Total Inhibition of Hepatic β-Lipoprotein Production in the Rat by Orotic Acid*

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SUMMARY

Lipoproteins of rat plasma have been characterized by lipid analysis, preparative ultracentrifugation, electrophoresis on paper, and immunoelectrophoresis with specific antisera to α-lipoprotein, β-lipoprotein, and whole rat plasma. Similar techniques were used to characterize the lipoproteins released by livers perfused in situ with a suspension of erythrocytes in a medium initially free of rat plasma proteins. Normal rat livers released α-lipoprotein, β-lipoprotein, albumin, and other plasma proteins during the perfusion.

When rats were fed orotic acid as 1% of their diet, the plasma β-lipoprotein concentration fell within 7 days to less than 1% of normal. It rebounded to normal within 48 hours following withdrawal of orotic acid or supplementation of the diet with 0.17% of adenine. When perfused in situ, livers from orotic acid-fed rats released α-lipoprotein, albumin, and other plasma proteins but no detectable β-lipoprotein. They released smaller amounts of cholesterol and phospholipid than normal livers and no triglyceride, although they contained 10 times normal amounts of triglycerides.

The fatty liver produced in rats by orotic acid appears to result from an inhibition of synthesis or release of hepatic β-lipoprotein, supporting the concept that β-lipoprotein has a specific role in the normal transport of triglyceride out of the liver.

Lipids are thought to be released from liver and transported in blood as part of specific lipoprotein complexes. We have, therefore, examined the effects of orotic acid feeding on lipoproteins in plasma and in a medium, initially free of rat plasma proteins, after its perfusion through livers in situ. Lipoproteins were characterized by lipid analyses, paper electrophoresis, preparative ultracentrifugation, and immunoelectrophoresis, with antisera specific for α-lipoprotein, β-lipoprotein, and whole plasma.

EXPERIMENTAL PROCEDURE

Rats and Diets—Male rats of the National Institutes of Health Osborne-Mendel strain were maintained individually in wire-bottomed cages and supplied with food and water ad libitum. The basal diet (R1) (8), was a balanced semisynthetic mixture of glucose monohydrate (68%), casein (20%), corn oil (5%), DL-methionine (0.3%), choline chloride (0.2%), plus adequate amounts of vitamins and minerals. In a fat-free basal diet, used in one study, corn oil was omitted and the glucose monohydrate content was increased to 75%. Blood for analysis was obtained from the tip of the tail (small samples) or from the aorta under ether anesthesia. In all cases, the anticoagulant (25 μl per ml of blood) was 4% (w/v) sodium EDTA, pH 7.0. Analyses and fractionation were begun on the same day that blood was drawn.

Perfusion of Livers in Situ—The procedure for perfusion in situ by the method of Mortimore (9, 10) has been previously described (5). Plasma-free perfusing medium was prepared as follows. Blood was drawn with heparinized syringes from adult male rats. Erythrocytes were sedimented by centrifugation, the plasma anduffy coat were discarded, and the erythrocytes were washed four times with 3 to 4 volumes of ice-cold 0.15 M NaCl-0.1% (w/v) glucose. The washed cells were suspended in 3 volumes of Krebs-Ringer bicarbonate buffer, pH 7.4, containing 5% (w/v) crystalline bovine serum albumin (Armour) and 0.1% (w/v) glucose; the mixture was filtered, and 70 ml were transferred to each oxygenator reservoir of the perfusion apparatus.

A portion, 26 ml, of this perfusing medium was flushed through the livers and discarded before return flow to the reservoir was permitted, to ensure that the circulating medium was initially free of rat plasma proteins. After circulation was established, 250 μl of H2O (2 mM) were added to each reservoir. Livers were perfused for 6 hours at 37° at a flow rate of 13 ml per min. Perfusing medium was sampled (3 ml) as soon as circulation was es-
tablished (zero time) and at intervals of 2 hours thereafter. All analyses of perfusate were made after removal of erythrocytes by centrifugation. To portions of perfusate used for analysis of lipoproteins (paper electrophoresis, immunoelectrophoresis, or preparative ultracentrifugation) were added 25 μl per ml of 4% (w/v) sodium EDTA, pH 7. Analyses of perfusates were begun within 24 hours after termination of perfusion. Following 6 hours of perfusion, livers were flushed with 20 ml of cold 0.9% NaCl; weighed, frozen in liquid nitrogen, and powdered in a stainless steel beaker. Portions of each powder were weighed for subsequent lipid analyses.

Lipoprotein Separation by Preparative Ultracentrifugation—Lipoprotein fractions of plasma or perfusate were separated in a 40,000 rotor of a Spinco model L preparative ultracentrifuge. The density of plasma, perfusate, or of previously sedimented fractions was adjusted by addition of NaCl and KBr (11) and checked by pyknometer at 20°C. Samples were centrifuged at 40,000 rpm (143,500 x g) at 10°F for 16 hours at all density values except 1.21, when centrifugation was continued for 24 hours. Fractions recovered after slicing the cellulose nitrate tubes with a standard tube slicer were dialyzed for 16 to 24 hours against 150 or more volumes of 0.15 M NaCl-1 mM EDTA, pH 7.0, at 4°F and concentrated by further dialysis against Sephadex G-50 (12).

The lipoprotein fractions used as antigens were isolated from pooled serum from 20 to 50 rats by preparative ultracentrifugation at the density indicated. Each fraction was re-centrifuged once at the appropriate separation density and dialyzed.

Paper Electrophoresis—Lipoproteins were separated by electrophoresis in buffer containing albumin (13) on Whatman No. 1 paper strips, Grade EP 1, and stained with oil red O. Bovine serum albumin (Fraction V, Armour) was used.

Chemical and Radiochemical Determinations—Fatty acids, triglycerides, cholesterol, and phospholipids were determined in washed total lipid extracts of liver, plasma, and plasma lipoprotein fractions as previously described (5). Lipids of perfusate were similarly analyzed in total lipid extract prepared as follows. Methanol (13.3 ml), cooled in a Dry Ice-alcohol bath, and CHCl3 (26.6 ml) at 0°F, were rapidly added, with swirling, to 2 ml of perfusate chilled to 0°F in a glass stoppered bottle. The mixture was shaken vigorously for 1 min, then swirled occasionally during the next hour at room temperature. Water (40 ml) was layered over the mixture, and after standing for 2 to 3 days at 4°F, the lower CHCl3 layer was recovered with a cannula attached to a syringe. Use of chilled solvents was found necessary to disperse proteins and insure adequate lipid extraction when perfusate was prepared with bovine serum albumin as the only protein.

The procedure used to determine tritium in perfusate water and in liver and perfusate fatty acids has been described (5). The number of micromoles of fatty acid synthesized during a perfusion experiment was calculated by dividing the relative total activity of perfusate fatty acids plus liver fatty acids by 13.3 (5). Relative total activity was determined by dividing disintegrations per min in fatty acids by disintegrations per min per μg atom of perfusate water hydrogen. During the course of a 6-hour perfusion the specific activity of perfusate water fell by about 25% due mostly to exchange with unlabeled water in the humidified gassing mixture; in the calculations of fatty acid synthesis, an average value was used, i.e. the specific activity determined after 3 hours of perfusion.

Preparation of Antisera—For use as antigens β-lipoprotein was isolated from plasma in the 1.006 < d < 1.035 fraction and α-lipoprotein in the 1.035 < d < 1.21 fraction. The rabbit α-lipoprotein antigen injected intramuscularly in three equal doses at intervals of 3 weeks was 80 mg of protein in a Cheviot sheep (40 to 50 kg) and 35 mg in each of two rabbits (4 kg). β-Lipoprotein (20 mg of protein) was administered only once to two rabbits (4 kg). Blood was collected 10 days after the final dose, and the serum was frozen after the addition of 0.01% merthiolate.

Characterization of Antisera—Rabbit α-lipoprotein, like human α-lipoprotein (14), proved to be a far better antigen than β-lipoprotein and produced adequate anti-β-lipoprotein titer in both rabbits. α-Lipoprotein produced potent anti-α-lipoprotein antisera in two rabbits, but in both, as well as in the sheep, the antisera also reacted with β-lipoprotein. All five antisera are characterized in Table I. The rabbit antisera to α-lipoprotein could be freed of all anti-β-lipoprotein activity by absorption with β-lipoprotein without appreciable decrease in anti-α-lipoprotein titer. SR1 (Table I) reacted with albumin, γ-globulin, α- and β-lipoproteins, as well as several other unidentified plasma proteins and was used as an “anti-whole plasma.” Absorption of antibodies was carried out at equivalence as described previously (14).

With each antisera the precipitating antibodies were shown by immuno-electrophoresis to be almost entirely γG-immunoglobulin. Anti-human β-lipoprotein sera prepared in rabbits and sheep (14) reacted only very weakly with rat β-lipoprotein. Anti-human α-lipoprotein sera (14) failed to react with rat α-lipoprotein.

Imunochemistry—Immuno-electrophoresis was performed on Agarose-coated microscope slides, as described previously (12-15).

### Table I

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Sensitizing antigens</th>
<th>β-Lipoprotein</th>
<th>α-Lipoprotein</th>
<th>Albumin</th>
<th>Other proteins</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sheep</td>
<td>SR1........ α-Lipoprotein</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Rabbit</td>
<td>RR1........ α-Lipoprotein</td>
<td>+</td>
<td>+</td>
<td>±</td>
<td>±</td>
</tr>
<tr>
<td>RR2...... α-Lipoprotein</td>
<td>+</td>
<td>+</td>
<td>±</td>
<td>±</td>
<td></td>
</tr>
<tr>
<td>RR3...... β-Lipoprotein</td>
<td>+</td>
<td>−</td>
<td>±</td>
<td>−</td>
<td></td>
</tr>
<tr>
<td>RR4...... β-Lipoprotein</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td></td>
</tr>
</tbody>
</table>

* Sensitizing antigens were isolated from pooled rat plasma by preparative ultracentrifugation: α-lipoprotein, fraction of 1.006 < d < 1.21; β-lipoprotein, fraction of 1.006 < d < 1.035.
FIG. 1. Paper electrophoresis of whole plasma lipoproteins. Plasma (60 μl) was obtained from a 200-g rat fed basal diet (−OA) or basal diet plus 1% erotic acid for 7 days (+OA). β-LP, β-lipoprotein; α-LP, α-lipoprotein. See "Experimental Procedure" for details.

Antisera RR3 and RR4 were capable of detecting β-lipoprotein in concentrations as low as 0.016 that of normal rat plasma and even lower amounts were detectable when samples were first concentrated by dialysis against Sephadex G-50. Antisera RR1 and RR2 could detect α-lipoprotein when present with a concentration 0.125 of normal. Antiserum RR3 gave an immunoprecipitin line with 0.25 of normal but not with normal plasma concentrations of albumin.

Examination of Liver Extracts for Soluble Plasma Lipoproteins
—Three livers were used: (a) a liver from a rat fed basal diet; (b) a liver, from a rat fed basal diet, perfused in situ for 4 hours at 37° with Krebs-Ringer bicarbonate buffer, pH 7.4, containing 5% bovine albumin (Fraction V, Armour) and 0.1% glucose (the perfusate (85 ml) was continuously recirculated at a rate of 2 ml per g of liver per min); (c) a fatty liver from a rat fed basal diet plus 2% erotic acid for 10 days.

All livers were flushed by perfusion with 20 ml of cold 0.15 M NaCl, and equal portions were homogenized with 2.5 volumes of cold 0.15 M NaCl or 2 M LiCl containing 6 mM EDTA, pH 7.0. To test for recovery of added lipoproteins, portions of liver were also homogenized with similar salt solutions containing 10% (v/v) of rat plasma (from a rat fed basal diet). Homogenates were centrifuged for 1 hour at 143,000 × g. The floating fat fraction was removed and dispersed in a small volume of cold 0.15 M NaCl by sonic treatment (Fraction 1). The clear supernatant fraction was recovered (Fraction 2). The sedimented pellet was resuspended in a small volume of 0.15 M NaCl, sonically disintegrated, centrifuged, and the clear supernatant fraction recovered (Fraction 3). Lipoproteins of d < 1.21 from Fraction 2 were concentrated by flotation in the ultracentrifuge followed by dialysis against 0.15 M NaCl and then Sephadex G-50 (Fraction 2a).

All fractions were examined for lipoproteins by immunoelectrophoresis with the use of antisera RR2, RR3, and RR4 (see Table I).

RESULTS

Plasma Electrophoresis

After electrophoresis on paper, plasma from a normal rat fed the basal diet showed a weakly staining β-lipoprotein band and a darker, usually double, α-lipoprotein band (Fig. 1). In plasma from a rat fed the same diet plus 1% erotic acid for 1 week, the β-lipoprotein band was no longer seen and the α-lipoprotein band was reduced in intensity.

Plasma Immunoelectrophoresis

The absence of β-lipoprotein but not α-lipoprotein from plasma of erotic acid-fed rats was confirmed by immunoelectrophoresis (Figs. 2 and 3). The method was capable of detecting β-lipoprotein concentrations lower than 1% of normal, as determined by serial dilution analysis of normal plasma. After erotic acid was added to the diet, the concentration of plasma β-lipoprotein began to decrease within 24 hours (Fig. 4), as did the concentration of plasma total fatty acids (3, 4). Within 7 days, β-lipoprotein was undetectable in 50% of the rats and the plasma total fatty acid concentration was 30% of normal. Continuing the erotic acid feeding produced no further change.

After erotic acid had been fed for 7 to 10 days, its withdrawal was followed within 48 hours by a return of the concentrations of β-lipoprotein and blood lipids to normal (Fig. 4). Similar results were observed when 0.25% adenine sulfate was added to the diet containing erotic acid (Fig. 4).

FIG. 2. Reappearance of β-lipoprotein in plasma of rat following withdrawal of erotic acid (OA). A 200-g rat was fed basal diet plus 1% erotic acid from day 1 until day 10, and thereafter basal diet alone. Plasma obtained on the day indicated was analyzed by immunoelectrophoresis (antisera to β-lipoprotein (RR2) on the left and antisera to α- and β-lipoprotein (RR4) on the right, in each case). All the precipitin lines on this and succeeding figures were unstained. All the lines seen here were subsequently shown to stain for lipid.
A few rats always retained very low but detectable titers of \(\beta\)-lipoprotein (Fig. 4). In these rats, and not in others, small areas on one or more lobes of the liver were observed to be less fatty in appearance. Occasionally one or two small lobes appeared nearly normal (for additional data see Group III, Table III).

Food intake, growth rate, and the gross appearance of the rats all showed little or no response to any of the above dietary alterations.

**Fractionation of Plasma by Preparative Ultracentrifugation**

*Normal Plasma*—The very low density fraction \((d < 1.006)\), isolated from normal plasma and concentrated by preparative ultracentrifugation, migrated as a discrete pre-\(\beta\)-lipoprotein band (Fig. 5). A small amount of fat was seen at the origin. This fraction \((d < 1.006)\) contained 36% of the total plasma fatty acids, 10% of the phospholipid, \(\beta\)-lipoprotein, and small but detectable amounts of \(\alpha\)-lipoprotein and albumin (Table II). \(\beta\)-Lipoprotein was also found by immunoelectrophoresis in the fraction of \(1.006 < d < 1.019\) and by immunoelectrophoresis and paper electrophoresis (Fig. 5) in the fraction of \(1.006 < d < 1.035\), together with 2% of the plasma total fatty acids. Of the fatty acids 61%, and of the phospholipids 88% were recovered in the fraction of \(d > 1.035\), with the bulk of plasma proteins and \(\alpha\)-lipoprotein (Fig. 5).

*Plasma from Orotic Acid-fed Rats*—In plasma from orotic acid-fed rats, absence of \(\beta\)-lipoprotein was associated with an absence of all migrating lipoprotein bands in fractions of \(d < 1.035\) (Fig. 5). The lipid in the \(d < 1.006\) fraction, representing 7.5% of the plasma fatty acids and 1% of the phospholipid (Table II), remained at the origin. The significant amount of protein which appeared in this fraction in this and other studies (17) may be explained by the presence of albumin and other plasma proteins (Table II). \(\alpha\)-Lipoprotein and more than 99% of plasma fatty acids were recovered in the fraction of \(d > 1.035\).

**Release of Lipids and Lipoproteins by Perfused Livers**

Data from hepatic perfusions involving three groups of rats are summarized in Table III.

Normal livers (Group I, Table III) synthesized more than 300 \(\mu\)moles of fatty acid, of which \(16\%\) was released to the perfusate. The rates of release of triglyceride, cholesterol, and phospholipid are shown at the top of Fig. 6. Most of the perfusate lipid was associated with a lipoprotein band having mobility on paper intermediate between \(\beta\) and \(\alpha\)-lipoprotein (Fig. 7) and similar in migration to the pre-\(\beta\)-lipoprotein of the very low density fraction \((d < 1.006)\) of normal plasma (see Fig. 5). By immunoelectrophoretic analysis (Fig. 8) the perfusate, which was free of detectable amounts of rat plasma proteins at zero time, had increasing amounts of \(\beta\)-lipoprotein, albumin, and other rat plasma proteins not further identified. The presence of \(\alpha\)-lipoprotein,
FIG. 5. Paper electrophoresis of lipoproteins from plasma fractions isolated by ultracentrifugation at increasing densities. A pool of plasma was obtained from nine rats fed basal diet (−OA) or basal diet plus 1% orotic acid for 14 days (+OA). Lipoprotein fractions, obtained sequentially by ultracentrifugation after addition of appropriate amounts of NaCl and KBr, were dialyzed and concentrated as described in "Experimental Procedures." β-LP, β-lipoprotein; pre-β-LP, α-LP, α-lipoprotein.

### Table II

**Distribution of lipids and proteins of plasma after ultracentrifugation at increasing densities**

Plasma fractions were obtained as described for Fig. 5.

<table>
<thead>
<tr>
<th>Addition to basal diet</th>
<th>Plasma fraction</th>
<th>Total fatty acids</th>
<th>Phospholipids</th>
<th>Total protein</th>
<th>Specific proteins</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>µmoles/ml plasma</td>
<td>% of total</td>
<td>µmoles/ml plasma</td>
<td>% of total</td>
</tr>
<tr>
<td>None</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>d &lt; 1.006</td>
<td>3.00</td>
<td>36.3</td>
<td>0.14</td>
<td>9.5</td>
</tr>
<tr>
<td></td>
<td>1.006 &lt; d &lt; 1.019</td>
<td>0.04</td>
<td>0.5</td>
<td>&lt;0.01</td>
<td>&lt;0.7</td>
</tr>
<tr>
<td></td>
<td>1.006 &lt; d &lt; 1.035</td>
<td>0.19</td>
<td>2.3</td>
<td>0.03</td>
<td>2.0</td>
</tr>
<tr>
<td></td>
<td>d &gt; 1.035</td>
<td>5.07</td>
<td>61.4</td>
<td>1.30</td>
<td>87.8</td>
</tr>
<tr>
<td>1% orotic acid (14 days)</td>
<td>d &lt; 1.006</td>
<td>0.25</td>
<td>7.5</td>
<td>0.01</td>
<td>1.4</td>
</tr>
<tr>
<td></td>
<td>1.006 &lt; d &lt; 1.019</td>
<td>&lt;0.01</td>
<td>&lt;0.3</td>
<td>&lt;0.01</td>
<td>&lt;1.0</td>
</tr>
<tr>
<td></td>
<td>1.006 &lt; d &lt; 1.035</td>
<td>0.04</td>
<td>1.2</td>
<td>&lt;0.01</td>
<td>&lt;1.0</td>
</tr>
<tr>
<td></td>
<td>d &gt; 1.035</td>
<td>3.05</td>
<td>91.3</td>
<td>0.67</td>
<td>98.7</td>
</tr>
</tbody>
</table>

* Determined by the method of Lowry, Rosebrough, Farr, and Randall (16).

† Detected by immunoelectrophoresis.

* Traces of β-lipoprotein were detected in the fraction of 1.055 < d < 1.063 but none in the fraction of d > 1.063.

As well as β-lipoprotein, was clearly shown by immunoelectrophoresis of the perfusate lipoproteins (d < 1.21) concentrated by flotation in the ultracentrifuge (Fig. 10). The β-lipoprotein, 98% of the triglyceride, and over 55% of the phospholipid were recovered in the perfusate fraction of d < 1.016 (Table IV). α-Lipoprotein and albumin were also detected in this fraction, although most were found in the fraction of d > 1.016 (Table IV).

The uniformly fatty livers from orotic acid-fed rats (Group II, Table III) contained 4 times the normal amounts of fatty acids and 10 times the normal amounts of triglycerides (5, 18). They synthesized only 36 µmoles of fatty acid during perfusion (5), of which only 0.3% was released to the perfusate. These livers released 0.32 as much phospholipid and 0.26 as much cholesterol as the control livers and no detectable triglyceride (bottom of Fig. 6). No lipoprotein was observed in the perfusate after electrophoresis on paper (Fig. 7). By immunoelectrophoretic analysis (Fig. 9), increasing amounts of albumin and other plasma proteins were observed as with normal liver (compare Figs. 8
TABLE III

Synthesis and release of lipids and lipoproteins by perfused livers

Livers of rats (270 to 300 g) were perfused in situ for 6 hours (see "Experimental Procedure"). Values are means of four to eight experiments.

<table>
<thead>
<tr>
<th>Group</th>
<th>Addition to basal diet (for 8 to 11 days)</th>
<th>Appearance of livers</th>
<th>Liver</th>
<th>Net release into perfusate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Lipids</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Triglycerides</td>
</tr>
<tr>
<td>I (8)</td>
<td>None</td>
<td>Normal</td>
<td>14.0</td>
<td>118</td>
</tr>
<tr>
<td>II (4)</td>
<td>1 or 2% erotic acid*</td>
<td>Uniformly fatty</td>
<td>15.5</td>
<td>456</td>
</tr>
<tr>
<td>III (4)</td>
<td>1% erotic acid</td>
<td>Variably fatty</td>
<td>17.2</td>
<td>403</td>
</tr>
</tbody>
</table>

* Calculated from the incorporation of \(^{3}H\), from \(^{3}H_2O\), into liver and perfusate fatty acids (5).

\(^{3}H_2O\) fraction of the liver plus perfusate fatty acid radioactivity recovered in perfusate after 6 hours.

\(^{3}H_2O\) detected by immunoelectrophoresis (see Figs. 8 to 10); number of plus signs indicates relative quantities.

\(^{3}H_2O\) figures in parentheses represent number of experiments.

* Orotic acid, 1% in two experiments and 2% in two experiments.

**Fig. 6.** Net release of lipids by livers perfused in situ. Rats were fed basal diet (minus erotic acid) or basal diet plus 1 or 2% erotic acid (plus erotic acid) for 8 to 11 days prior to perfusion of their livers. See "Experimental Procedure" for details and Table III for additional data (Groups I and II). Numbers in parentheses refer to number of rats; TG, triglycerides; PL, phospholipid; C, cholesterol.

DISCUSSION

Earlier work had revealed that when 1% of erotic acid was fed to rats, plasma lipid concentrations rapidly declined (3), release of hepatic lipid to the circulation was severely reduced (4, 5), and, after 3 days of feeding, triglycerides began accumulating in liver (18) within vesicles arising from the endoplasmic reticulum (19, 20). By 7 days, no release of triglycerides from liver could be demonstrated (5) (Fig. 6). Plasma lipid concentrations (4) and hepatic triglyceride release (5) returned to normal within 48 hours following either withdrawal of the oro-
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Fig. 7. Paper electrophoresis of hepatic perfusate lipoproteins. Perfusate (80 $\mu$l) was sampled at 2-hour intervals throughout the perfusion, as indicated. Rats were fed basal diet (−OA) or basal diet plus 1% orotic acid (+OA) for 10 days before their livers were perfused (Table III, Groups I and II, respectively). Normal whole rat plasma was included as an electrophoresis control. See "Experimental Procedure" for details.

Fig. 8. Immunoelectrophoresis of proteins released by normal liver perfused in situ. Perfusate was sampled at 2-hour intervals throughout the perfusion (Group I, Table III). Left trough, antiserum to $\beta$-lipoprotein (RR3); right trough, antiserum to whole rat plasma (RR1). The heavy lines nearest the well are $\beta$-lipoprotein and the line farthest from the well is albumin.

detect or addition of adenine to the orotate-containing diet; within 7 days all accumulated liver triglyceride was mobilized (7).

The present studies have shown that a decrease in plasma $\beta$-lipoprotein concentrations paralleled the orotate-induced decline in plasma lipid concentrations (Fig. 4). By 7 days, $\beta$-lipoprotein had virtually disappeared from plasma. It reappeared when plasma lipid concentrations rebounded to normal following orotic acid withdrawal or adenine supplementation.

TABLE IV

Distribution of lipids and proteins in hepatic perfusates after ultracentrifugation (density = 1.016)

<table>
<thead>
<tr>
<th>Perfusate</th>
<th>Fraction</th>
<th>Distribution of lipids</th>
<th>Distribution of proteins</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Triglycerides</td>
<td>Cholesterol</td>
</tr>
<tr>
<td>Minus orotic</td>
<td>d &lt; 1.016</td>
<td>98.3</td>
<td>60.7</td>
</tr>
<tr>
<td>acid</td>
<td>d &gt; 1.016</td>
<td>1.7</td>
<td>39.3</td>
</tr>
<tr>
<td>Plus 1% orotic acid</td>
<td>d &lt; 1.016</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td>d &gt; 1.016</td>
<td>0.0</td>
<td>100.0</td>
</tr>
</tbody>
</table>

* Detected by immunoelectrophoresis.
† Percentage of total lipid recovered in d < 1.016 fraction + d > 1.016 fraction. For all lipids total recovery was 87 to 90% of the amount found in the whole perfusate.
‡ No triglyceride was found.

In perfused livers, total inhibition of hepatic triglyceride release induced by feeding orotic acid was correlated with a total block in production of $\beta$-lipoprotein. Livers from orotic acid-fed rats continued to produce other plasma proteins, including albumin*"
and α-lipoprotein that contained phospholipids and cholesterol but no triglyceride. These observations support previous suggestions of a specific role for β-lipoprotein in the transport of triglyceride from liver (12, 21).

Patients with α-beta-lipoproteinemia, a rare human disease (22) characterized by a total absence of serum β-lipoprotein, have fatty livers (23), like those from orotic acid-fed rats, that are unable to release any triglyceride (12). In addition these patients cannot form chylomicrons although their intestinal mucosal cells may become engorged with fat, and thus they exhibit a severe malabsorption of fat and fat-soluble vitamins. This is not found in orotic acid-fed rats as indicated by the following observations: (α) orally administered fat can produce a transient hyperlipemia; (β) normal amounts of fat-soluble vitamins (vitamin A and tocopherols) and essential fatty acids (linoleic and arachidonic) were found in the fatty livers and blood of rats fed 1% of orotic acid for 19 months, during which time plasma lipid concentrations were always depressed. While patients with α-beta-lipoproteinemia apparently have a defect in transporting fat both out of the intestinal wall and out of liver, only the latter defect is found in orotic acid-fed rats.

Rubin and Pendleton (24), with the use of liver slices from orotic acid-fed rats, reported an inhibition of amino acid incorporation into a low density fraction of the medium, and similar results were obtained with perfused livers by Roheim et al. (17). These and other studies (25, 26) have made it clear, however, that orotate does not inhibit total incorporation of amino acids into liver and plasma proteins, as do ethionine (27, 28), puromycin (29), and carbon tetrachloride (30, 31), which also inhibit hepatic lipid release. By a mechanism not yet defined, orotate more specifically affects either synthesis or release of β-lipoprotein. Our failure to detect any β-lipoprotein in an orotate-induced fatty liver suggests that its synthesis is blocked, or that lipoprotein accumulates in a form not detectable by the extraction and immunochemical methods used.

Roheim et al. (17), after perfusing a normal liver with a 14C-labeled protein fraction of d > 1.21 isolated from plasma of orotic acid-fed rats, recovered 0.2% of the perfuse radioactivity in a fraction of d < 1.019. They concluded that orotic acid-fed rats, like normal rats (32), have in plasma an apoprotein of low density lipoproteins. In the present studies, no immunohemical evidence was obtained for a plasma apoprotein, nor was such a protein required in the perfusion medium for the transport of lipid from liver (32). Normal livers released lipid as well as α- and β-lipoprotein without a noticeable lag when perfused with a medium that contained no rat plasma proteins (Figs. 6 to 8).

Work from several laboratories had previously suggested liver as a source of the protein moieties of plasma lipoproteins. Incorporation by liver of amino acids into perfusate proteins with flotation densities less than 1.21 was first shown by Radding, Bragdon, and Steinberg (33), and also by Haft, Roheim, White and Eder (34), and Wilcox, Fried, and Heimberg (35). Per fusate proteins of lowest density generally showed the highest specific activity. Radding and Steinberg (36) found that one of the labeled proteins released by liver slices and isolated in the 1.063 < d < 1.21 fraction of the medium produced a peptide "fingerprint" pattern very similar to that of plasma lipoproteins isolated at the same density. At least partial immunological similarity between lipoproteins in rat plasma and those in rat
liver perfusates and incubated homogenates was suggested by the work of Marsh and Whereat (37) and Marsh (38).

The present studies with normal rats have clearly established that liver can synthesize and release both α- and β-lipoprotein immunochemically indistinguishable from the lipoproteins normally found in rat plasma. During the course of a 6-hour experiment, perfusate, initially free of rat plasma protein, contained increasing amounts of α- and β-lipoproteins that resem-

bled plasma lipoproteins in their reactivity with specific antisera and in their relative lipid concentration, flotation density, and electrophoretic mobility (in Agarose). Triglycerides accumulated in liver and failed to enter the circulation when the liver failed to synthesize or release β-lipoprotein.

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Total Inhibition of Hepatic β-Lipoprotein Production in the Rat by Orotic Acid
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