Inhibition of lipid synthesis in isolated rat hepatocytes by serum lipoproteins

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ABSTRACT The incorporation of labeled acetate into lipids was studied in rat hepatocytes isolated after treatment of liver with collagenase and hyaluronidase. About 60% of the lipid radioactivity was in free cholesterol and 13% was in triglycerides. Acetate incorporation was markedly inhibited when human serum lipoproteins were present in the incubation medium. Very low, high, and low density lipoproteins, at concentrations of 1.0 mg/ml, inhibited acetate incorporation by 70, 55, and 35%, respectively. Chylomicrons, at similar concentrations, did not inhibit acetate incorporation. The distribution of radioactivity into lipid classes was unchanged by the addition of lipoproteins. Lipoproteins did not produce a nonspecific toxic effect on hepatocytes, since their addition did not alter the rate of leucine incorporation into protein. The addition of the delipidated protein from low density lipoprotein or of lecithin in amounts comparable to those present in inhibitory concentrations of lipoproteins failed to diminish acetate incorporation. Artificial cholesterol–lecithin emulsions containing small amounts of free cholesterol did not inhibit lipid synthesis. Although the mechanism for the inhibition of acetate incorporation by lipoproteins is unclear, such effects may play some physiological role in the control of lipid biosynthesis in the liver.

SUPPLEMENTARY KEY WORDS chylomicrons · control of lipid synthesis · apolipoprotein · protein synthesis

THE SYNTHESIS of hepatic lipids is largely under dietary and hormonal control. High fat diets, fasting, and diabetes are associated with diminished hepatic fatty acid synthesis (1). Diets enriched with cholesterol clearly inhibit cholesterol synthesis in the livers of both animals and man (2, 3). Dietary fat intake might regulate the rate of hepatic lipid synthesis by producing alterations in the concentrations or compositions of chylomicrons and other serum lipoproteins that perfuse the liver. The circulating lipoproteins, in turn, may exert a feedback control on the synthetic rate of their peptide or lipid components in the liver. In fact, a recent study by Weis and Dietschy (4) suggests that the dietary regulation of hepatic cholesterol synthesis is mediated by the concentration of circulating lymph chylomicrons. Little information is available, however, concerning the effects of serum lipoproteins in vitro on lipid synthesis by the liver.

In this study the incorporation of labeled acetate into cellular lipids was inhibited by the addition of serum lipoproteins, at concentrations physiological for the rat, to an incubation mixture containing rat hepatocytes isolated by an enzymatic method (5). Comparable concentrations of chylomicrons did not affect acetate incorporation.

MATERIALS AND METHODS

Materials

Collagenase (type I) and hyaluronidase (type I) were obtained from Sigma Chemical Co., St. Louis, Mo. Potassium penicillin G and streptomycin sulfate were purchased from E. R. Squibb & Sons, New York. Digitonin was a product of Fisher Scientific Co., Pittsburgh, Pa., and cholesterol was obtained from Eastman Organic Chemicals, Rochester, N.Y. Bovine serum albumin was purchased from Armour Pharmaceutical Co., Kankakee, Ill., and egg lecithin from Nutritional Biochemicals Co., Cleveland, Ohio. PPO (2,5-diphenyloxazole) was a product of Packard Instrument Co., Downers Grove, Ill. POPOP (*p*-bis[2-(5-phenyloxa-

Abbreviations: VLDL, very low density lipoprotein, d 1.006; LDL, low density lipoprotein, d 1.019–1.063; HDL, high density lipoprotein, d 1.063–1.21; SDS, sodium lauryl sulfate; TCA, trichloroacetic acid; FFA, free fatty acids.

zolyl)]-benzene), sodium acetate-l-^{14}C (2.0 mCi/mmole), and uniformly labeled L-leucine-^{14}C (260 mCi/mmole) were obtained from New England Nuclear, Boston, Mass.

Isolation of Liver Cells

Male Sprague-Dawley rats (250–300 g), fed ad lib., were used in all experiments, except (as indicated) in several initial studies, where female rats were used. Liver cells were isolated by a modification of the method of Howard and Pesch (5). Rats were anesthetized with ether, 5–10 ml of blood was removed from the abdominal aorta, and the portal vein was perfused with 20 ml of ice-cold enzyme solution, which contained 120 U/ml of collagenase and 0.1% hyaluronidase in modified Hanks buffer (Hanks solution [6] without bicarbonate or calcium). The liver was rinsed in ice-cold saline, finely minced with scissors in the presence of 1 ml of enzyme solution, and transferred to a plastic flask with an additional 30 ml of enzyme solution. The flask was then incubated for 60 min at 37°C with constant shaking at 120 oscillations/min. After the addition of 40 ml of ice-cold modified Hanks solution, the contents of the flask were filtered through 10X silk mesh. The filtrate was transferred to 12-ml plastic centrifuge tubes and centrifuged in a model CL International centrifuge at 4°C for 2 min at 50 *g*. The supernatant was withdrawn and the cells were gently dispersed in 15 vol of ice-cold modified Ca^{2+}–Hanks buffer (modified Hanks buffer with 1 mM $CaCl_2$) by blowing air through a Pasteur pipet. The cells were recentrifuged in the cold at 20 *g* for 2 min and the supernatant was withdrawn. Except in studies in which different incubation media were compared, the cells were resuspended in 3 vol of modified Ca^{2+}–Hanks buffer. Cell isolations and incubations were carried out in Nalgene plastic vessels. To minimize bacterial contamination, all containers were autoclaved and solutions were passed through a Millipore filter (0.45 μ) before use.

Cell counts with a hemocytometer indicated that about 3×10^7 cells were isolated from a single rat liver. Cellular intactness was evaluated by the permeability of cells to 0.2% toluidine blue stain. Between 50 and 90% of the isolated cells were impermeable to this dye. Howard and Pesch (5) have used impermeability to vital stains as an index of cell viability.

Lipid Synthesis and Extraction

Each incubation flask contained $1.5–3.0 \times 10^6$ cells, suspended in 0.65 ml of modified Ca^{2+}–Hanks buffer, and 4.0 mg of penicillin G, 1.25 mg of streptomycin, and 1.5 μCi of acetate-^{14}C added in a volume of 0.125 ml. The final volume was adjusted to 3.0 ml with modified Ca^{2+}–Hanks buffer. Acetate incorporation was initiated by the addition of the cells. Flasks were incubated at 37°C and shaken at 120 oscillations/min. After 50 min of incubation, incorporation of labeled acetate was stopped by the addition of 0.2 ml of 10% sodium acetate solution to the flasks, which were then placed on ice. In one flask cells were incubated in the usual manner, except that the labeled acetate was added after the addition of the sodium acetate solution; this was the blank. The contents of each flask were transferred to 12-ml centrifuge tubes, and the flasks were rinsed with 0.5 ml of 0.15 M NaCl. Cells were separated from the medium by centrifugation at 3000 *g* for 10 min at 4°C. The cellular pellet was dispersed in 1.5 ml of chloroform–methanol 2:1 with the aid of a Vortex mixer, brought to a final volume of 10 ml, allowed to sit overnight, and filtered through fine glass wool. The medium was lyophilized, dispersed in chloroform–methanol, and then treated exactly as was the cellular pellet.

Lipids were extracted by the method of Folch, Lees, and Sloane Stanley (7). Volumes were readjusted to 10 ml by the addition of chloroform–methanol 2:1, and 2 ml of 0.15 M sodium acetate was added to each tube. The two-phase system was shaken five times for 30 sec during the next 30 min and was centrifuged at 3000 *g* for 10 min. The aqueous epiphase was discarded and 1.5 ml of upper phase (chloroform–methanol–water 3:48:47) was added. After the centrifuge tubes were shaken and centrifuged, the aqueous epiphase was again discarded. The extraction with upper phase was repeated twice. Aliquots of the lower phase were then transferred to a scintillation vial, evaporated to dryness on a hot plate at 56°C without charring, and counted after the addition of 10 ml of scintillation fluid (4 g of PPO and 100 mg of POPOP dissolved in 1 liter of toluene). Samples were counted on an Ansitron liquid scintillation counter with an efficiency of 73% for ^{14}C. All results are expressed as total counts per minute in each incubation flask.

Distribution of Radioactivity in Lipids

Extracted lipids were taken to dryness under nitrogen, dissolved in chloroform, and applied along with appropriate standards to silica gel G thin-layer plates, which were developed in hexane–ether–acetic acid 70:20:1. The plates were sprayed with a solution of rhodamine 6G, lipids were identified, and areas corresponding to each lipid were scraped into scintillation vials. Scintillation fluid was added and the vials were counted. Labeled free cholesterol was also isolated by the digitonin method of Crawford (8), modified by the addition of 1 ml of carrier cholesterol (0.4 g/100 ml) to 1 ml of lipid extract dissolved in ethanol–ether in

order to obtain a precipitate on the addition of 2 ml of 1% digitonin. The digitonide precipitate was washed as described by Crawford, and counted.

Leucine Incorporation

Incorporation of leucine-^{14}C into protein was carried out by a modification of the method of Capuzzi and Margolis (9). Cell suspensions of isolated hepatocytes were prepared as described above. Incubation conditions were identical to those for acetate incorporation, except that 1.5 μCi of leucine-^{14}C was added to the incubation medium instead of acetate-^{14}C. After 50 min of incubation the flasks were placed in ice, and 0.20 ml of unlabeled 2% L-leucine solution was added to each. The contents of the flasks were centrifuged at 2000 *g* for 10 min to separate the cells from the medium. The packed cells were lysed by the addition of 0.2 ml of 0.15 M NaCl, 0.1 ml of 30% albumin, and 0.1 ml of 10% Triton. Cell lysates were individually applied with Pasteur pipets to No. 3 Whatman filter paper disks of 2.3-cm diameter. The disks were dried with a hair dryer, placed in a beaker of cold 10% TCA, and heated in a solution of 5% TCA at 90°C for 15 min. The disks were then washed three times with 5% TCA–70% ethanol at 25°C, twice with 95% ethanol at 25°C, once with 95% ethanol–ether–chloroform 2:2:1 for 15 min at 37°C, and three times with 95% ethanol–acetone 1:1 at 55°C. The disks were dried and counted in vials containing 10 ml of scintillation fluid.

Preparation of Liver Slices

Liver slices were prepared using the Stadie-Riggs microtome (Thomas). Slices (150–200 mg) were added to incubation flasks containing 4.0 mg of penicillin G, 1.25 mg of streptomycin, and 1.5 μCi of acetate-^{14}C; the volume was adjusted to 3.0 ml by addition of modified Ca^{2+}–Hanks buffer. After incubation for 60 min as previously described, incorporation of labeled acetate was stopped by the addition of 0.2 ml of 10% sodium acetate solution. The slices were transferred to a test tube containing 0.4 ml of water and were homogenized with a Teflon pestle. The homogenates were dispersed in 15 ml of chloroform–methanol 2:1, and lipids were extracted as described above.

Preparation of Lipoproteins and Chylomicrons

Three preparations of LDL were employed, two from patients with hypercholesterolemia and one from a normal subject. Serum from normal subjects was utilized to prepare two samples of both VLDL and HDL. Rat HDL was prepared from pooled rat serum. Lipoproteins were isolated using the Beckman L2-50 ultracentrifuge by the method of Havel, Eder, and Bragdon (10). In addition, one sample of LDL was prepared by the method of Margolis and Langdon (11). After initial isolation, the lipoproteins were purified by recentrifugation and dialyzed for 24 hr against 0.15 M NaCl containing 0.3 mM EDTA at pH 7.6 (saline–EDTA). LDL was delipidated by the method of Granda and Scanu (12), using SDS. The soluble product contained 97% of the initial protein but no measurable cholesterol (<0.01 mg/ml). After 10 days of dialysis this suspension contained 0.7 mg/ml of SDS, as determined by the assay of Karush and Sonenberg (13).

Two chylomicron samples were isolated from the plasma of patients with type V hyperlipoproteinemia. An equal volume of saline–EDTA was layered over plasma which was centrifuged at 107,000 *g* for 40 min. The chylomicrons, which migrated to the top of the centrifuge tubes, were collected and resuspended in saline–EDTA with a 22-gauge needle. The chylomicrons were then centrifuged, resuspended, and dialyzed against saline–EDTA for 24 hr.

Addition of Cholesterol to an Aqueous Medium

Cholesterol was dissolved in ethanol, propanol, or dioxane, and small amounts (2 μl/ml) of the organic solvent were dispersed in a modified Ca^{2+}–Hanks solution containing lecithin (1 mg/ml). By this modification of the method of Festenstein (14), cholesterol (100 μg/ml) was dispersed in a lecithin suspension.

RESULTS

Conditions for Lipid Synthesis

Table 1 compares acetate incorporation into lipids in different media. Incorporation was greater in modified Ca^{2+}–Hanks buffer than in Tris–KCl buffer, the optimal medium for acetate incorporation in liver cells isolated with a tissue press technique ("mechanical cells") (15). The addition of cofactors, absolutely required for acetate incorporation in "mechanical cells," caused a slight increase in acetate incorporation by "enzyme cells" incubated in Tris–KCl medium; but the cofactors inhibited incorporation in the modified Hanks buffer, both in the presence and absence of calcium. 1 mM calcium enhanced lipid synthesis in "enzyme cells," whereas this concentration of calcium inhibited acetate incorporation in "mechanical cells."[1] Bicarbonate was not added to the modified Hanks buffer because Capuzzi and Margolis (15) found that bicarbonate was inhibitory to lipid synthesis in "mechanical cells." However, preliminary data from our laboratory indicate that bicarbonate stimulates acetate incorporation in "enzyme cells."

[1] Lipson, L. G., D. M. Capuzzi, and S. Margolis. Unpublished results.

TABLE 1 EFFECT OF INCUBATION MEDIA ON INCORPORATION OF ACETATE-^{14}C INTO TOTAL CELLULAR LIPID

Incubation Medium	Incorporation
	cpm per flask
Tris–KCl,* Ca^{2+}	3,600
Tris–KCl, Ca^{2+}, cofactors†	5,500
Modified Hanks	14,400
Modified Hanks, cofactors	5,800
Modified Ca^{2+}–Hanks	22,900
Modified Ca^{2+}–Hanks, cofactors	10,300

Cells were isolated from female rats. Each flask contained 3.0×10^6 cells. Experimental conditions were identical to those described in Methods, except that cells were suspended and incubated in the appropriate buffer. The concentration of Ca^{2+} was 1 mM.

* 0.10 M KCl, 0.02 M Tris, pH 7.6.

† Cofactors were added in a volume of 0.1 ml to give the following final concentrations: $MgCl_2$, 3.3 mM; $MnCl_2$, 0.1 mM; sodium succinate, 10 mM; coenzyme A, 0.03 mM; sodium citrate, 3.3 mM; nicotinamide, 6.7 mM; $NADP^+$, 0.23 mM; glucose-6-phosphate, 1.7 mM.

Acetate incorporation into lipids was consistently greater in female (Table 1) than in male rats, which were used in the remainder of the reported experiments. The formation of radioactive lipids from acetate was particularly rapid in pregnant rats.

Time Course of Acetate Incorporation into Lipid

As shown in Fig. 1, the rate of incorporation of acetate-^{14}C into cellular lipids was rapid during the first 45 min of incubation. After 45 min, radioactivity in cellular lipids gradually declined, but the release of labeled lipids into the incubation medium continued. Although Fig. 1 illustrates a fairly typical experiment, the incorporation after 1 hr of incubation was variable. In some experiments active incorporation of acetate continued for 2 hr, although a sharp decrease in rate was always apparent after 45 min of incubation.

Labeled lipids may appear in the medium because of their physiological release in lipoproteins or because of progessive cellular damage with time. The former possibility is supported by earlier studies by Capuzzi and Margolis (9) in "mechanical cells," which synthesize and release low density lipoproteins into the incubation medium. Peptide-labeled low density lipoproteins were released from "mechanical cells" at a linear rate for 3 hr although incorporation of leucine-^{14}C into cellular proteins stopped after 1 hr of incubation. These cells also continued to release labeled lipids into the incubation medium after the cessation of acetate incorporation into cellular lipids (15). On the other hand, loss of the capacity of cells to incorporate acetate into lipids during incubation at 37°C (Table 2) suggests progressive cellular damage with time. A resultant increase in cellular permeability may cause

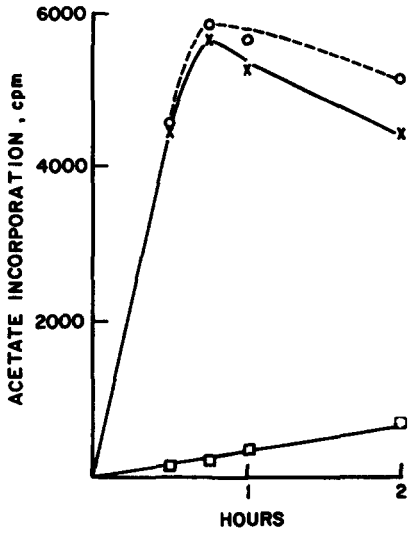

FIG. 1. Time course of acetate-^{14}C incorporation into lipids. Radioactivity of lipids in cells, ×—×; medium, □—□; and cells + medium, ○- - -○. Each flask contained 3.0×10^6 cells. Experimental conditions were identical to those described in Methods except for the duration of incubation.

previously synthesized lipids to leak into the incubation medium from such damaged cells.

Distribution of Radioactivity in Cellular Lipid Classes

As indicated in Table 3, 60% of the acetate incorporated into total lipids by "enzyme cells" was recovered in free cholesterol. Good agreement with this value was obtained when radioactivity was determined by precipitation of free cholesterol with digitonin. The distribution of label among lipid classes was much different in "enzyme cells" than in "mechanical cells," where phospholipids, fatty acids, and monoglycerides were the primary lipids synthesized from labeled acetate (15).

Effect of Lipoproteins and Chylomicrons on Acetate Incorporation

Fig. 2 illustrates that the addition of VLDL, LDL, or HDL to the incubation medium inhibited incorporation of acetate into total lipids. VLDL was the most and LDL the least inhibitory lipoprotein; 1.0 mg/ml of VLDL, HDL, and LDL decreased acetate incorporation by 70, 55, and 35%, respectively. At comparable concentrations chylomicrons did not inhibit, and in fact, they stimulated acetate incorporation slightly. These results were reproducible with lipoproteins and chylomicrons isolated from all subjects. LDL isolated either by preparative ultracentrifugation or by dextran sulfate precipitation diminished acetate incorporation to a similar degree. In a single experiment, rat serum HDL, 2.5 mg/ml, reduced the incorporation of acetate into lipids by 65%. This indicates that rat

TABLE 2 EFFECT OF PREINCUBATION ON INCORPORATION OF ACETATE-^{14}C INTO CELLULAR LIPIDS

Preincubation Time	Incorporation
min	*cpm per flask*
0	7600
30	420
60	190
120	30

Each flask contained 3.0 × 10^6 cells. Experimental conditions were identical to those described in Methods, except that acetate-^{14}C was added to the incubation medium after the indicated preincubation time. Acetate incorporation was measured 50 min after the addition of acetate-^{14}C.

TABLE 3 DISTRIBUTION OF CELLULAR RADIOACTIVITY INTO LIPID CLASSES IN "ENZYME" AND "MECHANICAL CELLS"

Lipid Classes	"Enzyme Cells"	"Mechanical Cells"*
	%	%
Cholesterol	60 ± 6†	9
Triglycerides	13 ± 2.0	9
Fatty acids	4 ± 1.3	23
1,2-Diglycerides	5 ± 1.5	6
Monoglycerides	5 ± 0.8	17
Cholesteryl ester	6 ± 0.7	2
Phospholipids	6 ± 1.4	35

Extracted lipids were resolved by thin-layer chromatography as indicated in Methods.

* Values are those of Capuzzi and Margolis (15).

† Mean ± SEM, n = 5.

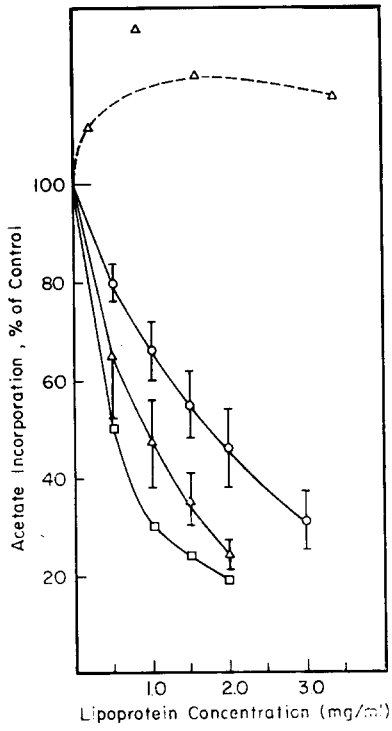

FIG. 2. Effect of lipoproteins on the incorporation of acetate-1-^{14}C into cellular lipids. Radioactivity with VLDL, □—□; HDL, △—△; LDL, ○—○; and chylomicrons, △- - -△. Each point is the mean (±SEM where indicated) of six experiments for LDL, four experiments for HDL, and two experiments for VLDL and chylomicrons. Each incubation flask contained 1.5 × 10^6 cells. Experimental conditions were identical to those described in Methods except for the addition of lipoprotein. All flasks for each experiment contained the same amount of the buffer (saline–EDTA) used for addition of the lipoproteins.

lipoproteins also inhibit lipid synthesis in isolated rat hepatocytes.

Since the distribution of radioactivity among lipid classes was unchanged in the presence of lipoproteins, VLDL, LDL, and HDL produced a generalized inhibition of lipid synthesis. Digitonin precipitation confirmed that this depression was not specific for cholesterol. The medium contained only trace amounts (< 5%) of the total lipid radioactivity. Thus, the presence of lipoproteins did not diminish radioactivity of cellular lipids by stimulating release of lipid into the medium.

Also studied were the effects of lipoproteins and chylomicrons on acetate incorporation by liver slices. At high concentrations (5 mg/ml), VLDL, HDL, and LDL inhibited the incorporation of acetate into lipids. However, the effects of lipoproteins on acetate incorporation in liver slices were variable, and less inhibition was noted than when lipoproteins were added to isolated hepatocytes. Chylomicrons either stimulated incorporation of acetate slightly in liver slices or had no effect.

The lipoproteins could reduce the incorporation of acetate in a nonspecific manner by lowering the pH of the incubation medium, especially since modified Hanks solution is poorly buffered because of the absence of bicarbonate. However, the addition of dialyzed lipoproteins produced no change in the pH of the incubation mixture of buffer plus isolated cells. Moreover, human LDL inhibited lipid synthesis to the same extent in the presence or absence of 4 mM bicarbonate buffer, pH 7.6.

The inhibitory effect of lipoproteins on the incorporation of acetate-^{14}C could result from hydrolysis of triglyceride by a lipase which leaked from the liver cells into the incubation medium. The released fatty acids might inhibit lipid synthesis directly, or oxidation of the fatty acids could dilute the specific activity of acetyl CoA derived from the labeled acetate. The titration method of Dole and Meinertz (16) was used to measure fatty acids in the incubation medium of flasks containing cells and LDL (3 mg/ml) at time zero and after 50 min of incubation. The concentrations of FFA were 0.01 and 0.03 μeq/ml at 0 and 50 min of incubation, respectively. Lipolysis of about 1% of the triglycerides present on the LDL would account for this rise in fatty acid concentration. Acetate incorporation was inhibited by 60% in this experiment, although

little or no inhibition was produced by incubation of liver cells with albumin that contained similar concentrations of FFA.

An experiment was done to test the possibility that breakdown of lipoprotein lipids to fatty acids and their consequent oxidation to acetate could produce spurious inhibition by dilution of the specific activity of the acetate pool. The inhibitory effect of LDL (1.5 mg/ml) was compared in the presence of the usual concentration of total acetate (0.75 μmole/ml) and in the presence of 7.5 μmoles of acetate/ml. LDL inhibited acetate incorporation by 45 and 37%, respectively. Both of these values fall within the range of the standard error of the mean for inhibition by this concentration of LDL. In contrast, an inhibition of less than 10% would be expected at the higher acetate concentration if the effect were produced by dilution of the specific activity of the acetate-^{14}C.

These results are consistent with those of Felts and Berry (17), who reported that only about 0.5% of the labeled triglyceride fatty acids on chylomicrons and 5% of labeled FFA were converted to CO_2 by isolated rat liver cells. Furthermore, a lipase which released fatty acids from lipoproteins should also hydrolyze chylomicron triglycerides (18, 19); yet chylomicrons did not inhibit acetate incorporation, while HDL, LDL, and VLDL inhibited to a striking extent.

Experiments were carried out to determine which lipoprotein component was responsible for the inhibitory effect on lipid synthesis. One possible mechanism is a nonspecific inhibition of acetate incorporation by protein. As shown in Fig. 3, the addition of albumin caused a marked fall in acetate incorporation into cellular lipids, but high concentrations of albumin were required. Acetate incorporation was not inhibited by 0.8 mg/ml of albumin, whereas LDL protein at this concentration diminished acetate incorporation by 70%. These results indicate that the inhibition of lipid synthesis observed with lipoprotein was not the result of a nonspecific protein effect.

Since the protein moiety of the lipoproteins could account for the depression of acetate incorporation, two experiments were carried out with delipidated LDL peptide, solubilized by the presence of SDS. In these experiments, incorporation of acetate was compared after identical amounts of protein (1.3 mg/ml), as either delipidated or native LDL, were added to the incubation medium. In one experiment the delipidated LDL stimulated acetate incorporation slightly; in the other, incorporation declined modestly when delipidated LDL was added. Native LDL inhibited the labeling of cellular lipids by 90% in both experiments. These results suggest that the apolipoprotein moiety of LDL was not responsible for the depression of lipid synthesis

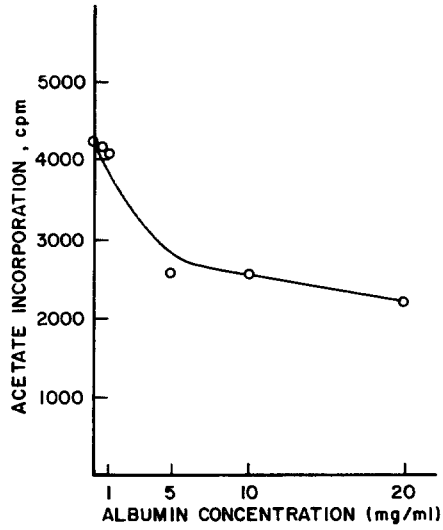

FIG. 3. Effect of albumin on incorporation of acetate-^{14}C into cellular lipids. Each flask contained 2.5 × 10^6 cells. Experimental conditions were identical to those described in Methods except for the addition of albumin.

obtained with fully lipidated LDL. The presence of SDS (0.7 mg/ml) in the suspension of delipidated LDL did not account for the maintenance of lipid synthesis, since SDS, when added alone in comparable concentrations, completely inhibited acetate incorporation. Apparently, binding of SDS to the delipidated protein prevented its inhibitory effect on acetate incorporation.

Effects of Artificial Lipid Emulsions

Artificial lipid emulsions were prepared to determine whether a specific class of lipids bound to lipoprotein was responsible for its inhibitory effect on lipid synthesis. Fig. 4 indicates that phospholipid (lecithin) concentrations of 1.5 mg/ml were necessary for any significant inhibition of lipid synthesis, and concentrations of 10 mg/ml were required to produce effects comparable to those noted with concentrations of lipoprotein that contained 0.4 mg/ml of lecithin. It seems unlikely, then, that the phospholipid component of LDL could account for the observed inhibition.

Since lecithin concentrations of 1.0 mg/ml did not inhibit lipid synthesis, this concentration of lecithin was used to disperse cholesterol in the incubation medium. To prepare cholesterol–lecithin emulsions, cholesterol was first dissolved in an organic solvent (ethanol, propanol, or dioxane) that is miscible with water. Small amounts of the organic solvent, which contained the dissolved cholesterol, were then dispersed in modified Ca^{2+}–Hanks buffer with lecithin (1.0 mg/ml) added. Table 4 indicates that even small amounts of dioxane, ethanol, or propanol decreased

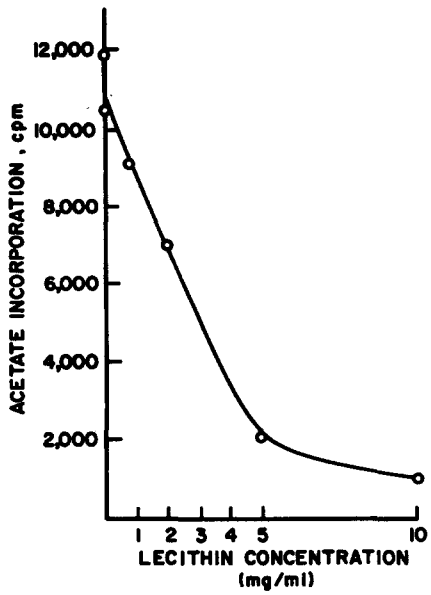

FIG. 4. Effect of lecithin concentration on incorporation of acetate-^{14}C into cellular lipids. Each flask contained 3.0 × 10^6 cells. Experimental conditions were identical to those described in Methods except for the addition of lecithin. The indicated amount of lecithin for each flask was added from a suspension of lecithin, 3 g/100 ml, which was dispersed by stirring in modified Ca^{2+}–Hanks buffer at 37°C followed by readjustment of the pH to 7.6.

lipid synthesis. The addition of cholesterol (100 μg/ml) produced no further inhibition, but in fact increased acetate incorporation into lipids. In contrast, when this amount of cholesterol was present in association with VLDL, acetate incorporation was inhibited by 55%.

Effect of Lipoproteins and Chylomicrons on Leucine Incorporation

As indicated in Table 5, at concentrations that are maximally inhibitory for acetate incorporation, lipoproteins did not significantly alter leucine incorporation into cellular protein. Chylomicrons stimulated leucine incorporation by about 33%. These results suggest that lipoproteins do not diminish lipid synthesis by a direct toxic effect on cellular metabolism.

TABLE 4 EFFECT OF CHOLESTEROL:LECITHIN EMULSIONS ON INCORPORATION OF ACETATE-^{14}C INTO LIPID

Additions	Incorporation
	cpm per flask
None	4100
Dioxane	1900
Dioxane and cholesterol	3400
Propanol	1800
Propanol and cholesterol	2400
Ethanol	1200
Ethanol and cholesterol	1400

Each flask contained 2.5 × 10^6 cells and lecithin (1.0 mg/ml). Experimental conditions were otherwise identical to those described in Methods. The concentrations of the additions were: cholesterol, 100 μg/ml; dioxane, 8 mM; propanol, 27 mM; and ethanol, 33 mM.

TABLE 5 EFFECT OF LIPOPROTEINS AND CHYLOMICRONS ON INCORPORATION OF LEUCINE-^{14}C INTO CELLULAR PROTEIN

Additions	Concentration	Incorporation
	mg/ml	*cpm per flask*
None		2590
VLDL	1.5	2950
HDL	5	2450
LDL	5	2170
Chylomicrons	5	3450

Each flask contained 2.0 × 10^6 cells. Experimental conditions were identical to those described in Methods except for the indicated additions. In the flask without lipoprotein, an equal amount of saline–EDTA was added.

DISCUSSION

The addition of lipoproteins to the incubation medium caused a dramatic decrease in the amount of label incorporated into cellular lipids by isolated rat liver cells. Since lipoproteins did not enhance the release of labeled lipids, the fall in radioactivity in cellular lipids must reflect a decrease in the rate of acetate incorporation. Concentrations of lipoproteins that are physiological for the rat, 0.8 mg/ml of VLDL, 0.6 mg/ml of LDL, and 1.7 mg/ml of HDL (10, 20), diminished acetate incorporation into all lipid classes by 65, 25, and 70%, respectively. Moreover, small changes in lipoprotein concentration in this range produced major differences in the amount of lipids synthesized from labeled acetate.

Little is known about the effects of lipoproteins on hepatic lipid synthesis. Siperstein and Fagan (21) reported that cholesterol synthesis was inhibited in liver slices obtained from rats injected with LDL (d = 1.019) 2.5 hr earlier. Boyd and Onajobi (22) noted that cholesterol synthesis by isolated rat liver microsomes was decreased by the addition of HDL, which sequestered squalene. Such studies do not provide an explanation for the inhibition of acetate incorporation into all lipid classes noted with VLDL, LDL, and HDL in our studies. It is possible that these lipoproteins produced a completely nonspecific interference with the metabolism of isolated hepatocytes. This seems unlikely, because at least one parameter of liver cell activity, incorporation of labeled amino acids into protein, was unaffected by the presence of lipoprotein.

Since lipoproteins of different density and com-

position produced similar effects on lipid synthesis, a moiety common to all these lipoproteins was considered to be the factor responsible for the depression of lipid synthesis. Phospholipid (lecithin) probably does not account for the observed inhibition, as lecithin concentrations of 1.5 mg/ml were necessary for any significant effect. Albumin depressed acetate incorporation only at high concentrations, and one specific protein moiety, the apolipoprotein of LDL, did not affect lipid synthesis. Free cholesterol, 100 mg/ml, did not inhibit lipid synthesis when added as an emulsion with lecithin, although acetate incorporation was diminished by 55% when VLDL was added in concentrations which bound similar amounts of cholesterol. Because they are so nonpolar, it was not possible to disperse cholesteryl esters in amounts comparable to those present in inhibitory concentrations of lipoproteins.

The degree of inhibition produced by VLDL, LDL, and HDL does not correlate with the concentration of any specific component of these lipoproteins. The most inhibitory lipoprotein was VLDL, which has the lowest concentrations of protein and cholesterol but carries large amounts of triglycerides. Yet, triglycerides alone cannot be the inhibitory factor, since chylomicrons failed to decrease acetate incorporation. Neither apolipoprotein A of HDL nor the B apolipoprotein of LDL can be specifically responsible for the depression of lipid synthesis. Although no specific component of the lipoproteins is apparently solely responsible for the inhibition, some unique orientation of lipids or proteins on the surface of the lipoproteins could be the determining factor. Unfortunately, information concerning the structure and surface properties of lipoproteins is insufficient to evaluate this consideration.

If each lipid component of the lipoprotein regulated its own synthesis, the cumulative effect could account for a generalized inhibition of lipid synthesis. Such a mechanism would presumably require the transfer of lipids from lipoprotein to hepatocyte. Rapid exchange of cholesterol and phospholipids has been demonstrated between lipoproteins and red blood cells (23–25). The mechanism of this exchange is obscure, but Gurd (26) proposed that cholesterol exchange required a collision between the donor lipoprotein and the acceptor cell. A similar exchange may occur between lipoproteins and liver cells, but this has not yet been demonstrated. In contrast, cholesteryl esters and triglycerides exchange poorly between different plasma lipoproteins (27–29).

It is difficult to explain the finding that chylomicrons, at concentrations comparable to the other lipoproteins, did not inhibit incorporation of acetate into lipids. Chylomicrons also exchange phospholipids and cholesterol with other lipoproteins (28), and Green and Webb (30) demonstrated that isolated hepatocytes take up labeled fatty acids from chylomicrons. Chylomicrons contain much less protein than VLDL, HDL, or LDL, but the effects of these lipoproteins do not correlate with either protein concentration or a specific apolipoprotein.

However, chylomicrons have a diameter of 75–500 nm (31) and are therefore much larger than HDL, LDL, and VLDL, which have diameters of 4–13 nm, 22 nm, and 30–70 nm, respectively (32–34). Perhaps their bulkiness prohibits the uptake of chylomicrons by hepatocytes, or their size may prevent sufficient surface contact with liver cells to trigger appropriate feedback mechanisms. Also to be considered is the possibility that VLDL, HDL, and LDL may release some toxic substance that is not present in chylomicrons or is bound more tightly to chylomicron particles. If such a toxic substance is present, it does not affect amino acid incorporation into protein. Finally, lipoproteins may bind some substrate or cofactor needed for lipid synthesis, acetate-^{14}C for example, that is not similarly bound by chylomicrons.

The observation that chylomicrons do not depress acetate incorporation in isolated liver cells is especially interesting, since Weis and Dietschy (4) elegantly demonstrated in vivo that physiological concentrations of rat lymph chylomicrons inhibited the hepatic incorporation of labeled acetate into cholesterol. The inhibiting effect of circulating chylomicrons seems to account for the depression of cholesterol synthesis noted in liver slices obtained from rats fed a high cholesterol diet (2).

If such a feedback mechanism were operative in isolated hepatocytes, chylomicrons should inhibit total lipid synthesis, since 60% of the acetate was incorporated into cholesterol. There are some possible explanations for this discrepancy. In our experiments, rat liver cells were exposed to human chylomicrons, whereas Weis and Dietschy (4) used rat chylomicrons. Our chylomicrons were isolated from serum, not from lymph, and the lipid and protein compositions of lymph chylomicrons are modified upon entry into the general circulation. Also, since they were not recentrifuged after initial isolation, the chylomicron fraction employed by Weis and Dietschy (4) may have contained some contaminating VLDL (35), the most inhibitory lipoprotein in our studies. It is possible, although unlikely, that small concentrations of VLDL accounted for the effects observed by these authors. The most likely explanation, however, is that the inhibitory effect of lipoproteins on lipid synthesis reported in the present studies is unrelated to the specific depression of cholesterol synthesis by chylomicrons noted by Weis and Dietschy.

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References

1. Nikkilä, E. A. 1969. Control of plasma and liver triglyceride kinetics by carbohydrate metabolism and insulin. *Advan. Lipid Res.* **7:** 63–134.
2. Tomkins, G. M., H. Sheppard, and I. L. Chaikoff. 1953. Cholesterol synthesis by liver. III. Its regulation by ingested cholesterol. *J. Biol. Chem.* **201:** 137–141.
3. Frantz, I. D., Jr., H. S. Schneider, and B. T. Hinkelman. 1954. Suppression of hepatic cholesterol synthesis in the rat by cholesterol feeding. *J. Biol. Chem.* **206:** 465–469.
4. Weis, H. J., and J. M. Dietschy. 1969. Failure of bile acids to control hepatic cholesterogenesis: evidence for endogenous cholesterol feedback. *J. Clin. Invest.* **48:** 2398–2408.
5. Howard, R. B., and L. A. Pesch. 1968. Respiratory activity of intact, isolated parenchymal cells from rat liver. *J. Biol. Chem.* **243:** 3105–3109.
6. Hanks, J. H., and R. E. Wallace. 1949. Relation of oxygen and temperature in the preservation of tissues by refrigeration. *Proc. Soc. Exp. Biol. Med.* **71:** 196–200.
7. Folch, J., M. Lees, and G. H. Sloane Stanley. 1957. A simple method for the isolation and purification of total lipides from animal tissues. *J. Biol. Chem.* **226:** 497–509.
8. Crawford, N. 1958. An improved method for the determination of free and total cholesterol using the ferric chloride reaction. *Clin. Chim. Acta.* **3:** 357–367.
9. Capuzzi, D. M., and S. Margolis. Metabolic studies in isolated rat liver cells. II. Biosynthesis of serum low density lipoproteins and its regulation. *Lipids.* In press.
10. Havel, R. J., H. A. Eder, and J. H. Bragdon. 1955. The distribution and chemical composition of ultracentrifugally separated lipoproteins in human serum. *J. Clin. Invest.* **34:** 1345–1353.
11. Margolis, S., and R. G. Langdon. 1966. Studies on human serum β_1-lipoprotein. I. Amino acid composition. *J. Biol. Chem.* **241:** 469–476.
12. Granda, J. L., and A. Scanu. 1966. Solubilization and properties of the apoprotein of the very low and low density lipoproteins of human serum. *Biochemistry.* **5:** 3301–3308.
13. Karush, F., and M. Sonenberg. 1950. Long-chain alkyl sulfates: colorimetric determination of dilute solutions. *Anal. Chem.* **22:** 175–177.
14. Festenstein, G. N. 1955. The effect of ergosterol and ergocalciferol on the anaerobic glycolysis of rat liver slices. *Biochem. J.* **59:** 605–609.
15. Capuzzi, D. M., and S. Margolis. Metabolic studies in isolated rat liver cells. I. Lipid synthesis. *Lipids.* In press.
16. Dole, V. P., and H. Meinertz. 1960. Microdetermination of long-chain fatty acids in plasma and tissues. *J. Biol. Chem.* **235:** 2595–2599.
17. Felts, J. M., and M. N. Berry. 1971. The metabolism of free fatty acids and chylomicron triglyceride fatty acids by isolated rat liver cells. *Biochim. Biophys. Acta.* **231:** 1–7.
18. Fielding, C. J. 1970. Human lipoprotein lipase. I. Purification and substrate specificity. *Biochim. Biophys. Acta.* **206:** 109–117.
19. Higgins, J. A., and C. Green. 1967. Properties of a lipase of rat liver parenchymal cells. *Biochim. Biophys. Acta.* **144:** 211–220.
20. Camejo, G. 1967. Structural studies of rat plasma lipoproteins. *Biochemistry.* **6:** 3228–3241.
21. Siperstein, M. D., and V. M. Fagan. 1964. Studies on the feedback regulation of cholesterol synthesis. *Advan. Enzyme Regul.* **2:** 249–264.
22. Boyd, G. S., and F. D. Onajobi. 1969. Control of cholesterol biosynthesis by a plasma apolipoprotein. *Nature* (London). **221:** 574–575.
23. James, A. T., J. E. Lovelock, and J. P. W. Webb. 1959. The lipids of whole blood. I. Lipid biosynthesis in human blood in vitro. *Biochem. J.* **73:** 106–115.
24. Lovelock, J. E., A. T. James, and C. E. Rowe. 1960. The lipids of whole blood. II. The exchange of lipids between the cellular constituents and the lipoproteins of human blood. *Biochem. J.* **74:** 137–140.
25. Sodhi, H. S., and N. Kalant. 1963. Hyperlipemia of antiserum nephrosis. III. Plasma lipoprotein. *Metab. Clin. Exp.* **12:** 420–427.
26. Gurd, F. R. N. 1960. Association of lipids with proteins. *In* Lipid Chemistry. D. J. Hanahan, editor. John Wiley & Sons, New York. 208–325.
27. Roheim, P. S., D. E. Haft, L. I. Gidez, A. White, and H. A. Eder. 1963. Plasma lipoprotein metabolism in perfused rat livers. II. Transfer of free and esterified cholesterol into the plasma. *J. Clin. Invest.* **42:** 1277–1285.
28. Minari, O., and D. B. Zilversmit. 1963. Behavior of dog lymph chylomicron lipid constituents during incubation with serum. *J. Lipid Res.* **4:** 424–436.
29. Kruger, F. A., D. G. Cornwell, G. J. Hamwi, and J. B. Brown. 1958. Iodinated triolein: a lipid tracer for the study of plasma lipoprotein metabolism. *Federation Proc.* **17:** 258. (Abstr.)
30. Green, C., and J. A. Webb. 1964. The uptake of chylomicron fatty acids by isolated liver cells. *Biochim. Biophys. Acta.* **84:** 404–411.
31. Bierman, E. L., T. L. Hayes, J. N. Hawkins, A. M. Ewing, and F. T. Lindgren. 1966. Particle-size distribution of very low density plasma lipoproteins during fat absorption in man. *J. Lipid Res.* **7:** 65–72.
32. Hazelwood, R. N. 1958. The molecular weights and dimensions of some high density human serum lipoproteins. *J. Amer. Chem. Soc.* **80:** 2152–2156.
33. Beischer, D. E. 1954. Electron microscopy of human plasma lipoprotein separated by ultracentrifugation. *Circ. Res.* **2:** 164–168.
34. Lindgren, F. T., and A. V. Nichols. 1960. Structure and function of human serum lipoproteins. *In* The Plasma Proteins. Vol. 2. F. W. Putnam, editor. Academic Press, New York. 1–58.
35. Gustafson, A., P. Alaupovic, and R. H. Furman. 1965. Studies of the composition and structure of serum lipoproteins: isolation, purification, and characterization of very low density lipoproteins of human serum. *Biochemistry.* **4:** 596–605.