JCI The Journal of Clinical Investigation

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J Clin Invest. 1960;39(10):1560-1569. https://doi.org/10.1172/JCI104177.

Research Article

STUDIES ON THE SYNTHESIS AND SECRETION OF SERUM LIPOPROTEINS BY RAT LIVER SLICES

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(Submitted for publication May 23, 1960; accepted June 19, 1960)

There is at present a great deal of interest in factors determining the levels of serum lipids because of their possible relation to atherosclerosis. Since the serum lipids circulate only in the form of lipid-protein complexes, the mechanism of production of these complexes and their delivery into and removal from the serum must be understood before there is a sound basis for defining the homeostatic mechanisms controlling the levels of individual lipid components. While factors influencing the metabolism of any one of the individual lipid moieties may influence the serum levels of that moiety, such an effect must ultimately be expressed indirectly through an alteration in the rate or pattern of metabolism of the lipid-protein complexes of which it is a part.

The present experiments were undertaken in order to define an *in vitro* system in which lipoprotein biosynthesis could be studied without the many complicating variables encountered in whole-animal studies. It is generally accepted that the liver is the principal source of the serum lipids (1–3) and it has been assumed, but not proved, that the liver is also the source of the protein moiety of the serum lipoproteins. We have previously described the incorporation by perfused rat liver and by rat liver slices of labeled amino acids into proteins with densities similar to those of the serum lipoproteins (4, 5). The studies reported here provide evidence that the high density lipoproteins synthesized and secreted by rat liver slices are identical with the high density lipoproteins of normal rat serum. The time course of synthesis of both the protein and the lipid moieties in the total lipoprotein fraction, the rate of appearance of radioactive lipoprotein in the medium, and the net changes in protein and lipid in liver slice and medium are presented. The effects of cholesterol feeding, Triton injection and the nephrotic state are also described.

MATERIALS AND METHODS

DL-leucine-1-C^{14} (5.3 mc per mmole) and sodium acetate-1-C^{14} (3.5 mc per mmole) were obtained from the Nuclear-Chicago Corp. A complete mixture of uniformly labeled C^{14}-amino acids was prepared from a thermophilic strain of *Chlorella* grown in a synthetic medium with $C^{14}O_2$ as the sole source of carbon, according to the method described by Catch (6). The cells were lysed and the lipids were extracted with organic solvents. The precipitated proteins were washed several times with 10 per cent trichloroacetic acid (TCA) and then with organic solvents. The washed proteins were hydrolyzed in 5.5 N HCl, the excess HCl was removed by evaporation to dryness, and the hydrolysate was passed over a column of Dowex-50 in the H^+ form. The amino acids, eluted with 4 M NH_4OH, had a specific radioactivity of 25 mc per mmole of ninhydrin color (referred to leucine).

Twice-recrystallized trypsin and chymotrypsin were obtained from Armour Laboratories. Triton WR-1339 (oxyethylated tertiary octyl phenol formaldehyde polymer) was obtained from Winthrop Laboratories.

Tissue incubations. Male Osborne-Mendel rats, maintained on Purina Laboratory Chow *ad libitum* and weighing approximately 250 g at the time of sacrifice, were used. Rats with the nephrotic syndrome were prepared [1] by the injection of nephrotoxic serum using the methods described by Baxter and Goodman (7). The rats were decapitated, the livers were rapidly removed and chilled, and slices were prepared for incubation using a Stadie-Riggs microtome. In most experiments 500 mg of liver slices was incubated in 5 ml of fresh rat serum prepared from blood drawn from control rats under ether anesthesia. The slices were incubated for up to 4 hours at 37° C under an atmosphere of 95 per cent O_2–5 per cent CO_2. In one set of experiments, in order to permit measurement of net changes in the medium, liver slices were incubated in Krebs' bicarbonate medium.

Isolation of lipoprotein fractions. At the end of the incubation the liver slices were removed from the medium which was then adjusted to the desired density. In order

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[1] We acknowledge with thanks the assistance of Dr. James C. Allen and Dr. James H. Baxter in making these animals available.

to minimize solution volumes, adjustments of density were made by addition of the appropriate amounts of solid KBr calculated according to the following equation:

$$V_0 d_0 + x = d(V_0 + \bar{V}x),$$

where x = amount of KBr in grams, V_0 = initial volume in milliliters, d_0 = initial density, d = final (desired) density, and $\bar{V}$ = apparent specific volume of KBr at the final density (8).

In studies of the time course of lipoprotein synthesis aliquots of tissue slices and medium were removed at intervals. The tissue slices were homogenized in 0.9 per cent saline in a glass homogenizer. These saline homogenates and the aliquots of medium were centrifuged for 1 hour at 100,000 × G in a Spinco model L preparative ultracentrifuge. Lipids floating to the top of the tube were removed with a tube slicer (9) and the clear supernatant was decanted, leaving behind cellular debris. The supernatant solution was then adjusted to density 1.21 and centrifuged for 48 hours at 114,000 × G. The upper 2.5 ml of the tube volume was isolated by means of the tube slicer. In the course of these studies it was found that, although all of the lipoproteins were concentrated into the upper 2.5 ml of the centrifuge tube by 48 hours of centrifugation at 114,000 × G, the layer immediately below the lipoproteins was not completely free of protein. This may reflect the presence of proteins with density close to 1.21 or the presence of tissue debris with density near 1.21, possibly fragments of cell wall. The presence of such non-lipoprotein proteins extending into the upper layer would alter the observed specific radioactivities for the lipoprotein fractions. To minimize possible errors of this kind the serum medium in the later studies was adjusted to density 1.21 and carefully layered under KBr solution of equal density so that the lipoproteins migrated to the top of the tube through a protein-free medium. In addition, the isolated lipoprotein fractions were passed through Millipore filters (0.4 μ pore diameter) to remove any particles of low density cell debris prior to analysis.

In the studies designed to characterize the protein moieties and relate them to serum lipoproteins the serum medium was first adjusted to density 1.063 and centrifuged at 114,000 × G for 24 hours. The low density lipoproteins (*D < 1.063 fraction*) were separated and the infranatant solution was adjusted to density 1.21. After centrifugation for 48 hours at 114,000 × G the upper layer (*D 1.063–1.21 fraction*) was separated. The proteins in the infranatant layer, presumably free of lipoproteins, are referred to as the *D > 1.21 fraction* or the *residual proteins.*

Proteolytic degradation and characterization by peptide pattern. The work of Sanger and Tuppy (10) and that of Ingram (11) has shown that the digestion of proteins with pure proteolytic enzymes of well defined specificity results in the production of a highly reproducible mixture of peptide degradation products. These usually can be well separated on paper using chromatography in one direction and high-voltage electrophoresis in the perpendicular direction. The resulting peptide pattern, developed by the use of ninhydrin staining, is characteristic for each protein and has been termed a "fingerprint" by Ingram (11). We employed a modification of this method [2] described by Katz, Dreyer and Anfinsen (12).

The ultracentrifugally isolated lipoproteins were delipidated by extraction with acetone-ethanol and then ether. The delipidated protein was suspended in 0.2 ml of 6 M urea in 0.2 M NH_4HCO_3 at pH 8.2 and allowed to remain overnight at 4° C. Then 2 vol of 0.2 M NH_4HCO_3 was added to lower the urea concentration to 2 M, and 25 μg of trypsin was added. After incubation for 2 hours at 30° C, 12.5 μg of chymotrypsin was added, and the incubation was continued for an additional 4 hours. The digest was then passed over a column of Dowex-50 (2 per cent cross-linkage; 50 to 100 mesh) in the H^+ form, and several column volumes of water were passed through. The peptides were eluted with 4 M NH_4OH and the eluate was lyophilized. Aliquots of the peptide mixture were spotted on sheets of Whatman no. 3 filter paper and the peptides were separated along one axis by descending chromatography using *n*-butanol: acetic acid: water (4:1:5) and then by high-voltage electrophoresis (13) in the perpendicular axis. Because the electrophoresis tank could not accommodate the full sheet it was necessary to cut each chromatogram in half and run the electrophoresis in two stages. Fingerprints obtained from lipoproteins synthesized in the presence of a complete mixture of C^{14}-labeled amino acids were autoradiographed by placing them in contact with Eastman Kodak type KK industrial X-ray film in cassettes. These were stored at − 15° C for 1 to 2 months at the end of which time the film was developed by standard methods.

Measurement of protein and protein radioactivity. Protein in the fractions analyzed was precipitated with 5 per cent TCA, washed once with 5 per cent TCA at 90° C, twice with cold TCA, once with ethanol: acetone, 1:1 (vol/vol), at 60° C, twice with cold 1:1 ethanol: acetone and once with ether. In the case of the D > 1.21 fraction, the TCA precipitate was resuspended and adjusted to a pH of approximately 6 before addition of ethanol-acetone because of the appreciable solubility of serum albumin in acidic ethanol-acetone. The dried protein precipitate was dissolved in 1 N NaOH. One aliquot of the alkaline solution was used for measurement of total protein by the method of Lowry, Rosebrough, Farr and Randall (14). For measurement of the small amounts of lipoprotein-protein in incubation media (less than 20 μg) it was necessary to scale this method down, using the Beckman spectrophotometer with microcell attachment for readings of optical density. A second aliquot was transferred to a counting vial and dried at 130° C for 4 hours. One ml of 1 N Hyamine in methanol (Rohm and Haas, Philadelphia, Pa.) was added to the dried cake in the bottom of the vial and it was allowed to stand overnight at room temperature. The next day the remaining caked material was broken up with a spatula, and Hyamine remaining on the spatula was rinsed back

[2] We acknowledge with thanks the generous assistance of Dr. Arnold Katz in our application of this technique.

into the vial with methanol. The vial was then heated at 40 to 60° C for 4 hours, after which 15 ml of 0.6 per cent 2,5-diphenyloxazole in toluene was added with 1 to 2 ml additional methanol. This procedure leaves a residual white sediment in the vial, presumably of NaOH, but the extraction of labeled protein by Hyamine appears to be complete (15). This was demonstrated in the range from 10 to 200 μg of protein sample with a sample of C^{14}-labeled protein of known specific radioactivity. Radioactivity was measured in a Packard liquid scintillation spectrometer.

Measurement of lipids and lipid radioactivity. The pooled ethanol-acetone and ether extracts were taken to dryness on a steam bath and redissolved in chloroform: methanol, 2:1 (vol/vol). The chloroform-methanol extract was washed three times according to the method of Folch and co-workers to remove nonlipid materials (16). An aliquot for radioassay was taken to dryness in a counting vial to which was added 15 ml of 0.6 per cent 2,5-diphenyloxazole in toluene, and the vial was counted in the liquid scintillation spectrometer. In some experiments the incorporation of acetate-1-C^{14} into the nonsaponifiable lipid fraction was measured (17). Cholesterol was determined by the method of Sperry and Webb (18) and phospholipids were determined by the method of Stewart and Hendry (19).

RESULTS

Identification of newly synthesized and secreted D 1.063–1.21 lipoproteins with normal serum D 1.063–1.21 lipoprotein. The net amount of lipoprotein appearing in an inorganic medium during liver slice incubations was extremely small—too small to permit direct chemical and physical characterization. An indirect but highly specific method was used to show that the newly synthesized D 1.063–1.21 lipoproteins were identical with those normally present in rat serum. The approach used was based on the uniqueness of the peptide pattern obtained by digestion of a protein with pure proteolytic enzymes of well defined specificity (10–12). Newly synthesized lipoproteins labeled throughout the peptide chain were prepared by incubating rat liver slices in serum with a complete mixture of uniformly C^{14}-labeled amino acids of high specific radioactivity. If the newly synthesized D 1.063–1.21 lipoproteins are structurally identical with the normal serum lipoproteins in that density class, then there should be radioactivity associated with every peptide derived from the serum lipoproteins, and the radioactivity in the peptide mixture should be limited exclusively to these peptides. The peptide pattern or fingerprint, as developed with ninhydrin staining, demonstrates exclusively the peptides derived from the lipoproteins in the serum incubation medium; the quantities of lipoprotein added to the serum medium during incubation are negligible relative to the quantities in the serum. In other words, the lipoproteins in the serum medium serve here as unlabeled carrier material for the small amounts of highly labeled lipoprotein produced during the incubation.

In Figure 1b is shown a photograph of the ninhydrin-positive spots in a fingerprint of the D 1.063–1.21 lipoprotein isolated from the serum medium after incubation of liver slices with C^{14}-labeled amino acids. In Figure 1c is shown a fingerprint of D 1.063–1.21 lipoprotein of normal rat serum. Comparison of these indicates the reproducibility of the peptide pattern and also shows that the incubation with liver slices did not introduce any detectable amounts of protein differing structurally from those present in this class in normal serum. In Figure 1a is shown a photograph of an autoradiogram made from the fingerprint shown in Figure 1b. While the resolution in the central group of peptides in the autoradiogram is not adequately shown, direct comparison with the fingerprint shows that to every peptide spot developed with ninhydrin there is a corresponding radioactive spot developed on the autoradiogram. Furthermore, there are no radioactive spots not associated with a corresponding ninhydrin spot. It should be noted that the *intensities* of the spots in the autoradiogram are not expected to parallel the intensities of the ninhydrin spots. Assuming stoichiometric splitting of susceptible bonds, the intensities of the spots in the autoradiogram will be roughly proportional to the lengths of the peptides, since all the amino acids are labeled. On the other hand, the ninhydrin color yield of the peptides will depend upon the number of free amino groups and the particular amino acid composition. Only the locations of the spots should be considered. It can be concluded that the newly synthesized D 1.063–1.21 lipoprotein-proteins secreted into the medium are structurally identical with normal rat serum D 1.063–1.21 lipoprotein-proteins.

Peptide pattern of D < 1.063 lipoproteins. A peptide pattern and autoradiogram were prepared from labeled D < 1.063 lipoproteins prepared ex-

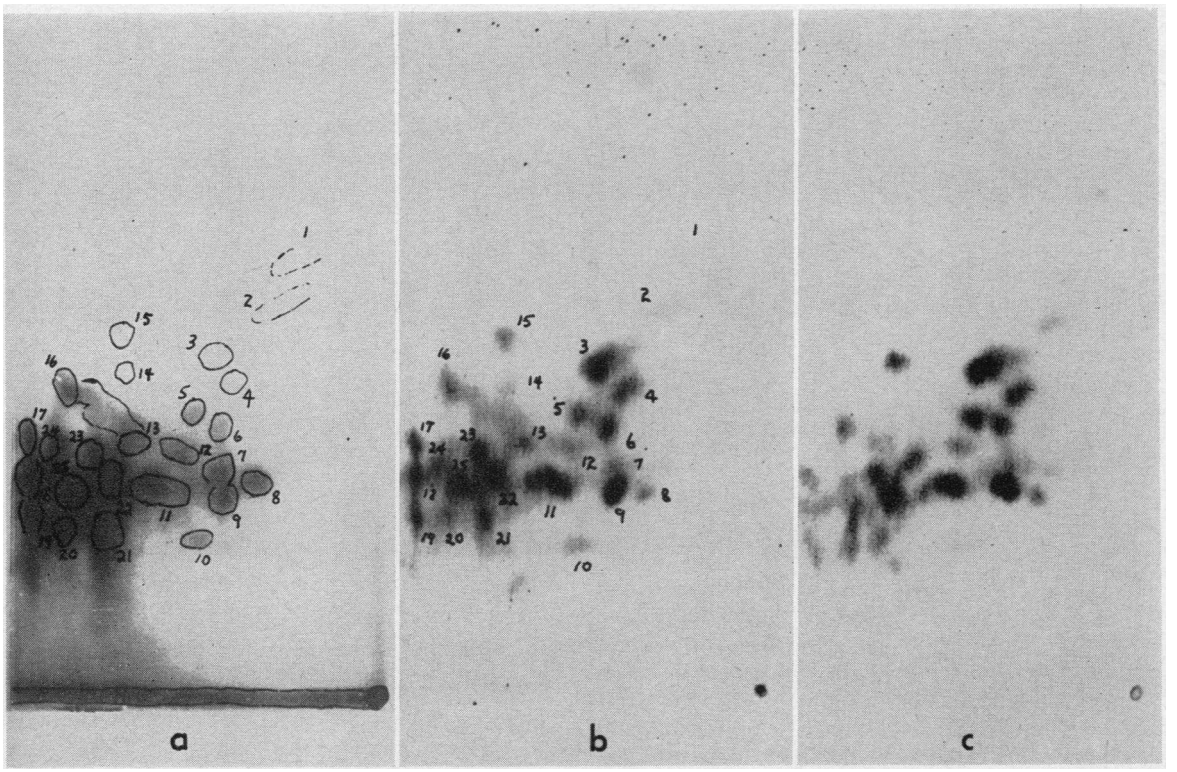

FIG. 1. a) AUTORADIOGRAM OF PEPTIDE PATTERN SHOWN IN b. The better-defined spots in the X-ray film have been outlined on an overlay and numbered to indicate correspondence with ninhydrin-positive spots in the fingerprint. b) PEPTIDE PATTERN OR "FINGERPRINT" OBTAINED FROM D 1.063–1.21 LIPOPROTEIN ISOLATED FROM THE SERUM MEDIUM AFTER INCUBATION FOR 4 HOURS AT 37° C WITH RAT LIVER SLICES AND A COMPLETE MIXTURE OF RANDOMLY LABELED C^{14}-AMINO ACIDS AS DESCRIBED IN THE TEXT. (Paper chromatography in the horizontal direction, high voltage electrophoresis in the vertical direction.) A few peptide spots, which ran near the front, are not shown since the paper had to be divided prior to electrophoresis. These spots not shown also correlated well with radioactive spots in the autoradiogram. c) PEPTIDE PATTERN OF D 1.063–1.21 LIPOPROTEIN-PROTEIN ISOLATED FROM NORMAL RAT SERUM THAT HAD BEEN INCUBATED AS ABOVE BUT IN THE ABSENCE OF LIVER SLICES.

actly as described above for the D 1.063–1.21 lipoproteins. There were approximately 24 peptide spots in the peptide pattern as developed with ninhydrin. The pattern of these peptides isolated from the serum medium at the end of incubation with liver slices was indistinguishable from that obtained from D < 1.063 lipoproteins isolated from normal rat serum. Thus, as in the case of the D 1.063–1.21 lipoproteins, there was no evidence for the addition of significant amounts of new D < 1.063 lipoproteins to the medium as the result of incubation with liver slices.

Autoradiography revealed the presence of radioactivity in 17 of the 24 detectable ninhydrin-staining peptides, but there were 10 to 15 radioactive spots not correlated with a ninhydrin positive spot. The incomplete identity between peptide pattern and autoradiogram may be due to technical difficulties such as incomplete proteolytic degradation or inadequate exposure time. Alternatively, the result may truly indicate that the newly synthesized lipoproteins appearing in the medium are not identical with or represent only a small fraction of the D < 1.063 lipoproteins found in normal rat serum. The presence of radioactivity in the large majority of the peptides derived from circulating low density lipoprotein suggests, but does not prove, that some of the newly synthesized and secreted lipoproteins do represent normal low density serum lipoproteins. Support for this interpretation comes from the studies of Marsh and Whereat, reported while this work was in progress (20, 21). These authors, using immunological techniques, have shown that rat liver slices incubated in an inorganic medium secrete low density lipoproteins that are im-

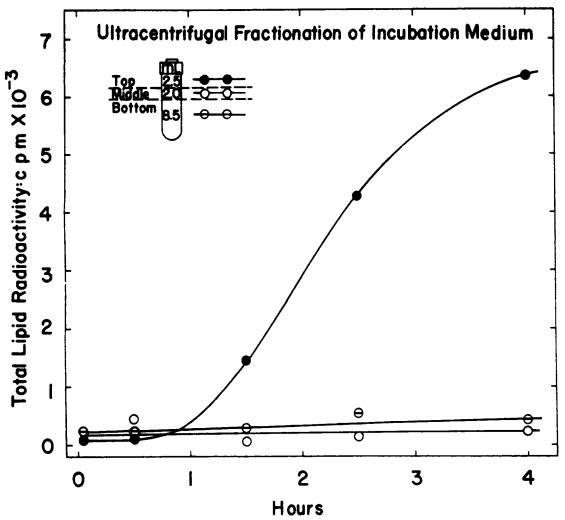

FIG. 2. TOTAL LIPID RADIOACTIVITY IN MEDIUM LIPOPROTEINS ($D < 1.21$) AS A FUNCTION OF TIME OF INCUBATION. Rat liver slices were incubated in Krebs' bicarbonate buffer containing 1-C^{14}-acetate and 1-C^{14}-leucine. Upper left: the key here indicates the manner in which the centrifuge tube was divided for analysis. After centrifugation as described under Methods, the upper 2.5 ml was taken as one fraction, the next 2.0 ml as a second fraction and finally the bottom 8.5 ml as a third fraction. Analyses of these fractions are shown separately according to this key in Figures 2, 3 and 5.

munochemically the same as low density lipoproteins found in normal rat serum.

Time course of lipoprotein synthesis. In all of the following studies the total lipoprotein fraction ($D < 1.21$) was isolated without subfractionation into low and high density groups. When rat liver slices were incubated in bicarbonate buffer with 1-C^{14}-acetate and 1-C^{14}-leucine, lipoproteins labeled both in the lipid and protein moieties appeared in the medium. In zero time control samples, there was negligible radioactivity in both lipid and protein moieties. As shown in Figure 2 there was an initial lag period in the appearance of labeled lipid in the lipoproteins of the medium after which the radioactivity of this fraction continued to increase for up to 4 hours. Little or no labeled lipid was found in the $D > 1.21$ fraction, indicating that the lipoproteins were effectively concentrated into the upper 2.5 ml of the tube by the flotation technique used.

The time course for appearance of labeled lipid in the tissue lipoproteins ($D < 1.21$ fraction) is shown in Figure 3. The incorporation of lipid radioactivity into these intracellular lipoproteins showed less of an initial time lag but fell off more rapidly, perhaps because of exhaustion of the small amount of labeled acetate used. Evidently transfer of label to the medium continued to occur even though the incorporation of label into intracellular lipoproteins after 1.5 hours of incubation was minimal. It should be noted that the total amount of lipid radioactivity in the intracellular lipoproteins was 15 to 20 times greater than the amount appearing in the lipoproteins of the medium. Effective flotation of tissue lipoproteins was demonstrated by the minimal amounts of lipid radioactivity found in the middle and bottom layers of the tube (Figure 3).

The specific radioactivity of the protein moiety of the lipoproteins in the medium and in the liver slices is shown in Figure 4 as a function of time. After an initial lag period, similar to that seen for appearance of lipid radioactivity, the specific radioactivity of the lipoprotein-protein in the medium increased almost linearly with time for 4 hours. Incorporation into the protein of the slice lipoproteins began more rapidly, and the specific radioactivity of this fraction was higher than that of the lipoproteins in the medium except at the 4-hour point. The data are consistent with the expected precursor-product relation between the intracel-

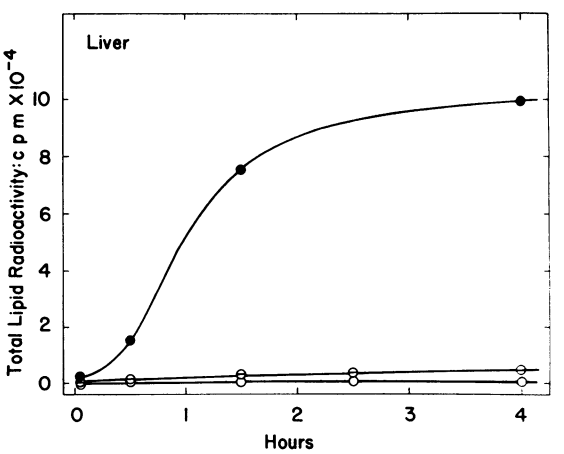

FIG. 3. TOTAL LIPID RADIOACTIVITY IN TISSUE SLICE LIPOPROTEINS ($D < 1.21$) AS A FUNCTION OF TIME OF INCUBATION. Rat liver slices were incubated in Krebs' bicarbonate buffer containing 1-C^{14}-acetate and 1-C^{14}-leucine. Results are shown for counts in the upper 2.5 ml (●——●), the middle 2.0 ml (○——○) and the bottom 8.5 ml (⊖——⊖) of the tube (key as in Figure 2).

lular lipoproteins and the lipoproteins of the medium.

Net changes in lipoprotein levels during incubation. The amounts of both protein and phospholipid in the lipoprotein fraction of the tissue slices increased during the first 1.5 hours of incubation but then showed no further increase at 4 hours. The amount of lipoprotein-protein increased from 470 μg per g of liver at zero time to 730 μg per g at 1.5 hours; the lipoprotein-phospholipid increased from 160 μg per g of liver at zero time to 230 μg per g at 1.5 hours. No attempts were made to characterize structurally the lipoproteins in the tissue slice. To what extent these represent newly synthesized plasma proteins and to what extent they represent exclusively intracellular lipoproteins is not known. Despite these measurable increases in tissue slice lipoprotein there was no demonstrable increase in the amount of lipoprotein in the medium. It should be noted that the concentration of lipoprotein in the medium was extremely low, about 1 μg per ml of medium. Losses due to denaturation or adsorption or losses during isolation cannot be ruled out. The fact that the *specific radioactivity* of the medium lipoprotein-proteins continued to rise linearly with

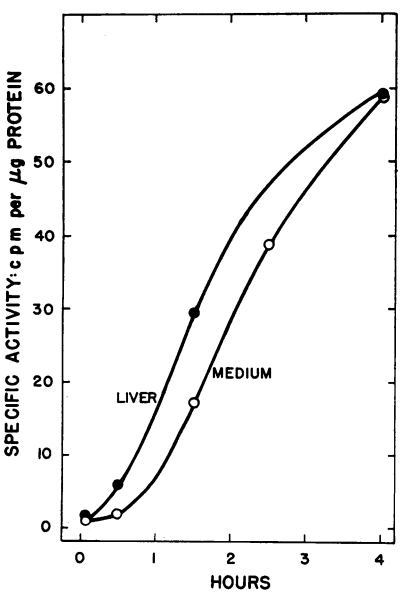

FIG. 4. SPECIFIC RADIOACTIVITY OF THE PROTEIN MOIETY OF THE D < 1.21 LIPOPROTEINS. In liver slices (●——●) and in medium (○——○) during incubation in Krebs' bicarbonate buffer containing 1-C^{14}-leucine and 1-C^{14}-acetate.

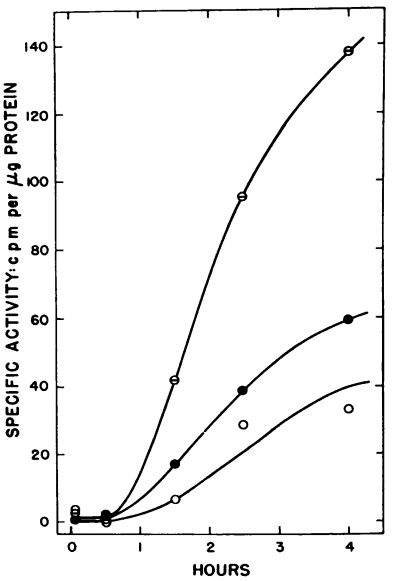

FIG. 5. SPECIFIC RADIOACTIVITY OF THE PROTEIN MOIETY OF THE MEDIUM PROTEINS IN THREE DIFFERENT LAYERS OF THE CENTRIFUGE TUBE AFTER CENTRIFUGATION AT DENSITY 1.21. Rat liver slices were incubated in Krebs' bicarbonate buffer containing 1-C^{14}-leucine and 1-C^{14}-acetate for the times indicated (key as in Figure 2).

no concomitant rise in their concentration might also be due to re-entry of medium lipoproteins into the slice or exchange of intracellular and extracellular lipoproteins.

Observations on the ultracentrifugal separation of lipoproteins from infranatant proteins. The flotation technique used in these studies for isolation of lipoproteins is essentially that described by Havel, Eder and Bragdon (22), a standard procedure that has been used by many other workers studying serum lipoproteins. That the lipoproteins are effectively concentrated into the upper 2.5 ml of the tube by this procedure is shown by the data of Figures 2 and 3 discussed above. The 2.0 ml of solution immediately below this top fraction, however, was found to contain protein. In other words, there was no protein-free zone clearly separating the lipoprotein fraction from the residual fraction. The concentration of protein in the middle layer was actually greater than that in the top layer. The extent to which the top layer was contaminated with what appear to be slowly sedimenting non-lipoprotein proteins could not be determined directly. However, the specific radioactivities of the proteins in top, middle and bot-

tom layers were separately measured, and the results are shown in Figure 5. In the incubation medium the bottom fraction was found to have the highest specific radioactivity, most likely due to newly synthesized and secreted serum albumin (23). The lipoprotein fraction at the top was next highest in specific radioactivity and the protein of the middle layer the lowest. Thus the top and bottom fractions were separated by a fraction of lower specific radioactivity, indicating that the lipoprotein fraction at the top was probably not contaminated by material of high specific radioactivity. No finer separation of the middle layer was attempted, and it may be that the protein of this fraction is indeed separated from the top layer by a narrow protein-free zone. The best evidence that this may be the case is the fact that the peptide patterns of the D 1.063–1.21 fraction from the medium, described above, and their autoradiograms coincide with the pattern of serum D 1.063–1.21 lipoproteins. On the other hand the data do not exclude the possibility that the gradient of sedimenting proteins may have extended up into the top 2.5 ml. These results and the results of Avigan, Eder and Steinberg (24) show that the routine methods used for preparative isolation of lipoproteins, while adequate for most purposes, must be carefully re-examined in each specific application and particularly in experiments involving measurements of lipoprotein-protein specific radioactivity. In the following experiments the layering technique and filtration step described under Methods were employed to minimize contamination by slowly sedimenting proteins or by particulate matter.

Relation between rates of cholesterol biosynthesis and rates of lipoprotein synthesis and secretion. Implicit in many discussions of cholesterol metabolism is the assumption that changes in the rate of cholesterol biosynthesis are reflected in changes in the rate of lipoprotein production and thus in serum lipoprotein levels. With the system described here it was possible to explore the question by inducing changes in the rate of cholesterol synthesis by appropriate pretreatment of the intact rat and then direct measurement of the rate of lipoprotein synthesis in liver slices incubated in serum *in vitro*. Stimulation of cholesterol synthesis in the liver was induced by intravenous injection of Triton WR-1339 (25). Inhibition of cholesterol synthesis was produced by feeding a diet containing 1 per cent cholesterol (26).

Liver slices from control rats and from rats pretreated as described were incubated in a serum medium to which were added both acetate-1-C^{14} and leucine-1-C^{14}, to permit simultaneous measurement of cholesterol synthesis and lipoprotein-protein synthesis. As shown in Table I, the rate of incorporation of radioactivity into total liver cholesterol was increased approximately 150 per cent by injection of Triton and decreased approximately 90 per cent by cholesterol feeding. Despite these large changes in the rates of cholesterol synthesis there were no significant changes in the rates of incorporation of radioactivity into the protein moiety of secreted lipoproteins. The rate of appearance of labeled cholesterol in the medium lipoproteins was not measured in these experiments, and no conclusions can be drawn regarding the influence of changes in biosynthetic rate on the rate of transfer of labeled sterol to the medium. The assumption is made that the rate of exchange would be similar in control and experimental liver slice incubations, since the medium contained equal concentrations of lipoprotein available for exchange reactions. In order to

TABLE I

Changes in rate of cholesterol synthesis without change in rate of lipoprotein-protein synthesis *

Group	No. of rats	Tissue non-saponifiable lipid radioactivity	Medium lipoprotein-protein radioactivity	Lipoprotein-protein radioactivity Residual protein radioactivity
		cpm X 10^{-3}/g liver		
Control	6	100 ± 16	6.9 ± 1.6	1.6 ± 0.18
Triton-treated	6	250 ± 82	8.4 ± 1.5	1.8 ± 0.34
Cholesterol-fed	6	11 ± 0.54	6.8 ± 1.1	2.1 ± 0.39

* Liver slices (500 mg) were in each case incubated for 2.5 hours at 37° C under 95% O_2:5% CO_2 in 5 ml of normal rat serum containing 10 μc sodium acetate-1-C^{14} and 15 μc DL-leucine-1-C^{14}. Nonsaponifiable lipids were isolated from the liver slices only; lipoproteins were isolated from the medium only, as described in the text.

check on the possibility that the pretreatment given the rats might have altered the general pattern of protein biosynthesis, and thus have masked an effect on lipoprotein biosynthesis, the specific radioactivity of the residual protein fraction was determined. For each group the specific radioactivity of the lipoprotein in the medium relative to the specific radioactivity of the residual protein fraction is shown in the last column of Table I. The differences are not statistically significant.

Comparison of rates of lipoprotein synthesis in normal and in nephrotic rats. Nephrotoxic serum nephritis was produced in a group of rats by injection of antikidney serum prepared in rabbits by the method of Baxter and Goodman (7). These animals develop marked proteinuria, hypoalbuminemia and strikingly elevated serum lipid levels. Serum lipid levels measured in two of the four rats studied were, respectively: total cholesterol, 640 and 336 mg per 100 ml; phospholipids, 632 and 386 mg per 100 ml; triglycerides, 3,150 and 1,380 mg per 100 ml. Liver slices from nephrotic rats and from normal rats were incubated with leucine-1-C^{14} in normal rat serum and the radioactivity appearing in the lipoprotein-proteins of the medium ($D < 1.21$ fraction) was measured. Results are expressed in terms of the specific radioactivity of the lipoprotein-proteins. Since, as shown in separate studies, the amount of lipoprotein in the serum medium does not change measurably during incubation these values are proportional to the *total amount* of radioactivity appearing in the medium lipoproteins. The specific radioactivity of the proteins in the residual fraction was also determined. As shown in Table II, the incorporation into both fractions was, with nephrotic rat liver slices, almost double the incorporation observed with normal rat liver slices. One possible interpretation of these results is that the true rate of synthesis of lipoproteins and also of the non-lipoprotein proteins, principally albumin (23), is increased in the nephrotic rat liver. An increased net synthesis of albumin by liver slices from nephrotic rats has been reported previously by Marsh and Drabkin (27). Overproduction of both would be quite compatible with the observed serum lipid concentrations, since the albumin is being lost at a rapid rate in the urine while the lipoproteins, being much larger, are not so lost. On the other hand there are no data to rule out differences in pool size of precursor amino acids or pool size of preformed, unlabeled intracellular proteins and lipoproteins.

TABLE II

Lipoprotein synthesis in liver slices from normal and nephrotic rats *

	Specific radioactivity of medium proteins	
	D <1.21 fraction	D >1.21 fraction
	cpm/mg	
Normal rats	760	300
	940	380
Mean	850	340
Nephrotic rats	1,100	550
	1,600	710
	1,600	570
	1,800	600
Mean	1,520	600

* Incubation and isolation procedures as described in footnote to Table I.

DISCUSSION

The present studies demonstrate that it is possible to explore the mechanism of lipoprotein biosynthesis and some of the factors controlling it by using an *in vitro* system. Little or nothing is known at present about the mechanisms by which the apparently quite specific lipid-protein complexes found in the serum are put together. The fact that virtually all of the lipid of serum lipoproteins can be stripped from the protein moiety by extraction with organic solvents indicates that the binding of lipid to protein is loose, largely the result of non-covalent linkages. It would be of great interest to explore the steps involved in the organization of the lipoprotein molecule, and the system described here may be valuable in such studies.

It has been unequivocally shown that the liver slice *in vitro* synthesizes and secretes high density lipoproteins (D 1.063–1.21) identical with those found in the serum. Thus it is established that the liver is a source, although not necessarily the only source, of the protein moiety as well as of the lipid found in this class of serum lipoproteins. The data with respect to low density lipoproteins, while suggestive, are inconclusive. Marsh and Whereat, on the other hand, have shown that at least some of the low density lipoproteins secreted

by rat liver slices are identical immunochemically with serum low density lipoproteins (20, 21).

Using the *in vitro* liver slice system it has been possible to study factors influencing rates of synthesis of lipoproteins. It was shown that the rate of synthesis of cholesterol can be varied over a wide range without altering the rate of synthesis of the protein moiety of the lipoproteins. Evidently the synthesis of new cholesterol is not rate-limiting in the production of lipoprotein molecules under these conditions. Studies by Haft and colleagues (28) on the incorporation of labeled lysine into lipoproteins by perfused rat liver have led to a similar conclusion. They found that depression of cholesterol synthesis by feeding of cholesterol or stimulation of cholesterol synthesis by pretreatment with X-rays did not cause parallel changes in synthesis and secretion of lipoprotein-protein into the perfusate. It should be noted, however, that these *in vitro* studies cover only a relatively short interval of time and that the preformed cholesterol available in the tissue may have been adequate to support lipoprotein production at a normal rate over this short interval of time. The possibility that alterations in the rate of cholesterol synthesis may ultimately alter the rate of lipoprotein production if studied over longer periods of time cannot be ruled out.

Liver slices taken from rats with the nephrotic syndrome incorporated labeled amino acids into lipoproteins more rapidly than did liver slices from normal rats. The rate of synthesis of the non-lipoprotein-proteins (residual proteins) was likewise increased. Marsh has recently reported that the *net* synthesis of low density lipoproteins by perfused livers from nephrotic rats is much greater than the rate seen with normal rat livers and that albumin synthesis is likewise increased, although to a lesser extent (29). The results are compatible with the hypothesis that the hyperlipoproteinemia of nephrosis is in part due to overproduction of lipoproteins.

SUMMARY

Rat liver slices incubated *in vitro* incorporated labeled amino acids into the protein moiety and labeled acetate into the lipid moiety of lipoproteins. The high density lipoproteins (D 1.063–1.21) secreted into the medium were shown to be identical with the normally circulating high density lipoproteins, according to the peptide-pattern or "fingerprint" method of Ingram. Depression of the rate of cholesterol synthesis by prefeeding of cholesterol or acceleration of the rate of cholesterol synthesis by pretreatment with Triton did not significantly alter the rate of incorporation of amino acids into lipoproteins. The rate of synthesis and secretion of the protein moiety of lipoproteins as well as other proteins was elevated in liver slices of nephrotic rats.

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