%, 80%, and 90% each, and 180 min in 100%. Infiltration was carried out at 4°C overnight in a complete Aquon mixture consisting of Aquon 2.5 ml,
For all cell fractions, hydrolysis was much greater (per flask) to 2-ml incubation mixtures containing one or another liver cell fraction. All liver cell fractions aqueous solution of Tween 40 (1 mg of Tween added in solution in 50 µl) was assessed by the addition of the substrate cholesteryl-7α3H oleate (New England Nuclear Corp.). The substrate (2 µg of cholesteryl-3H oleate) was added in 25 µl of acetone via a Hamilton syringe. At the end of the incubations, the mixtures were extracted with 20 volumes of CHCl3-CH3OH, 2:1, containing 0.5 mg each of free cholesterol and cholesteryl oleate as carrier. The lipid extract was separated into free and ester cholesterol fractions (7), and each fraction was assayed for 3H so as to determine the percent of hydrolysis. For the no-enzyme control, 0.6% of the 3H was found in the free cholesterol fraction, and this amount was subtracted from the amount found in the other samples.

In addition to preserving the lipid radioactivity in the tissue, this method of tissue preparation also provides satisfactory preservation of ultrastructure and good localization of the label. These characteristics are illustrated in Fig. 1, which represents a portion of the lymph fistula rat’s small intestine obtained 27 hr after feeding cholesterol-3H. Fairly numerous grains were found over the microvilli, which have a high cholesterol content (16, 17). Only occasional grains were seen over the plastic infiltrating the interstitial spaces and the gut lumen.

Preparation of Subcellular Fractions

For each experiment, the livers of four to five rats were pooled and homogenized in 0.25 M sucrose. The plasma membrane fraction was obtained from the whole homogenate with the use of a combined flotation and sedimentation method described before (18). The plasma membrane preparation so obtained is only slightly contaminated with mitochondria (4% as assessed by cytochrome c oxidase content) and is contaminated 10-20% with microsomes (using glucose-6-phosphatase as a micromolar marker) (18). Mitochondria, microsomes, and supernatant fractions were obtained from a portion of the whole homogenate by differential centrifugation. Mitochondria were first sedimented at 10,000 g av for 15 min, and following resuspension they were sedimented at 6,600 g av for 10 min. Microsomes were sedimented at 100,000 g av for 60 min. The post-microsomal supernatant either was used as a single fraction or was divided into the upper layer containing floating fat and a clear “de-fatted” lower layer.

Cholesteryl Ester Hydrolysis In Vitro

In a preliminary experiment, the hydrolysis of cholesteryl-7α3H oleate (New England Nuclear Corp.) was assessed by the addition of the substrate in solution in 50 µl of acetone, or dispersed in an aqueous solution of Tween 40 (1 mg of Tween added per flask) to 2-ml incubation mixtures containing one or another liver cell fraction. All liver cell fractions actively hydrolyzed the labeled cholesteryl oleate. For all cell fractions, hydrolysis was much greater with the substrate added in acetone than dispersed in Tween.

A more quantitative study was then conducted in which 1-ml incubation mixtures containing small amounts of a liver cell fraction in Tris-HCl buffer pH 7.3 were incubated with substrate at 37°C for 15-60 min. The substrate (2 µg of cholesteryl-3H oleate) was added in 25 µl of acetone via a Hamilton syringe. At the end of the incubations, the mixtures were extracted with 20 volumes of CHCl3-CH3OH, 2:1, containing 0.5 mg each of free cholesterol and cholesteryl oleate as carrier. The lipid extract was separated into free and ester cholesterol fractions (7), and each fraction was assayed for 3H so as to determine the percent of hydrolysis. For the no-enzyme control, 0.6% of the 3H was found in the free cholesterol fraction, and this amount was subtracted from the amount found in the other samples.

Incubations were also conducted in a similar manner but with 14C-labeled tripalmitin as substrate, in order to assess lipolytic activity. The extent of hydrolysis of the labeled tripalmitin was determined by separating FFA (free fatty acids) from neutral lipids by solvent partition as described by Borgström (19).

Other Procedures

Radioassay was carried out in a Packard liquid scintillation counter with 0.5% diphenyloxazole in toluene as scintillation solvent. Protein was measured by the biuret method (20) or by the method of Lowry et al. (21).

RESULTS

Liver Perfusion Study

During the 1st hr after the injection of chylomicrons containing labeled cholesteryl ester, a large proportion of the labeled cholesterol is found in the liver in the form of intact, unhydrolyzed cholesteryl ester (1, 4). A study was conducted to determine whether this intact labeled cholesteryl ester could be washed out of the liver by perfusing the liver with buffer under relatively physiological conditions. The results of this study are shown in Table III. Only 3-9% of the total cholesterol-3H in the liver, and only 3-14% of the cholesteryl-3H ester in the liver, was washed out during perfusions of 30-35 min duration.

Electron Microscopic–Radioautographic Study

As indicated in Table IV, six rats were injected with either 1 or 2 ml of labeled chylomicrons,

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FIGURE 1  Segment of proximal jejunum, obtained 27 hr after feeding of cholesterol-$^3$H in oil. The tissues were dehydrated and embedded in Aquon. There is satisfactory preservation of ultrastructure, and the distal ends of microvillar filaments show marked electron opacity. The radioautographic reaction is seen over intracellular organelles and over microvilli. Only occasional grains are found over the plastic. $\times$ 8,900. Scale line (all figures) = 1 $\mu$. 

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TABLE III
Recovery of Cholesterol-3H in Liver and in Perfusates

<table>
<thead>
<tr>
<th>Time after chylomicron injection</th>
<th>Cholesterol-3H recovered (dpm X 10^6)</th>
<th>Liver Cholesterol-3H washed out by perfusion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Start perfusion</td>
<td>End perfusion</td>
<td>Total Liver</td>
</tr>
<tr>
<td>min</td>
<td>min</td>
<td>Liver</td>
</tr>
<tr>
<td>A. Rats whose livers were not perfused:*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>18</td>
<td>20</td>
<td>200</td>
</tr>
<tr>
<td>35</td>
<td>512</td>
<td>48</td>
</tr>
<tr>
<td>78</td>
<td>496</td>
<td>22</td>
</tr>
<tr>
<td>B. Rats whose livers were perfused:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>27</td>
<td>62</td>
<td>498</td>
</tr>
<tr>
<td>47</td>
<td>77</td>
<td>517</td>
</tr>
<tr>
<td>73</td>
<td>106</td>
<td>557</td>
</tr>
</tbody>
</table>

* The livers were removed and extracted at the times indicated.
† One rat.
§ Two rats for each time with good agreement between the duplicate samples (mean values tabulated).
¶ Three rats for each time with good agreement between individual samples (mean values tabulated). One rat liver of each group of three was perfused with dilute human whole plasma (plasma-saline = 1:1) instead of Krebs-Ringer-bicarbonate buffer.

and the livers were removed after three time intervals. For the rats injected with the smaller lipid load, 75% of the injected cholesterol-3H was recovered in the liver after 30 min, with the same ratio of labeled free to esterified cholesterol as was present in the injected chylomicrons. Slow hydrolysis of the labeled cholesteryl ester then occurred within the liver, together with a decrease in the recovery of labeled cholesterol in the liver (presumably by transport of labeled sterol out of the liver to peripheral tissues [1, 4]). A similar pattern was seen with the rats injected with the larger load of lipid, except that the uptake of chylomicron cholesterol by the liver occurred more slowly, as did hydrolysis within the liver and removal of cholesterol-3H from the liver.

The results of the radioautographic study are illustrated in Figs. 2 through 12. As discussed in the Methods section, the method used for tissue preparation gave a satisfactory preservation both of ultrastructure and of lipid radioactivity within the tissue. At the earliest time interval studied (27 min), chylomicrons were encountered in the space of Disse only very rarely. The parenchymal liver cells did not differ appreciably in appearance from noninjected controls, except for an increase in intracellular lipid droplets which were most prominent in the vicinity of the sinusoids (Figs. 2, 5, and 6). In many cells, the Golgi apparatus was quite prominent and contained numerous granules presumed to be lipoproteins (Figs. 5 and 11).

In the livers of rats injected with the larger dose of chylomicrons, phagocytosed chylomicrons were seen in Kupffer cells both at 27 and at 67 min after injection (Figs. 7 and 8). This finding was more rarely encountered after injection of the smaller dose. No phagocytosis of chylomicrons by parenchymal cells was seen at any time interval, irrespective of the dose injected.

At the early time interval (27 or 30 min) after injection of the labeled chylomicrons, the radioautographic reaction was concentrated in the region of the sinusoidal cell boundary (Figs. 2–4, 6). Because of the size and shape of the developed grains, it was not possible to decide whether the grains were associated with the cell membrane proper, with the cytoplasmic matrix of the microvilli, or with material localized between the

TABLE IV
Hepatic Recovery of Cholesterol-3H after Chylomicron Injection, in the Animals Used for the Radioautographic Study

<table>
<thead>
<tr>
<th>Time after chylomicron injection</th>
<th>Labeled Cholesterol-3H (dpm X 10^6)</th>
<th>Recovery of injected 3H</th>
</tr>
</thead>
<tbody>
<tr>
<td>Start perfusion</td>
<td>End perfusion</td>
<td>Total Liver</td>
</tr>
<tr>
<td>min</td>
<td>ml</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>27</td>
<td>2</td>
</tr>
<tr>
<td>2</td>
<td>39</td>
<td>1</td>
</tr>
<tr>
<td>3</td>
<td>67</td>
<td>2</td>
</tr>
<tr>
<td>4</td>
<td>75</td>
<td>1</td>
</tr>
<tr>
<td>5</td>
<td>210</td>
<td>2</td>
</tr>
<tr>
<td>6 (12 hr)</td>
<td>1</td>
<td>32</td>
</tr>
</tbody>
</table>

* The chylomicron suspension contained 25 mg lipid and 678 X 10^6 dpm per ml, with 70% of the 3H present as cholesteryl ester.
† Assuming a plasma volume of 3.5% of body wt.
Figures 2–8  Sections of liver obtained 27 or 30 min after the injection of chylomicrons containing cholesterol$^3$H (70% ester) into rats. Figs. 2 and 6 are from the rat injected with the low dose (Rat No. 2), while Figs. 3–5, 7, and 8 are from the rat given the high dose (Rat No. 1).

Figure 2  Portion of liver cell showing the radioautographic reaction mainly over the sinusoidal cell boundary. Note the prominent electron-opaque, tight junctions of the bile capillaries. $\times$ 12,000.
FIGURE 3 The radioautographic grains are seen mainly over the sinusoidal region; intracellularly, the grains are over mitochondria and endoplasmic reticulum. × 9,000.
microvilli. The radioautographic reaction was hence simply designated as being concentrated in the region of the sinusoidal cell boundary. This localization was found after injection of either the low (Figs. 2 and 6) or the high dose (Figs. 3 and 4) of chylomicrons. In addition, at this time interval some radioautographic grains were present intracellularly, and were seen over lipid droplets (Figs. 2 and 6), mitochondria, and endoplasmic reticulum (Figs. 3, 5–7). The lysosomes and the Golgi apparatus were usually not labeled (Figs. 5 and 6). In the livers of the rats injected with the larger dose of chylomicrons, more grains were seen over the Kupffer cells (Figs. 7 and 8) than were seen in the liver of rats injected with the smaller dose (Fig. 6). The silver grains over the Kupffer cells were seen mainly over lysosomes (Figs. 7 and 8) which contained lipid droplets of various sizes.

At later time intervals, the anatomic distribution of label differed from that seen at the early time interval. Thus, with progressively longer time intervals, relatively more and more of the grains were seen over the cytoplasmic organelles, although label was still present also over the sinusoidal microvilli (Figs. 9 and 10). Grains were seen also over the bile canaliculi (Figs. 9–11) and over the Golgi apparatus (Fig. 11). All of the radioactivity found in the tissue at later (as well as earlier) time intervals was shown, chromatographically, to be present in free and esterified cholesterol; this finding has been well documented previously (1, 4). The change in the distribution of label with time occurred roughly parallel with the hydrolysis of the labeled cholesteryl ester within the liver (Table IV). By 210 min, the radioautographic reaction was seen mainly intracellularly, over mitochondria and endoplasmic reticulum (Fig. 12).
FIGURE 5 The intracellular localization of the radioautographic grains over mitochondria, endoplasmic reticulum, and at the boundary of lipid droplets. The lysosomes are not labeled. × 17,500.

Cholesteryl Ester Hydrolysis with Subcellular Fractions

The radioautographic study demonstrated that label was concentrated in the region of the sinusoidal cell boundary at early time intervals when most of the label was present as intact cholesteryl ester, whereas label was widely distributed intracellularly at later time intervals...
FIGURE 6 Sinusoidal region of three parenchymal cells and portions of Kupffer cells. Most of the label is seen over the sinusoidal cell boundary of the parenchymal cells, while the Kupffer cells are not labeled. Intracellular silver grains are over lipid droplets, mitochondria, and endoplasmic reticulum; the lysosomes are not labeled. × 11,000.
when most of the cholesteryl ester had been hydrolyzed. An experiment was, therefore, conducted to determine whether plasma membrane preparations could catalyze cholesteryl ester hydrolysis. The results of this study are shown in Table V. All of the subcellular fractions tested (soluble supernatant, mitochondria, microsomes, and plasma membranes) effectively catalyzed the hydrolysis of labeled cholesteryl oleate. The highest specific activity (activity per mg protein) was seen with the microsomes. The enzymatic activity of the plasma membrane preparation was only slightly less than that of the microsomes. In contrast, the plasma membranes were much less effective than the microsomes in catalyzing the hydrolysis of labeled tripalmitin. Thus, after 60-min incubations with either 1.68 mg of protein of microsomes or 2.26 mg of protein of plasma membranes, the hydrolysis of labeled tripalmitin was 17.7% with the microsomes and 6.9% with the plasma membranes.

Subcellular Recovery of Labeled Chylomicron Cholesterol

A final experiment was conducted to determine whether the label which was seen to be concentrated over the sinusoidal cell boundary at the early time interval could be recovered in the plasma membrane fraction after subcellular fractionation. Two groups of rats were injected intravenously with chylomicrons containing newly absorbed \(^3\text{H}\)-labeled cholesterol (55% as ester cholesterol). The livers of the first group were removed after about 30 min, whereas those of the second group were removed after about 65 min. The livers were rinsed in iced isotonic saline, minced, and then homogenized. Portions of each whole homogenate were extracted. Most of each homogenate was processed so as to isolate washed plasma membranes, mitochondria, and microsomes, and the soluble supernatant fractions. Each fraction was extracted and analyzed by chromatography and radioassay.

The results of this study are shown in Table VI. Some of the labeled cholesterol, and particularly much of the labeled cholesteryl ester, was recovered in the floating fat layer of the soluble supernatant. It seems likely that during the processes of homogenization and subcellular fractionation much of the labeled cholesteryl ester was dislodged from the region of the sinusoidal cell boundary where it was located in vivo, and hence recovered in the floating fat layer.

DISCUSSION

The radioautographic study reported here required the development of a method of tissue preparation which would retain radioactive cholesterol and cholesteryl ester within the tissue. The method used was effective in this regard, and also provided a satisfactory preservation of ultrastructure and good localization of label within the microscopic sections. In sections of liver and intestine prepared with this method, the tight junctions in the vicinity of the bile capillaries and the bases of the intestinal microvilli were unusually electron opaque. The results of the radioautographic study provided considerable information about the anatomic localization of newly absorbed cholesterol during the hepatic uptake and metabolism of chylomicron cholesterol. Chylomicrons containing \(^3\text{H}\)-labeled cholesterol, mainly (70%) present as cholesteryl ester, were injected intravenously into intact rats. After 30 min, most of the labeled cholesterol had been cleared from the circulation and was found in the liver. The ratio of free to esterified labeled cholesterol in the liver at this time was the same as in the injected chylomicrons, a finding consistent with the previous demonstration (4) that hepatic uptake of chylomicron cholesteryl ester occurs without ester hydrolysis. Owing to the different kinetics of the uptake and metabolism of chylomicron triglyceride and cholesteryl ester (as discussed below), it seems that only part of the cholesteryl ester reached the liver in the form of intact chylomicrons, while part of the uptake of sterol occurred after the disaggregation of the chylomicron particle. In previous studies, chylomicrons were clearly seen in the space of Disse 10 min after the injection of a large load (22, 23). In the present study, 30 min after the injection of a smaller load chylomicrons were seen only rarely. At this time interval, however, the radioautographic reaction was found to be concentrated in the region of the sinusoidal cell boundary of the hepatic parenchymal cells. This finding suggests that, at this time interval when most of the label was present as intact cholesteryl ester, the labeled cholesterol was largely present in the liver associated in some manner with the plasma membrane of the hepatic cells. In the liver, the newly absorbed labeled cholesteryl ester under-
FIGURES 7 and 8  Portions of Kupffer cells with numerous lysosomes which contain lipid material in the form of droplets of various sizes. The radioautographic reaction is seen over some of the lysosomes. Fig. 7, $\times 18,000$; Fig. 8, $\times 21,000$. 
FIGURES 9-11 Sections of liver obtained 67 min after injection of labeled chylomicrons into rat No. 3.

Figure 9 The presence of label over intracellular organelles and persistence of label over the sinusoidal cell boundary. There are only a few grains over the Kupffer cell. × 11,000.
FIGURE 10 Fewer grains are found over the sinusoidal cell boundary (compared to the earlier time interval). Most of the grains are located intracellularly and also over bile canaliculi. Note the prominent electron opacity of the region of the tight junctions. × 11,000.
went a slow and steady hydrolysis. Concomitant with this, there occurred a change in the anatomic distribution of labeled cholesterol within the liver. Thus, with progressively longer time intervals, the radioautographic reaction became relatively more widely distributed over the cytoplasmic organelles. At later time intervals, when most of the cholesterol ester had been hydrolyzed, the labeled cholesterol was found to be widely and generally distributed intracellularly. These findings suggest that the hydrolysis of chylomicron cholesterol esters and the entry of chylomicron cholesterol into the intracellular compartment may be related phenomena; the possible nature of this relationship is discussed below. The findings also suggest that chylomicron cholesterol becomes widely distributed intracellularly in the form of free cholesterol (after cholesterol ester hydrolysis) rather than in the form of cholesterol ester.

Generally similar findings were obtained with the rats injected with larger or smaller loads of chylomicron lipid. The rats injected with the larger load showed a slower hepatic uptake of chylomicron cholesterol from the circulation, and hence a slower rate of cholesterol ester hydrolysis within the liver. These findings were anticipated, since the larger load (50 mg of lipid) was in the range of injected lipid which has been reported to be associated with a decreased fractional rate of clearance of chylomicrons from the circulation (24). The only significant difference due to the injected load of lipid was seen in the Kupffer cells. In the rats injected with the larger dose, the Kupffer cells contained phagocytosed chylomicrons, and label was found present over the lysosomes, which contained lipid droplets of various size. In contrast, the Kupffer cells from the rats injected with the smaller dose contained fewer phagocytosed chylomicrons, and much less label in their lysosomes. No phagocytosis of chylomicrons by parenchymal cells was seen at any time, regardless of the dose injected. These findings are in agreement with those reported previously (22, 23), when, even after large loads of chylom-
Figure 12  Liver of rat (No. 5) 210 min after injection of labeled chylomicrons. The radioautographic reaction is seen mostly intracellularly, over mitochondria and endoplasmic reticulum. × 8,000.
TABLE V
Cholesteryl Oleate Hydrolysis by Subcellular Fractions In Vitro

<table>
<thead>
<tr>
<th>Cell fraction</th>
<th>Amount assayed (mg protein)</th>
<th>Time of incubation (min)</th>
<th>Hydrolysis (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma membranes</td>
<td>0.57</td>
<td>60</td>
<td>3.7</td>
</tr>
<tr>
<td>Plasma membranes</td>
<td>1.13</td>
<td>60</td>
<td>5.2</td>
</tr>
<tr>
<td>Plasma membranes</td>
<td>2.26</td>
<td>60</td>
<td>9.8</td>
</tr>
<tr>
<td>Plasma membranes</td>
<td>2.26</td>
<td>15</td>
<td>4.4</td>
</tr>
<tr>
<td>Microsomes</td>
<td>0.84</td>
<td>60</td>
<td>4.2</td>
</tr>
<tr>
<td>Microsomes</td>
<td>1.68</td>
<td>60</td>
<td>10.0</td>
</tr>
<tr>
<td>Microsomes</td>
<td>1.68</td>
<td>15</td>
<td>4.3</td>
</tr>
<tr>
<td>Mitochondria</td>
<td>1.66</td>
<td>60</td>
<td>3.4</td>
</tr>
<tr>
<td>Mitochondria</td>
<td>1.66</td>
<td>30</td>
<td>3.0</td>
</tr>
<tr>
<td>Soluble supernatant*</td>
<td>0.86</td>
<td>60</td>
<td>2.1</td>
</tr>
<tr>
<td>Soluble supernatant*</td>
<td>1.72</td>
<td>60</td>
<td>3.5</td>
</tr>
<tr>
<td>Soluble supernatant*</td>
<td>1.72</td>
<td>15</td>
<td>2.1</td>
</tr>
</tbody>
</table>

* “De-fatted” (see Methods).

The results of this study clarify the role of the hepatic reticuloendothelial cells (the Kupffer cells) in the hepatic uptake and metabolism of chylomicron cholesterol. Previous studies by Friedman, Byers, and their colleagues (25, 26), and by Neveu et al. (27) and Bernick and Patek (28) suggested that the hepatic reticuloendothelial system may play an important role in the normal disposition of chylomicron cholesterol. In contrast, studies by DiLuzio (29) and by Ashworth, Di-Luzio, and Riggi (30) suggested that the hepatic parenchymal cells are predominantly involved in the uptake and metabolism of chylomicron cholesterol and triglyceride by the liver. The present study demonstrates that the Kupffer cells play only a very minor role in the normal metabolism of chylomicron cholesterol in the liver. Under physiological conditions, when the load of chylomicron lipid presented to the liver is small, only a very small amount of chylomicron cholesterol seems to be taken up by the Kupffer cells.

Table VI
Recovery of Cholesterol-3H, after Chylomicron Injection, in Liver Subcellular Fractions

<table>
<thead>
<tr>
<th>Sample*</th>
<th>% dpm per µg protein</th>
<th>Cholesterol-3H, µg (total) per mg protein</th>
<th>% dpm per µg cholesterol</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>A. Group No. 1</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Whole homogenate</td>
<td>1,810</td>
<td>52</td>
<td>5.9</td>
</tr>
<tr>
<td>Plasma membranes</td>
<td>12,150</td>
<td>23</td>
<td>49.</td>
</tr>
<tr>
<td>Mitochondria</td>
<td>2,990</td>
<td>34</td>
<td>10.</td>
</tr>
<tr>
<td>Microsomes</td>
<td>5,090</td>
<td>38</td>
<td>26.</td>
</tr>
<tr>
<td>Soluble supernatant (F)†</td>
<td>5,370</td>
<td>77</td>
<td>4.8</td>
</tr>
<tr>
<td>Soluble supernatant (“Defatted”)</td>
<td>1,610</td>
<td>73</td>
<td>—</td>
</tr>
</tbody>
</table>

| **B. Group No. 2**                |                      |                                         |                          |
| Whole homogenate                  | 2,630                | 31                                      | 5.3                      | 496                      |
| Plasma membranes                  | 17,200               | 7                                       | 55.                      | 313                      |
| Mitochondria                      | 8,150                | 28                                      | 14.                      | 382                      |
| Microsomes                        | 7,480                | 25                                      | 26.                      | 288                      |
| Soluble supernatant (F)‡          | 2,410                | 70                                      | 3.9                      | 622                      |
| Soluble supernatant (“Defatted”)  | 790                  | 60                                      | —                        | —                        |

* In Group No. 1, four rats were injected with a total of 5.3 ml of labeled chylomicrons, and the livers were removed after 25-34 min. In Group No. 2, four rats were injected with 6.0 ml total, and livers removed after 62-66 min. The chylomicron suspension contained 17 mg of total lipid and 4.15 X 10⁶ dpm of cholesterol-3H (55% ester) per ml.
† Containing the floating fat layer.
§ The symbol (—) means not analyzed.
The role of the Kupffer cells probably increases with increasing fat load, since relatively more phagocytosed lipid particles, together with more labeled chylomicron cholesterol, was seen in the Kupffer cells of the rats injected with the larger load of chylomicron lipid.

Considerable information is available about the metabolism of chylomicron triglyceride in the liver. In contrast to chylomicron cholesterol, chylomicron triglyceride fatty acids are mainly cleared from the circulation by extrahepatic, peripheral tissues. The available data suggest that the liver is responsible for the direct clearance of only about one-third of injected chylomicron triglyceride (31-34). Studies with doubly-labeled triglycerides have indicated that the initial uptake of lipoprotein or chylomicron triglyceride by the liver occurs without hydrolysis of triglyceride ester bonds (32, 33, 35-38). Most of the intact chylomicron triglyceride found in the liver soon after the injection of labeled chylomicrons appears to be present in the extracellular spaces of the liver. Thus, a considerable portion of the intact (unhydrolyzed) triglyceride can be washed out of the liver by perfusing the liver with saline for short periods (37, 38). It has accordingly been suggested that intact chylomicron triglyceride taken up by the liver initially remains in the spaces of Disse, or attached to the outer surface of the plasma membrane of the cells, and that triglyceride hydrolysis occurs prior to fatty acid entry into the cells. Felsa has suggested that this hydrolysis takes place mainly in peripheral tissues (38), whereas other workers have presented considerable evidence that hydrolysis occurs to a significant extent in the liver itself (37, 39-41). The latter conclusion is consistent with the finding that chylomicrons can bind to isolated liver cells, and can then undergo triglyceride hydrolysis while so bound (42, 43). More recently, Higgins has described the effects of a variety of parameters on the binding of chylomicrons to isolated liver cells (44). It has been suggested that hydrolysis of chylomicron triglyceride in the liver is catalyzed by a lipase present in the hepatic cell plasma membrane. The occurrence of a significant lipolytic activity in plasma membrane preparations has been demonstrated (43, 45) and its properties have been partially characterized (45).

Under normal in vivo conditions, chylomicron triglyceride taken up by the liver is metabolized fairly rapidly, with a considerable amount of triglyceride hydrolysis occurring in the interval 10-30 min after chylomicron injection (24, 32, 33). The hydrolysis of newly absorbed cholesteryl ester in the liver occurs much more slowly than that of chylomicron triglyceride, since most of the chylomicron cholesteryl ester is still present in the liver as intact, unhydrolyzed molecules after 30-45 min. The form in which the intact cholesteryl ester is present in the liver at these time intervals seems to differ markedly from that of the intact chylomicron triglyceride (which had been transiently present at shorter time intervals). Thus, as reported here, only a very small fraction (of the order of 10%) of the labeled cholesteryl ester found in the liver 27-73 min after the injection of labeled chylomicrons could be washed out of the liver by prolonged liver perfusion. These results indicate that the intact cholesteryl ester was not present freely in the extracellular compartment, or loosely associated with the outer surface of the liver cell membrane. Moreover, although cholesterol label was seen to be concentrated in the region of the sinusoidal cell boundary at the early time intervals studied, the label was not found associated with any particular structures such as chylomicron-like particles. The combined perfusion and radioautographic results suggest that, after its initial uptake by the liver, the intact cholesteryl ester is largely present associated in some fairly strong manner with the plasma membrane of the hepatic cells. The details of this association remain to be defined. If this hypothesis is correct, however, it should be noted that the results presented in Table VI indicate that the association of the cholesteryl ester with the plasma membrane is not sufficiently strong to be able to withstand the effects of homogenization and subcellular fractionation.

As discussed above, a temporal relationship was seen between the hydrolysis of cholesteryl ester within the liver and the more widespread distribution of labeled cholesterol within the intracellular compartment. These findings can be explained in two possible ways. First of all, it is possible that intact cholesteryl ester molecules cannot penetrate the hepatic cell membrane and enter the intracellular compartment. This explanation would require that cholesteryl ester hydrolysis occur at the plasma membrane, with the subsequent entry of the liberated free cholesterol into the hepatic cell. In favor of this explanation is the present finding that isolated plasma membrane
preparations displayed enzymatic activity for the hydrolysis of cholesteryl esters. Against this explanation, however, is the finding that the level of cholesteryl ester hydrolase activity in the plasma membrane preparations did not appear to be sufficient to account for the observed rates of chylomicron cholesteryl ester hydrolysis in rat liver (7). Further study of the properties of the cholesteryl ester hydrolase in the plasma membrane will be required in order to obtain more insight into this question.

An alternative explanation is available for the observed temporal relationship. It is, for example, possible that intact cholesteryl ester can penetrate the hepatic cell membrane, but that the rate of penetration of these very nonpolar molecules is very slow. In this view, chylomicron cholesteryl ester would become associated with the plasma membrane, and would remain at this site for some time because of the very slow rate of entry through the plasma membrane and into the cell. On entering the intracellular compartment, the cholesteryl ester would be hydrolyzed fairly rapidly by the soluble and membrane-bound cholesteryl ester hydrolases present intracellularly. A similar possibility for the intracellular uptake of triglycerides, which envisaged the transfer of intact triglyceride molecules but not intact chylomicrons, has been considered (23).

The data available do not permit us to choose between these alternative explanations. It is, moreover, certainly possible that both explanations are partly correct. Thus, chylomicron cholesteryl ester, taken up intact by the liver and associated in some manner with the plasma membrane, may be partly hydrolyzed at the plasma membrane, and may partly penetrate the membrane without hydrolysis and then undergo hydrolysis, during its slow passage from the region of the plasma membrane to other areas within the intracellular compartment.

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REFERENCES

22. French, J. E. 1963. In Biochemical Problems of