Radioactive lipoproteins in the very low density lipoprotein (VLDL) density range were taken up by rat liver in vivo. The radioactivity became associated with an intracellular particle of $d = 1.11$ that did not correspond to lysosomes, endoplasmic reticulum, or plasma membrane as determined by marker enzyme distribution. Radioactive VLDL remnants could be released from these particles by passage through a hydraulic press, hypotonic shock, or sonication. The release of radioactivity from the particles by one of these methods became more complete with increasing time after injection. The injection of colchicine inhibited the breakdown of the VLDL triglyceride and cholesterol ester and caused an accumulation of radioactive material in the $d = 1.11$ particles. In contrast, injected chloroquine inhibited breakdown of VLDL triglyceride and cholesterol ester and caused an accumulation in lysosomes.

We have concluded VLDL remnants are metabolized in liver by an endocytosis-lysosomal digestion pathway and that the $d = 1.11$ particles are endocytic vesicles. The existence of a releasable pool of VLDL within endocytic vesicles makes it possible to examine the internalized remnant.

Endocytosis is believed to be important in the metabolism of a large number of substances such as insulin (1), human chorionic gonadotropin (2), epidermal growth factor (3), asialoglycoproteins (4), and low density lipoproteins (5). This process involves the binding of the ligand to a specific surface receptor, the internalization of the ligand-receptor complex into an endocytic vesicle, fusion of the endocytic vesicle with a primary lysosome, and hydrolysis of the ligand by lysosomal enzymes.

There are 3 types of experimental observations that indicate VLDL remnant remnants are also metabolized via endocytosis by liver. First, when doubly labeled VLDL remnants are perfused through rat livers, both labels disappear at identical rates indicating the VLDL remnant may be metabolized as a unit (6, 7). Second, chloroquine, a known inhibitor of lysosomal hydrolysis, inhibits breakdown of VLDL cholesterol ester and iodinated apoproteins by liver in vivo (8). Third, electron microscope autoradiography has shown that at later times after injection of $^{125}$I-VLDL, a number of autoradiographic grains are found over secondary lysosomes (9).

The process of endocytosis has been studied in more detail using other ligands, especially asialoglycoproteins. Tolleshaug et al. (4) have shown that labeled asialoglycoproteins accumulate in endocytic vesicles, and Dunn has reported this accumulation occurs because of the time required for endocytic vesicles to move to and fuse with lysosomes (10). Previous investigations in our laboratory have shown that there is a 10-min lag between uptake and hydrolysis of serum triglyceride (11). This lag might indicate an accumulation of labeled VLDL remnants in endocytic vesicles. Therefore, we carried out subcellular fractionation studies to test this hypothesis. If, indeed, the VLDL remnants accumulate in endocytic vesicles, this would: (a) verify that endocytosis is taking place; (b) show that the rate-limiting step in endocytosis of VLDL remnants was the same as for asialoglycoproteins; and (c) provide an explanation for the 10-min lag in hydrolysis of triglyceride.

**EXPERIMENTAL PROCEDURES**

**Materials**—Chloroquine and colchicine were obtained from Sigma. The $[3,10-^3$H]oleate (2 Ci/mmol in toluene) was from Amersham Corp. Chloroform and methanol were redistilled prior to use. Ether (anhydrous) was microanalysis grade and was obtained from Fisher.

**Animals**—Male Sprague-Dawley rats (Carworth Farms) were housed in wire bottom cages with free access to food (Purina Rat Chow) and water for 5–14 days. All animals were fasted for 48 h prior to use and weighed 180–250 g the day of the experiment. Rats were injected via a tail vein with labeled VLDL as described by Abrams and Cooper (12). The in vivo treatment with chloroquine was as described by Stein et al. (8).

Colchicine was prepared immediately before use by dissolving either 20 mg (low dosage) or 40 mg (high dosage) in 10 ml of 0.15 M NaCl. The low dosage was 0.5 mg/100 g body weight given intraperitoneally 1 h prior to injection of VLDL. The high dosage of 1 mg/100 g body weight was given 2 h prior to injection of VLDL (13).

Rats were injected with 1–2 μCi of labeled VLDL intravenously via a tail vein (12). This constituted less than 5% of the pool size of VLDL.

**Isolation of VLDL**—VLDL was isolated by a modification of the method of Redgrave et al. (14). The $d$ = 1.006-1.21 gradients were centrifuged for 22 h at 259,000 × $g$ (39,000 rpm) in a SW 41 Ti rotor (Beckman) at 4 ºC. One-ml fractions were collected from the gradient, and the upper 2 ml contained the VLDL. When human plasma was centrifuged on this gradient and total cholesterol was determined in each fraction, 3 peaks of cholesterol mass were observed. The peaks correspond to VLDL, LDL, and HDL as described by Redgrave et al. (14).

VLDL labeled in vivo by injection of $[^3$H]oleate was analyzed by thin layer chromatography. The plate was divided into 1-cm bands, scraped, and counted. The distribution of labeled lipids was 7.1 ± 0.9% (S.E.) in cholesterol ester, 91.3 ± 0.8% (S.E.) in triglyceride, and 2% or less in mono- and diglycerides, phospholipids, or fatty acids in 18 preparations analyzed. The average diameter was determined by electron microscopy and found to be $422 ± 14$ Å (S.E.) for 3 preparations.
Labeling of VLDL—Solvent was removed from 5 mCi of [9,10-3H]oleate or 50 μCi of [1,4C]oleate by evaporation under a stream of N2. The fatty acid was then incubated with 50 μl of 2% (w/v) ethanolic KOH for 10 min at 4 °C. A 1-ml aliquot of serum from a rat fasted 48 h was then added quickly. The serum was collected by bleeding a rat by aortic puncture, allowing the blood to clot 30 min at room temperature, and, after centrifuging at 1000 × g for 25 min. The labeled serum was injected into a second 24-h fasted rat and, 25 min later, the blood was removed by aortic puncture under light ether anesthesia. The serum used for puncture contained 0.4 ml of 67 mM EDTA, pH 7.4. Plasma was prepared by centrifuging at 1000 × g for 25 min. VLDL was isolated on a 1.006-1.21 gradient.

Preparation of Postnuclear Supernatants—Rats were sacrificed by decapitation at the desired time after injection, and the liver was removed quickly. The liver was rinsed and homogenized rapidly in 18-20 ml of cold 0.25 M sucrose-MOPS-1 mM EDTA, pH 7.4. All subsequent operations were carried out at 4 °C. The homogenate was centrifuged at 10,000 × g for 30 min at 4 °C. The supernatant was then injected with labeled VLDL and sacrificed 45 min later. The supernatant fractions were collected, their volumes were measured, and an aliquot treated with crystalline bovine serum albumin as standard.

Enzymatic Assays—Marker enzymes were assayed using glucose-6-phosphatase (15) and NADP-dependent glutamate dehydrogenase (21) as standards. The syringe used for puncture contained 0.15 M NaCl for 30 s followed by 150 ml of Karnovsky fixative. The liver was cut into 1-mm3 pieces, fixed for an additional 1.5 h in Karnovsky fixative to which 25 mg of CaCl2/50 ml had been added, and then washed twice with 0.15 M sodium cacodylate buffer containing 25 mg of CaCl2/50 ml. The pieces were then postfixed with 1% Oso4 (11 ml of 0.15 M Na cacodylate, 5 ml of 4% Oso4, 1 ml of 1% NaN3, and 1 ml of 0.1 M NaHCO3) for 20 min followed by three washes with triple distilled water. Dehydration involved sequential treatment with 25, 50, 75, 95, and 100% ethanol for 10 min with each solution. The pieces were treated with Spurr-resin (1:1) for 15 min after which another volume of Spurr was added, and the incubation was continued for an additional 10 min. The pieces were then made into a slab, using a Buchler grinder attached to a Brinkman MP13 multichannel pump. Gradients were formed in 38-ml (1 × 3 in) polyallomer tubes. Total volume of the gradient was 30 ml. Two different gradients were used, 18-38% sucrose (w/v) or 6-46% sucrose (w/v). All gradients were chilled to 4 °C prior to use.

Two or four ml of postnuclear supernatant fraction was layered onto a 30-ml gradient with a Pasteur pipette, and the sample was centrifuged in a SW 27 rotor for either 3 h (18-38% gradients) or 3.5 h (6-46% gradients) at 116,000 × g for 25 min. The labeled serum was injected into a second 48-h fasted rat and, 25 min later, the serum was collected by bleeding a rat under light ether anesthesia. The syringe used for puncture contained 0.15 M NaCl for 30 s followed by 150 ml of Karnovsky fixative. The liver was cut into 1-mm3 pieces, fixed for an additional 1.5 h in Karnovsky fixative to which 25 mg of CaCl2/50 ml had been added, and then washed twice with 0.15 M sodium cacodylate buffer containing 25 mg of CaCl2/50 ml. The pieces were then postfixed with 1% Oso4 (11 ml of 0.15 M Na cacodylate, 5 ml of 4% Oso4, 1 ml of 1% NaN3, and 1 ml of 0.1 M NaHCO3) for 20 min followed by three washes with triple distilled water. Dehydration involved sequential treatment with 25, 50, 75, 95, and 100% ethanol for 10 min with each solution. The pieces were treated with Spurr-resin (1:1) for 15 min after which another volume of Spurr was added, and the incubation was continued for an additional 10 min. The pieces were then made into a slab, using a Buchler grinder attached to a Brinkman MP13 multichannel pump. Gradients were formed in 38-ml (1 × 3 in) polyallomer tubes. Total volume of the gradient was 30 ml. Two different gradients were used, 18-38% sucrose (w/v) or 6-46% sucrose (w/v). All gradients were chilled to 4 °C prior to use.

Two or four ml of postnuclear supernatant fraction was layered onto a 30-ml gradient with a Pasteur pipette, and the sample was centrifuged in a SW 27 rotor for either 3 h (18-38% gradients) or 3.5 h (6-46% gradients) at 116,000 × g for 4 °C to pellet nuclei and unbroken cells. The postnuclear supernatant fraction was collected using a Pasteur pipette.

RESULTS

Subcellular Fractionation—When labeled VLDL is injected in vivo, radioactivity becomes associated with the cell particulate fraction. The subcellular location of this material was determined by homogenizing liver at various times after injection of labeled VLDL and preparing a postnuclear supernatant fraction. Four ml of this postnuclear fraction was layered onto 30 ml of a 6-46% sucrose gradient and centrifuged for 3.5 h at 116,000 × g at 4 °C. Fig. 1 shows the typical distribution obtained with these conditions and a similar one. Fig. 2 shows the distribution of radioactivity at different times after injection. There was a large peak of radioactivity in the middle of the gradient, at all times after injection, that did not correspond to any marker enzyme. At early times after injection, there was radioactivity at the top of the gradient which probably arose from bound VLDL since it decreased with time after injection. By 45 min after injection, a good deal of radioactivity was found in the lower part of the gradient and presumably represented incorporation of VLDL lipids into the cell structures that migrate to this part of the gradient, especially endoplasmic reticulum. Comparable results were obtained with a shallower 18-38% gradient (Fig. 3). It is not clear whether the small shifts in density of the middle peak represent time-dependent or experimental variation.

It is clear that, although the 5'-nucleotidase activity did not correspond to the peak of radioactivity in the middle peak, there was considerable overlap between the middle peak of radioactivity and 5'-nucleotidase activity in both gradients.

Identification of the Major Peak on the Sucrose Gradient—It seemed likely that the particle in the middle of the
were prepared for electron microscope autoradiography. The presence of attached ligands. To differentiate whether the membrane with altered density properties resulting from the croscope autoradiography. VLDL was labeled with spond. The peak could represent a subfraction of plasma membrane (extracellular), we employed electron microscopy to show that radioactivity was not associated with the plasma membrane containing bound VLDL remnants. The peak of VLDL radioactivity was associated with endocytic vesicles in the sucrose gradients. Asialo-orosomucoid was labeled with $^{125}$I by the method of Marshall et al. (26) and injected into rats. The rats were sacrificed at various times after injection, the liver was homogenized, and postnuclear supernatant fractions were prepared. These postnuclear fractions were then centrifuged on 18–38% sucrose gradients as before, and 1-ml fractions were collected from the gradients and counted. These results are shown in Fig. 4 for a rat sacrificed 3 min after injection, and virtually identical results were obtained with rats sacrificed at 1, 5, and 7 min. Most of the radioactivity was in a single peak near the position of the peak seen when VLDL was injected. Thus, it seems likely that the peak of VLDL radioactivity was associated with endocytic vesicles.

It is possible that the peak represents a special fraction of plasma membrane containing bound VLDL remnants. The radioactivity was clearly not associated with the total plasma membrane fraction since: (a) a large portion of the plasma membranes, as estimated from 5'-nucleotidase activity, was in the nuclear fraction which had a low specific radioactivity (data not shown); and (b) the pattern of 5'-nucleotidase activity and radioactivity on the sucrose gradient did not correspond. The peak could represent a subfraction of plasma membrane with altered density properties resulting from the presence of attached ligands. To differentiate whether the VLDL was in endocytic vesicles (intracellular) or still on the plasma membrane (extracellular), we employed electron microscope autoradiography. VLDL was labeled with $^{125}$I and injected into rats that were sacrificed 10 min later, and sections were prepared for electron microscope autoradiography. The distribution of grains in the extracellular spaces, bound to the cell membrane, and inside the hepatocytes was determined. It was found that 54% of grains were inside of hepatocytes. Since a large fraction of VLDL is intracellular, it would not have given rise to a special fraction of plasma membrane.

**Release of VLDL from Endocytic Vesicles**—Experiments were carried out to try to release VLDL remnants from isolated endocytic vesicles. Liver homogenates were prepared from rats sacrificed at various times after injection of labeled VLDL. An aliquot of the homogenates was centrifuged on a 6–45% sucrose gradient, and the fractions containing the radioactive peak were subjected to the treatments outlined in
was put through the French press once and then centrifuged associated with the particulate fraction. To evaluate the con-
dition in which labeled VLDL was added to unlabeled endo-
cytic vesicles prepared as described above, and the mixture
in the endocytic vesicle with time.

There was a substantial release of VLDL remnants (Table
3). One ml fractions were counted for radioactivity. Total radioactivity recovered was 2-4 μCi.

Table I. Treated fractions were then centrifuged on
a d 1.006-1.21 gradient (Table I). However, the time-
related differences are similar in both experiments, i.e. there
is increased release as a function of time after injection.

Composition of Radioactive Lipids in Endocytic Vesicles—
Rats were injected with labeled VLDL, sacrificed at various
times, and endocytic vesicles were prepared on 6-46% sucrose
gradients. The appropriate fractions from the gradient were
subjected to the treatment described. The final pellet was resuspended in 0.25 M sucrose, and 0.5 ml was
layered onto 30 ml of a 1.006-1.21 gradient. Data are ex-
pressed as a per cent of total recovered radioactivity (10,000-20,000
dpm) found in each fraction. Fractions were corrected for losses by
determining total counts before and after a cycle of passage through the French press, centrifugation, and resuspension of the resulting pellet.

Table II

Effect of time of passage through liver on the percentage of release of VLDL radioactivity from endocytic vesicles by various treatments.

Rats were sacrificed at the indicated time after injection of labeled VLDL, and the postnuclear supernatant fraction was prepared. Four ml was layered onto 30 ml of a 6-46% sucrose (w/v) gradient and centrifuged at 116,000 × g for 3.5 h at 4 °C. The fractions between 12-22 ml from the top of the gradient were subjected to the treatment indicated. Release was determined by centrifugation of treated ma-
terial on a d 1.006-1.21 gradient. The per cent of release as VLDL was calculated as radioactivity in the top 2 ml of the gradient divided by the total radioactivity (20,000-30,000 dpm) on the gradient.

Table III

Distribution of radioactive lipids in endocytic vesicles at various times after injection of in vivo labeled VLDL.

The distribution of radioactivity in the lipids of endocytic vesicles, isolated as described in the text, was determined by thin layer chromatography of a lipid extract. Each 4-cm lane was divided into 1-
mm bands that were scraped and counted. The per cent of total radioactivity in cholesterol ester and fatty acid was calculated as the
per cent of total counts (10,000-30,000 dpm) in the lane. Approximately 1% of lipidi radioactivity was in phospholipids. The injected VLDL was analyzed in a similar manner.

Table IV

Distribution of radioactive lipids in endocytic vesicles at various times after injection of in vivo labeled VLDL.

The distribution of radioactivity in the lipids of endocytic vesicles, isolated as described in the text, was determined by thin layer chromatography of a lipid extract. Each 4-cm lane was divided into 1-
mm bands that were scraped and counted. The per cent of total radioactivity in cholesterol ester and fatty acid was calculated as the
per cent of total counts (10,000-30,000 dpm) in the lane. Approximately 1% of lipidi radioactivity was in phospholipids. The injected VLDL was analyzed in a similar manner.

Table V

Distribution of radioactive lipids in endocytic vesicles at various times after injection of in vivo labeled VLDL.

The distribution of radioactivity in the lipids of endocytic vesicles, isolated as described in the text, was determined by thin layer chromatography of a lipid extract. Each 4-cm lane was divided into 1-
mm bands that were scraped and counted. The per cent of total radioactivity in cholesterol ester and fatty acid was calculated as the
per cent of total counts (10,000-30,000 dpm) in the lane. Approximately 1% of lipidi radioactivity was in phospholipids. The injected VLDL was analyzed in a similar manner.

Table VI

Distribution of radioactive lipids in endocytic vesicles at various times after injection of in vivo labeled VLDL.

The distribution of radioactivity in the lipids of endocytic vesicles, isolated as described in the text, was determined by thin layer chromatography of a lipid extract. Each 4-cm lane was divided into 1-
mm bands that were scraped and counted. The per cent of total radioactivity in cholesterol ester and fatty acid was calculated as the
per cent of total counts (10,000-30,000 dpm) in the lane. Approximately 1% of lipidi radioactivity was in phospholipids. The injected VLDL was analyzed in a similar manner.

Table VII

Distribution of radioactive lipids in endocytic vesicles at various times after injection of in vivo labeled VLDL.

The distribution of radioactivity in the lipids of endocytic vesicles, isolated as described in the text, was determined by thin layer chromatography of a lipid extract. Each 4-cm lane was divided into 1-
mm bands that were scraped and counted. The per cent of total radioactivity in cholesterol ester and fatty acid was calculated as the
per cent of total counts (10,000-30,000 dpm) in the lane. Approximately 1% of lipidi radioactivity was in phospholipids. The injected VLDL was analyzed in a similar manner.

Table VIII

Distribution of radioactive lipids in endocytic vesicles at various times after injection of in vivo labeled VLDL.

The distribution of radioactivity in the lipids of endocytic vesicles, isolated as described in the text, was determined by thin layer chromatography of a lipid extract. Each 4-cm lane was divided into 1-
mm bands that were scraped and counted. The per cent of total radioactivity in cholesterol ester and fatty acid was calculated as the
per cent of total counts (10,000-30,000 dpm) in the lane. Approximately 1% of lipidi radioactivity was in phospholipids. The injected VLDL was analyzed in a similar manner.

Table IX

Distribution of radioactive lipids in endocytic vesicles at various times after injection of in vivo labeled VLDL.

The distribution of radioactivity in the lipids of endocytic vesicles, isolated as described in the text, was determined by thin layer chromatography of a lipid extract. Each 4-cm lane was divided into 1-
mm bands that were scraped and counted. The per cent of total radioactivity in cholesterol ester and fatty acid was calculated as the
per cent of total counts (10,000-30,000 dpm) in the lane. Approximately 1% of lipidi radioactivity was in phospholipids. The injected VLDL was analyzed in a similar manner.
plasma VLDL as a function of time after injection (27) and with what would be expected for VLDL remnants in endocytic vesicles.

**Inhibitor Studies**—The involvement of endocytosis in the uptake of VLDL in vivo was tested by studying the effects of two inhibitors, namely colchicine and chloroquine. Colchicine, an inhibitor of microtubule polymerization, inhibits movement and/or formation of endocytic vesicles. Chloroquine inhibits various lysosomal enzymes, presumably by increasing intralysosomal pH (28). Table IV shows that the inhibitors caused a significant increase of radioactivity in liver at 45 min after injection without affecting uptake at 10 min. Most of the VLDL has been cleared from serum by 10 min and, therefore,

**TABLE IV**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>% injected dose in liver (mean ± S.E. (n))</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10 min</td>
</tr>
<tr>
<td></td>
<td>45 min</td>
</tr>
<tr>
<td>None</td>
<td>37.1 ± 1.9 (21)</td>
</tr>
<tr>
<td>Colchicine (0.5 mg/100 g, body weight)</td>
<td>39.3 ± 2.5 (2)</td>
</tr>
<tr>
<td></td>
<td>21.3 ± 3.0 (4)</td>
</tr>
<tr>
<td>Colchicine (1 mg/100 g, body weight)</td>
<td>38.1 (1)</td>
</tr>
<tr>
<td></td>
<td>28.0 ± 2.9 (5)</td>
</tr>
<tr>
<td>Chloroquine</td>
<td>39.6 ± 4.6 (6)</td>
</tr>
<tr>
<td></td>
<td>34.3 ± 1.6 (4)</td>
</tr>
</tbody>
</table>

**TABLE V**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>% injected dose in liver triglyceride (mean ± S.E. (n))</th>
<th>% injected dose of cholesterol ester in liver triglyceride (mean ± S.E. (n))</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>9.7 ± 2.3 (4)</td>
<td>46.1 ± 2.9 (2)</td>
</tr>
<tr>
<td>Colchicine</td>
<td>20.4 ± 2.2 (2)</td>
<td>63.9 ± 1.0 (4)</td>
</tr>
<tr>
<td>Chloroquine</td>
<td>23.8 ± 1.2 (2)</td>
<td>63.9 (1)</td>
</tr>
</tbody>
</table>

**FIG. 6** Distribution of radioactivity on 6-46% sucrose gradients of a postnuclear supernatant from a chloroquine-treated rat. A rat was treated with chloroquine. At 45 min after injection of labeled VLDL, a postnuclear supernatant fraction was prepared and centrifuged on a 4-46% sucrose gradient as described in Fig. 1. Fractions were collected and assayed for α-galactosidase activity and counted for radioactivity as in Fig. 1. The distribution of α-galactosidase in an untreated rat is also shown (●). Total radioactivity recovered was about 15,000 dpm.

**TABLE VI**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Per cent release</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>18.3</td>
</tr>
<tr>
<td>Colchicine (1 mg/100 g, body weight)</td>
<td>38.3</td>
</tr>
<tr>
<td>Chloroquine</td>
<td>63.8</td>
</tr>
</tbody>
</table>
Endocytosis of VLDL Remnants

somes are known to be. Rats were, therefore, treated with chloroquine or colchicine, and the postnuclear fractions were subjected to hypotonic shock. There was a clear difference in stability to hypotonic shock, as seen in Table VI, verifying that the chloroquine treatment lead to accumulation of radioactivity in the more fragile lysosomes and not in endocytic vesicles.

**DISCUSSION**

There is a progressive accumulation of radioactivity in the cell particulate fraction of liver after injection of labeled VLDL in vivo. The importance of endocytosis in the process is demonstrated by the effect of the inhibitors chloroquine and colchicine on this accumulation, i.e. they lead to an increased accumulation of unhydrolyzed triglyceride and cholesterol ester in the liver at later times after injection. Chloroquine has been shown to inhibit the degradation of a number of substances whose metabolism is believed to involve endocytosis. The current theory about the mode of action of chloroquine is that it accumulates in lysosomes and raises the intralysosomal pH so that lysosomal enzymes, that are most active at acid pH, are inactivated (28). Our data indicate the site of inhibition by chloroquine of endocytosis-mediated breakdown of VLDL is in lysosomes. The increase in total radioactivity in liver 45 min after injection of VLDL was due exclusively to an increase in radioactivity in the particulate fraction and was mainly in lysosomes as shown by its association with α-galactosidase on sucrose gradients and susceptibility to hypotonic shock.

Colchicine is an inhibitor of the breakdown of LDL (30), asialoglycoproteins (31) and epidermal growth factor (32). This drug inhibits polymerization of microtubules and the saltatory movements of cellular organelles (33). It would, therefore, be expected that internalized molecules or complexes would accumulate in endocytic vesicles in the presence of colchicine. This has been shown to be the case for asialoglycoproteins (31). The inhibition of LDL degradation by colchicine has been shown to be associated with the accumulation of endocytic vesicles containing LDL at the cell periphery as judged from indirect immunofluorescence (30). We saw accumulation of label in endocytic vesicles following colchicine treatment, in agreement with the findings with asialoglycoproteins (31).

We have concluded that endocytosis is a major pathway of breakdown of VLDL triglyceride and cholesterol ester in liver. At 5-20 min after injection of labeled VLDL, the radioactivity in the cell particulate fraction was primarily in a single peak at approximately \( d = 1.11 \) following sucrose gradient centrifugation of postnuclear supernatant fractions. We have tentatively identified this peak as endocytic vesicles. One of the major pieces of evidence for this assignment is that the position of marker enzymes for various hepatic organelles does not correspond to this peak. This reasoning presumes: (a) the marker enzyme is distributed uniformly in all organelles in which it exists; (b) all organelles of a given type constitute a single pool relative to the process in question. For example, the radioactivity in the peak at \( d = 1.11 \) could be a lysosomal subfraction with a density less than normal lysosomes \((d = 1.21)\) and containing little α-galactosidase activity, that took up VLDL preferentially. This possibility is unlikely, since the fraction was not as susceptible to hypotonic shock as lysosomes are known to be. Similarly, it is unlikely that this peak represents a special subfraction of endoplasmic reticulum that reincorporates radioactive fatty acids from the VLDL since little radioactive phospholipid is found in the \( d = 1.11 \) peak. The possibility that the peak is a special subfraction of plasma membrane is more likely, since it has a substantial 5'-nucleotidase activity, although its distribution on sucrose gradients is distinct. Electron microscope autoradiography was used to show that this is not the case, since the majority of ¹²⁵I-VLDL is intracellular at 10 min after injection and thus could not be on the plasma membrane. Therefore, the peak at \( d = 1.11 \) is probably endocytic vesicles.

The identification of the \( d = 1.11 \) peak as endocytic vesicles is supported by the findings of other groups studying the uptake of asialo glycoproteins: LaBadie et al. (24) observed that 13 min after injection of ¹²⁵I-asialofetuin, a large portion of the radioactivity was in a subcellular particle of \( d = 1.13 \) that did not correspond to any of the subcellular marker enzymes assayed, although it did overlap with the phosphodiesterase activity (a plasma membrane marker). These observations were extended by Tolleshaug et al. (4) who incubated hepatocytes with ¹²⁵I-asialofetuin for 10 min, washed to remove extracellular protein, and incubated at 37 °C. They observed a time-dependent movement of radioactivity from a particle of \( d = 1.136 \) to a more dense particle containing lysosomal enzymes. The radioactive peak at \( d = 1.136 \) contained 5'-nucleotidase activity, but there was a slight difference in distribution of the enzyme and radioactivity. When cells were incubated for 30 min at 10 °C, most of the ¹²⁵I-asialofetuin was bound to the plasma membrane and following homogenization and subcellular fractionation on a sucrose gradient, the radioactivity corresponded almost exactly to 5'-nucleotidase activity. Thus, the \( d = 1.136 \) peak is not a plasma membrane fraction but represents endocytic vesicles. They concluded that the movement of endocytic vesicles to lysosomes was a rate-limiting step in the catabolism of ¹²⁵I-asialofetuin by liver. Dunn et al. (10) showed this step had a \( t_1/2 \) of approximately 7 min. These findings are in agreement with observations by Hubbard and Stukenbrok (34) and Wall et al. (25) who followed the movement of either ¹²⁵I-asialo glycoproteins by electron microscope autoradiography or of asialoorosomucoid linked to horseradish peroxidase or lactosaminated ferritin by electron microscopy. These authors concluded that at early times after exposure to ligand (1-2 min), the ligand is internalized via coated vesicles into vesicles with a diameter of approximately 100 nm. By 5 min, the ligand was found in larger vesicles (200 nm) that had migrated to the Golgi-lysosome region of the hepatocyte. At 15 min, label began to appear in recognizable lysosomes. Thus, there is a 15-min lag between internalization and degradation that represents movement of labeled asialoglycoproteins in endocytic vesicles to lysosomes. It is of interest that LDL has recently been shown to follow a similar pathway of metabolism as asialoglycoproteins, with a similar time course (35). We found that radioactivity accumulates in a \( d = 1.11 \) fraction for the first 20 min after injection of VLDL which is analogous to the accumulation of ¹²⁵I-asialo glycoprotein radioactivity in a particle of \( d = 1.136 \). It is possible that the increase in fragility of the endocytic vesicles with time that we observed (Tables I and II) is caused by the formation of larger more fragile vesicles with increasing time after injection, as was described for asialo glycoproteins. Another reason for believing the \( d = 1.11 \) fraction is endocytic vesicles is the finding that colchicine leads to an accumulation of radioactivity in the fraction (Fig. 5). This agrees with the findings of Kolset et al. (31) using asialoglycoproteins.

Lipkin et al. (11) observed a 10-min lag in the hydrolysis of serum triglyceride taken up by liver. This probably results from the fact that most of the VLDL triglyceride is either extracellular or inside endocytic vesicles during this time and has not reached the primary site of hydrolysis in lysosomes.

**Acknowledgments**—We wish to thank Helga Beegen for the elec-
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REFERENCES