The Synthesis of Plasma Lipoprotein by Rat Liver*

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The immunochemical technique, first employed in the study of albumin synthesis in chicken liver slices by Peters and Anfinsen (3), has recently been successfully used with rat liver slices by Marsh and Drabkin (4) as well as by Campbell and Stone (5). The present work extends this technique to the problem of the biosynthesis of plasma lipoprotein by rat liver.

The liver has been shown to be the site of plasma cholesterol and phospholipid synthesis (6, 7). Miller et al. (8) observed that the plasma proteins (exclusive of gamma globulins), were labeled when radioactive amino acids were perfused through the isolated rat liver. Indirect evidence that the liver is capable of synthesizing plasma lipoproteins has recently been obtained by Radding et al. (9) who observed the incorporation of radioactive amino acids into lipoproteins of serum that had been incubated with rat liver slices. The present experiments demonstrate by the immunochemical method the net synthesis of low density lipoproteins by rat liver slices.

METHODS

Isolation of Rat Plasma Lipoproteins*—Very low density lipoproteins were isolated from heparinized rat plasma as follows. Chylomicrons were removed from 60 ml of rat plasma by the method of Rodbell (10). The infranatant plasma was centrifuged in portions of 10 ml in the Spinco No. 40 rotor at 36,000 r.p.m. for 16 hours. The uppermost milliliter was removed from each tube and all these top fractions were combined. The resulting solution was centrifuged for 16 hours at 36,000 r.p.m. The top 2 ml were diluted to 5.5 ml with 0.15 M NaCl and centrifuged for 16 hours at 36,000 r.p.m. in the Spinco No. 40.3 rotor. The top milliliter fractions were combined and recentrifuged twice at density 1.063.

The infranatant plasma solution from the first centrifugation at density 1.063 was adjusted to density 1.21 and again centrifuged for 16 hours at 36,000 r.p.m. The top milliliter portions were combined and recentrifuged twice at density 1.21 to isolate the high density (>1.063 and <1.017) lipoproteins.

The results of the quantitative precipitin reactions shown in Fig. 1 suggest that the protein moieties present in the very low density (<1.017) lipoprotein and in the low density (>1.017 <1.063) lipoprotein are closely related immunochemically. This is in agreement with the findings of Levine et al. (13). The quantitative difference shown in Fig. 1 may be related to the different molecular weights, representing different percentages of protein in the molecule, of the two groups of lipoproteins. The precipitin curves for the very low density lipoprotein shown in Fig. 1 were used as the standard curves for the estimation of the protein (Curve A-1) and cholesterol (Curve B-1) moieties of the low density lipoprotein in subsequent experiments with liver preparations.

The goat antiserum against low density lipoproteins was also tested for cross-reactivity with plasma high density lipoproteins (density >1.063 and <1.21). No precipitin reaction was observed when increasing amounts of high density lipoprotein were added to a constant amount of antiserum. However, when increasing amounts of antiserum were added to a constant amount of whole rat plasma and the cholesterol in the immune precipitate measured, the results shown in Fig. 2 were obtained. It is evident that the anti-low density lipoprotein antiserum does possess some reactivity against high density lipoprotein in view of the biphasic nature of the precipitin curve in whole plasma.

When studied by the Ouchterlony agar diffusion technique as described by Kornfeld (14), the goat antiserum tested against the density <1.017 lipoproteins showed two precipitin lines, indicating possible heterogeneity of the lipoproteins in this fraction. It did not react with a lipoprotein-free plasma solution.

Technique of Liver Perfusion with Washed Red Blood Cell Suspensions—The general method of rat liver perfusion described by Miller et al. (8) was used. The rats were not fasted before the experiment.
surgery. Nembutal (50 mg per kg) was used as the anesthetic agent. The liver was perfused in situ and bile was collected from a cannula in the bile duct. The oxygenation system for the blood consisted of a rotating round bottom flask with glass tubing sealed at the bottom to serve as an exit; 5% CO₂-95% O₂ was admitted to the flask during the perfusion.

The donor rat blood was heparinized but the red cells were washed twice with 0.15 M NaCl (heparin-free) and once with Krebs-Ringer bicarbonate solution containing 0.1% glucose (in most experiments) before resuspension in this medium to give a cell suspension with a hematocrit of 18 to 24.

In some experiments heparin was administered to the rat before ligature of the vena cava, but it was not found necessary to include heparin routinely. The usual perfusion rate was 10 ml per minute and the total volume of perfusing solution in the reservoir was 70 to 100 ml.

An initial sample of perfusing solution was taken for analysis after 3 or 4 minutes. Although some livers remained in good condition longer than 1 hour, as judged by gross appearance and continued bile production, a 1-hour period was used. A correction for changes in concentration due to evaporation during the perfusion was made by analyzing the initial and final red blood cell suspension for total hemoglobin content. The volume of supernatant fluid was estimated from the hematocrit. After removal of red cells by low speed centrifugation, all samples of supernatant fluid were centrifuged at 30,000 × g for 10 minutes before analysis for lipoprotein, to remove any residual red cells or red cell ghosts.

Specificity of Immunochemical Determination of Plasma Lipoprotein in Liver Extract or Perfusate—The question of whether the precipitate formed after the incubation of liver extracts or perfusates with anti-low density antiserum did, in fact, represent the precipitate formed after the incubation of liver extracts or red cell ghosts.

Four grams of liver slices, previously washed in Krebs-Ringer bicarbonate buffer for 45 minutes at 37°C, were homogenized in 30 ml of 0.15 M NaCl. After centrifugation at 25,000 × g for 20 minutes, aliquots of 3 ml of the supernatant solution were incubated for 16 hours with 0.3 ml of a 1:10 dilution of goat antiserum in 0.15 M NaCl. After removal of the precipitate by centrifugation, the supernatant solution was tested for antibodies by reaction with rat serum.
TABLE I

<table>
<thead>
<tr>
<th>Source of antigen*</th>
<th>Density of centrifugation</th>
<th>Lipoprotein-protein concentration µg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Slices: Top ml</td>
<td>1.063</td>
<td>16.4</td>
</tr>
<tr>
<td>Infranatant</td>
<td>1.065</td>
<td>15.8</td>
</tr>
<tr>
<td>Perfusate: Top ml</td>
<td>1.008</td>
<td>276</td>
</tr>
</tbody>
</table>

* Volumes of 10 ml of the sodium chloride soluble fractions of homogenized liver slices, or of the liver perfusion medium, were centrifuged for 16 hours in the Spinco No. 40 rotor at 36,000 r.p.m. The top milliliter was removed and the remaining 9 ml mixed before analysis for lipoprotein with antiserum.

TABLE II

<table>
<thead>
<tr>
<th>Medium</th>
<th>Sodium chloride soluble</th>
<th>Particles</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein, µg per g wet wt. per hour</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Initial (8)†</td>
<td>17</td>
<td>30</td>
<td>89</td>
</tr>
<tr>
<td>Increase per hour, aerobic (8)</td>
<td>+52</td>
<td>+10</td>
<td>+8</td>
</tr>
<tr>
<td>Increase per hour, anaerobic (3)</td>
<td>+31</td>
<td>-18</td>
<td>-12</td>
</tr>
</tbody>
</table>

| Cholesterol, µg per g wet wt. per hour |
| Initial (8)       | 0 | 11 | 78 | 89 |
| Increase per hour, aerobic (8) | +16 | +8 | -4 | +20 ± 3.0† |
| Increase per hour, anaerobic (3)   | +14 | -7 | -11 | -4 |

* Incubation conditions and treatment of samples before analysis were those described by Marsh and Drabkin (4). Thus, incubation mixtures were separated into three fractions: incubation medium, sodium chloride soluble extract of homogenized slices, and deoxycholate solution of particles from homogenized slices. Initial values represent preparations of slices preincubated for 30 minutes at 37° to reduce the lipoprotein content of the fresh slices. After resuspension of the slices in fresh medium, the incubation time was 2 hours. The increments were reasonably linear with time and the 2-hour values were divided in half to facilitate comparison of the slice experiments and those with isolated perfused liver. Anaerobic incubations were conducted under Nz.
† The numbers in parentheses represent the number of experiments.
‡ Standard error of the mean.

(b) Ultracentrifugation of liver extracts or perfusates resulted in concentration of the reacting (antigenic) material in the top portions of the centrifuge tube (Table I).

(c) The immune precipitate contained cholesterol. In experiments with liver slices incubated with acetate-1-C14 or mevalonic acid-2-C14, the cholesterol in the immune precipitate was found to be significantly labeled, as judged by isolation and purification as the digitonide after the addition of carrier cholesterol.

RESULTS

Net Synthesis of Plasma Low Density Lipoproteins by Liver Slices—The incubation medium employed and other procedural details were identical with those previously described for the net synthesis of albumin (4). The results of the experiments are given in Table II. Although these results are interpreted as indicating a net synthesis of low density lipoprotein, the possibility must be considered that, even though the antiserum contains a barely detectable titer against high density lipoproteins, the amount of antiserum used in these experiments may be sufficient to react with high density lipoprotein synthesized by the slices. The fact that most, though not all, of the reacting antigen which appears in the medium and the sodium chloride soluble fractions of the slices can be concentrated by ultracentrifugation at density 1.063 (Table I) is evidence against this possibility. However, it has not so far been possible to concentrate the low density lipoproteins present in the deoxycholate solution of the particles. Furthermore, a study of the quantitative precipitin curves obtained by reacting the deoxycholate solution of the particles with the antiserum reveals that considerable cross-reaction occurs, presumably with tissue lipoproteins which have been solubilized. The fact that slices incubated under anaerobic conditions show a considerable loss in the lipoprotein of the saline-soluble and particulate fractions strengthens the conclusion that net synthesis, and not passive transfer of lipoprotein from particles to incubation medium, occurs in the aerobic system. Because of the problems involved in the interpretation of the data for particulate lipoprotein, the synthesis of plasma lipoproteins by the isolated perfused liver was studied.

Net Synthesis of Low Density Plasma Lipoproteins in Isolated Rat Liver Perfused with Washed Red Blood Cells—Analysis for low density lipoprotein in aliquots of the initial and final supernatant fluid obtained during the perfusion gave the results shown in Table III. The values for net synthesis of the protein and cholesterol moieties compare favorably with those given in Table II for liver slices. Anaerobiosis greatly reduced the final lipoprotein content of the perfusion medium. Thus, it appears that the increment of lipoprotein in the perfusion fluid under aerobic conditions is not derived from red cells or from the washing out of preformed liver lipoprotein.

Labeling experiments with C14-amino acids in livers perfused with whole blood also indicated extensive incorporation of the label into the protein of low and high density lipoprotein. The

TABLE III

<table>
<thead>
<tr>
<th>Type of experiment</th>
<th>No. of experiments</th>
<th>Cholesterol µg*</th>
<th>Protein µg*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aerobic</td>
<td>9</td>
<td>+80 ± 13†</td>
<td></td>
</tr>
<tr>
<td>Anaerobic†</td>
<td>5</td>
<td>+80 ± 13†</td>
<td></td>
</tr>
<tr>
<td>Aerobic</td>
<td>5</td>
<td>+28 ± 6.7†</td>
<td></td>
</tr>
</tbody>
</table>

* Expressed as net increase per g (wet wt.) of liver in 1 hour at 37°.
† Standard error of the mean.
‡ Nz in the gas phase; 0.002 M KCN added to the perfusion fluid.
results were in accord with the recently published experiments of Radding et al. (9) and are therefore not recorded here.

In addition to the use of antiserum for the measurement of lipoproteins in the perfusion fluid, experiments were carried out in which direct measurements of the lipoproteins present were made with the analytical ultracentrifuge. At the conclusion of each experiment, the perfusion fluid supernatant, generally 30 ml, was adjusted to density 1.063 and centrifuged 16 hours at 36,000 r.p.m. in the Spinco No. 40 rotor. The top 1.5 ml of each tube were removed and the top fractions combined and recentrifuged. The top 1.2 ml of the final concentrate for each perfusate were then studied in the analytical ultracentrifuge (Table IV).

The net synthesis of low density lipoprotein, obtained by subtracting the anarocobic values from those obtained under aerobic conditions, was equivalent to 125 μg per g of liver perfused for 1 hour. Assuming a content of 14% cholesterol (calculated from the percentage of cholesterol found in isolated plasma low density lipoprotein), an average net synthesis of 17 μg of cholesterol per g of liver may be calculated. This figure is of the same order of magnitude as that obtained in liver slices (Table II) and perfused liver (Table III) by the immunochemical method. However, assuming that 15% of the molecule (Table IV) is protein, the corresponding value for lipoprotein-protein is 19 μg per g of perfused liver per hour. This is lower than the comparable figure of 79 μg obtained by immunochemical analysis (Table III).

The distribution of the lipoprotein S, values with respect to the presence or absence of heparin during the aerobic perfusion is shown in Table V. The characteristic effect of heparin (19) on the low density lipoprotein pattern is evident.

**DISCUSSION**

A net synthesis of low density plasma lipoprotein by rat liver slices and perfused rat liver has been demonstrated in these experiments. Four lines of evidence support the view that the immunochemical method used is specific for low density rat plasma lipoproteins. First, immunological absorption studies indicated that absorption of antiserum with liver extract diminished its titer against plasma lipoproteins (and vice versa). Second, the reacting liver antigen was greatly concentrated in the top fraction after preparative ultracentrifugation at density 1.063. Third, the immune precipitate contained cholesterol in amounts similar to those found in isolated plasma lipoprotein; and when radioactive acetate or mevalonate was added to the system, the cholesterol was labeled. Fourth, measurement of lipoprotein-cholesterol synthesis in the perfused rat liver by the independent method of analytical ultracentrifugation gave reasonable agreement with the results obtained by the immunochemical method.

One of the disadvantages of the immunochemical method, as presently employed, is the probable heterogeneity of the low density lipoproteins used as the immunizing antigen. Rodbell (16) has reported 3 different N-terminal amino acids in the protein moiety of the low density lipoproteins. It is possible, therefore, that one or more of the proteins present in this fraction need not be made in the liver (20). It should be noted, however, that experiments with kidney slices, intestine, and adipose tissue in vitro did not reveal net synthesis of lipoprotein when studied with anti-low density lipoprotein antiserum. The antiserum employed reacts with chylomicros, but not to any appreciable extent with high density lipoprotein. In view of the fact that very high ratios of antiserum to antigen (shown in Fig. 2) did precipitate additional lipoprotein-cholesterol, it is possible that the immune precipitate in these experiments included some high density lipoprotein along with the low density lipoprotein. The labeling studies of Radding et al. (9) indicate that the liver can synthesize protein moieties of both low and high density lipoprotein.

The failure of some observers (21) to demonstrate low density lipoprotein in liver homogenates may be explained by the very low concentrations present. In several experiments, we have homogenized liver (perfused to remove blood) and, after successive concentration of large volumes of homogenate in the preparative ultracentrifuge at density 1.063, have obtained values in the neighborhood of 20 μg per g of tissue as measured by the analytical ultracentrifuge. If the ratio of the lipoprotein concentration in plasma to that in liver is similar to the ratio for plasma albumin (4), the plasma low-density lipoprotein present in liver should have about 1/28 the concentration in plasma. Hillyard et al. (22) report a value of 0.74 mg per ml for this plasma fraction in the rat; therefore, a figure of 20 μg of plasma low density lipoprotein per g of liver appears reasonable.

Landon and Greenberg (23) have reported a turnover time of plasma cholesterol in the rat of approximately 36 hours. Due to the exchange of isotopic plasma cholesterol with liver and red cell cholesterol (24), calculation of rates of synthesis from this data is uncertain. If the total liver and blood cholesterol of a 100-g rat is taken as 20 mg, then the replacement rate of cholesterol would be 555 μg per hour. If this were entirely due to the activity of the liver, then the replacement rate per g of liver per hour would be 125 μg per g of liver per hour.

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**Table IV**

| Type of experiment* | No. of experiments | Low density lipoprotein content of perfusion fluid μg/g of liver
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Aerobic.............</td>
<td>9</td>
<td>199 ± 35†</td>
</tr>
<tr>
<td>Anaerobic...........</td>
<td>5</td>
<td>34 ± 29†</td>
</tr>
</tbody>
</table>

* Perfusions were carried out for 1 hour at 37° in the presence of 95% O₂-5% CO₂ (aerobic experiments) or 100% N₂ in the gas phase (anaerobic experiments). In the anaerobic experiments, the hemoglobin was completely reduced with Na₂S₂O₅ at the start of the perfusion.

† Standard error of the mean.

**Table V**

<table>
<thead>
<tr>
<th>S₁ range</th>
<th>Total lipoprotein</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No heparin</td>
</tr>
<tr>
<td>4-28</td>
<td>%</td>
</tr>
<tr>
<td>29-51</td>
<td>30</td>
</tr>
<tr>
<td>52-80</td>
<td>0</td>
</tr>
<tr>
<td>81-103</td>
<td>32</td>
</tr>
</tbody>
</table>

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These experiments were done in collaboration with Dr. Verne Schumaker, to whom the authors are very much indebted.

1 J. R. Marsh, unpublished experiments.
hour would be 110 μg. The data in Fig. 2 indicate that about 43% of the plasma cholesterol would be expected to react with the goat antiserum. Preliminary experiments with an antiserum prepared against high density lipoprotein by the liver has not been settled by the present experiments, the results of Radding et al. (9) suggest an affirmative answer. Preliminary experiments with an antiserum prepared against high density lipoprotein support this view, but further investigation of the problem will be required.

**SUMMARY**

With the aid of the immunochemical technique, net synthesis of low density plasma lipoproteins has been demonstrated in liver slices and in perfused liver of the rat. Goat antiserum against low density lipoproteins isolated from rat plasma was used to precipitate the low density lipoproteins of the liver preparations. From the analytical data, it was calculated that in liver slices, 70 μg of lipoprotein-protein and 20 μg of lipoprotein-cholesterol were synthesized per g of tissue per hour; in the perfused liver, the corresponding values were 89 μg (protein moiety) and 28 μg (cholesterol moiety).

With perfused liver, direct examination of perfusates by means of the analytical ultracentrifuge indicated a net synthesis of low density lipoprotein averaging 125 μg (total molecule) per g of liver per hour. In these perfusion experiments, the presence of heparin in the perfusion fluid caused a shift in pattern of low density lipoproteins in the direction of lower Sf values.

**Acknowledgments**—The authors are greatly indebted to Drs. Robert R. Marshak and Charles W. Raker of the School of Veterinary Medicine for their assistance with the goats and to Miss Francilla Sherry for technical assistance.

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