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Serum lipoprotein accumulation in the livers of orotic acid-fed rats

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ABSTRACT This study provides confirmation of previous observations that showed that rats fed a diet containing 1% orotic acid for 7 days develop a fatty liver and that there is an inhibition of the secretion of low density lipoproteins without altering general liver protein synthesis. Accumulated fat droplets (liposomes) are entrapped within rough endoplasmic reticulum vesicles. In this study, these vesicles have been shown to accumulate the apolipoproteins of low and very low density lipoproteins.

Inhibition of lipoprotein secretion was demonstrated by perfusion of livers from orotic acid-fed rats with a serum-free medium. Liposomes were isolated from these rats. Partially delipidated liposomes, but not similarly treated microsomes or cell sap, were found to form a precipitation line when reacted against anti-low density lipoprotein antiserum. Detergent solubilization of the liposome followed by density gradient centrifugation resulted in a peak at d 1.025 g/ml containing both lipid and protein. Acrylamide electrophoresis in 8 M urea after total delipidation demonstrated liposomal bands which co-electrophoresed with three of four very low density lipoprotein bands; there was no band corresponding to the very low density lipoprotein band which travels furthest in acrylamide electrophoregrams. However, acrylamide electrophoresis of the apoproteins of serum high density lipoprotein from orotic acid-fed animals revealed the presence of the latter band. The results indicate that liver liposomes from orotic acid-fed rats apparently contain the low density apoprotein and probably several other very low density lipoprotein peptides.

SUPPLEMENTARY KEY WORDS fatty liver · liposomes · lipoprotein secretion · apolipoprotein · very low density lipoprotein · low density lipoprotein

Rats fed a semisynthetic diet containing 1% orotic acid (OA) develop a fatty liver within 7 days (1). The fat accumulates as small droplets (liposomes)[1] 0.2–0.5 μ in diameter bounded by rough endoplasmic reticulum (5). Windmueller and Levy (6) have reported that livers from OA-treated rats, perfused with a serum-free medium, release no detectable serum β-lipoprotein into the medium. They suggest that the accumulation of fat is a result of an inability to mobilize lipid from the liver in the form of lipoprotein. The low levels of serum lipids found in OA-treated animals further substantiate this hypothesis (7).

Several lines of evidence suggest that the biochemical lesion caused by OA feeding is very discrete. Animals have been maintained on the diet for 250 days without evidence of untoward effects other than fatty liver. Animals raised from weanlings on the diet have mated and given birth to offspring (8). There appears to be no defect in the incorporation of labeled amino acids into either endogenous liver proteins (9) or serum proteins other than lipoproteins (10).

This paper is a report of our attempts to characterize the nature of this lesion. It seemed reasonable that if general liver protein synthesis was not disturbed by OA feeding, perhaps the protein moieties of LDL and VLDL were also being synthesized but were not being secreted either because of a defect in the assembly or secretion of these lipoproteins or both. The work presented here indicates that at least some of the proteins are synthe-

Abbreviations: chylo, serum chylomicrons; VLDL, serum very low density lipoprotein; LDL, serum low density lipoprotein; HDL, serum high density lipoprotein; SDS, sodium dodecyl sulfate; OA, orotic acid.

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[1] The term liposome, as used in this paper, refers to a neutral lipid-laden vesicle bounded by membrane probably derived from endoplasmic reticulum. It has been used in this sense in relation to the early changes seen in rat liver during the administration of ethionine and after the reversal of the ethionine-induced fatty liver by adenine administration (2–4).

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sized and that they, as well as the lipid, accumulate in the liposomes.

METHODS

Materials

Casein, Vitamin Diet Fortification, HMW salt mixture, choline chloride, orotic acid, and supplementary amino acids for perfusion were obtained from Nutritional Biochemicals Corp., Cleveland. Sodium dodecyl sulfate (SDS) and Tris(hydroxymethyl)aminomethane were obtained from Fisher Scientific Co., Chicago. SeaKem agarose was purchased from Bausch and Lomb, Rochester, N.Y., and bovine serum albumin, fraction V, was obtained from Armour Pharmaceutical Co., Chicago.

Animals

Adult male rats of the Sprague–Dawley strain, weighing 200–300 g, were used. After an overnight fast, the rats were fed for 7 days a semisynthetic diet containing sucrose (67.3%), corn oil (5%), casein (20%), Vitamin Diet Fortification Mixture (2.2%), HMW salt mixture (5%), and choline chloride (0.5%). Orotic acid at a level of 1% was added to the experimental diet at the expense of all other ingredients. Food and water were given ad lib. for the duration of the experiment. Care was taken to ensure that the rats were never without food.

Liver Perfusion

In situ liver perfusion was performed in an apparatus similar to that described by Mortimore (11). Livers were perfused with approximately 70 ml of a medium free of lipoproteins; it consisted of 20% washed rat red blood cells suspended in Krebs-Ringer bicarbonate buffer containing 4 g of bovine serum albumin and 200 mg of glucose per 100 ml of medium. Some perfusion media were supplemented with 3.20 mg of an amino acid mixture (12) and 1 μmole/ml of sodium linoleate. 40 ml of the perfusate was flushed through the liver before circulation was established. The perfusate was constantly equilibrated with a gas phase containing 95% O_2–5% CO_2, and the entire apparatus was maintained at 37°C.

Livers were perfused for 3 hr. 2-ml samples of the perfusate were taken 10 min after circulation was established and at 1-hr intervals thereafter. The red cells were removed by centrifugation, and the presence of LDL and VLDL was assessed by agarose–acrylamide electrophoresis and in some cases by gel diffusion against anti-LDL antiserum. After 3 hr of perfusion, the livers were removed and homogenized; their liposomes were isolated, partially delipidated, and analyzed for the presence of LDL antigen.

Isolation of Liposomes

The method used for isolating liposomes is a modification of that used by Rajalakshmi, Adams, and Handschumacher (13). In experiments in which livers were not perfused prior to subcellular fractionation, the animals were anesthetized by intraperitoneal injection of Seritol (sodium thiamylal). A needle was inserted through the heart into the aorta, and approximately 150 ml of saline at ambient temperature was perfused under slight positive pressure through the animal and out of an incision made in the inferior vena cava just above the diaphragm. Livers that were perfused were not flushed out with saline. The following procedures were all performed at 0°C.

When livers were perfused prior to analysis, each liver (12–15 g) was divided into two equal parts and each part was homogenized with four strokes of a loosely fitting Potter-Elvehjem homogenizer using enough buffer to bring the final volume to 40 ml. When the livers were not perfused first, a 20% homogenate (w/v) was made. The homogenizing medium consisted of 0.25 M sucrose, 0.05 M Tris-HCl, and 1.34 mM EDTA at pH 8.6.

The homogenates were centrifuged at 10,000 *g* for 30 min. The supernatants were removed, rehomogenized, and then centrifuged in a Spinco 60 titanium rotor at 50,000 rpm for 1 hr. The microsomes were recovered as a pellet at the bottom of the tube and the liposomes as a cake at the top, with the "cell sap" separating the two layers. The cell sap was collected without further purification, and the microsomes and liposomes were resuspended separately in the homogenizing medium and recentrifuged under the same conditions for another hour. Finally, the liposomes and microsomes were separately resuspended in the homogenizing medium without sucrose, so that the liposomes and microsomes from 1.6 g of rat liver were contained in 1 ml of medium. This method consistently yielded approximately 17 mg of microsomal and 0.36 mg of liposomal protein from each gram of rat liver.

Isolation of Liposomal Protein

To isolate the protein within the liposome it is necessary to lyse or solubilize the membrane surrounding the liposome and then to remove the overwhelming amount of fat present, while preserving the protein component of any constituent lipoprotein in its native state. Treatment with SDS was chosen because of its ability to solubilize membrane and because it has been shown to protect apolipoprotein. Three types of delipidation procedures were used in this study:

(*a*) Partial delipidation with ether and SDS was employed in those experiments aimed at seeking the presence of the LDL antigen by reaction of the partially

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delipidated products against anti-LDL antiserum. The method used for partial delipidation with ether was essentially that employed by Granda and Scanu (14). Liposomal preparations were made 0.2 M with respect to SDS by addition of crystalline SDS, and they were incubated at 37°C for 90 min. 10-ml aliquots were added to 150-ml Sorvall centrifuge bottles filled with cold, freshly distilled, peroxide-free diethyl ether. The bottles were rotated for 16 hr at 10–15 rpm on a multipurpose rotator, model 150 V (Scientific Industries, Inc., Springfield, Mass.), at 4°C. After centrifugation at 1,000 *g* for 10 min, the upper ether layer was aspirated off. The samples were then washed three times with fresh ether and dialyzed at room temperature against 50 vol of a buffer containing 0.01 M Tris–HCl, 0.27 mM EDTA, and 0.001% merthiolate, pH 8.6. Samples were dialyzed against daily changes of buffer until the concentration of SDS, as determined by the method of Karush and Sonenberg (15), was 1–10% of the amount of protein present. This usually required 10–12 days of dialysis. The samples were then concentrated approximately five times by either evaporation or pressure dialysis.

(*b*) Partial delipidation with SDS alone was employed in those experiments in which the relationship between liposomal protein and lipid was to be explored. This procedure was chosen because it removed less lipid than either of the other procedures detailed and yet appeared to separate the liposomal "lipoproteins" from the bulk of the "excess" neutral lipid. After incubation with SDS, as described above, the liposomes were centrifuged for 5 min at 2000 *g* in a 30.2 Spinco rotor. The lower aqueous layer containing approximately 90% of the total liposomal protein was separated from the floating fat with a tube cutter and recentrifuged at 2000 *g* for 5 min. The floating fat was recovered, resuspended in buffer, and recentrifuged under the same conditions. The subnatants from these two centrifugations were pooled. Sodium decyl sulfate has been shown to delipidate lipoproteins (16). The possibility arose that SDS might do likewise, and therefore, in order to insure a reproducible degree of delipidation, the SDS was removed by putting the samples through a Dowex 1 column which was previously equilibrated with the buffer in which the liposomes were suspended.

(*c*) Total delipidation with ether and ethanol was employed when the analysis of apoproteins and liposomal proteins was projected. A modification of the method used by Scanu, Pollard, Hirz, and Kothary (17) for serum LDL was applied to liposomal samples. Aliquots containing approximately 7 mg of liposomal protein were made 0.2 mM with respect to SDS by addition of crystalline SDS and incubated at 37°C for 90 min. The samples were then forcibly injected through a 26-gauge needle into a 150-ml Sorvall centrifuge bottle containing redistilled ethanol and peroxide-free diethyl ether (3:1, v/v) at −17°C. After rotating at 10–15 rpm on a multipurpose rotator at −17°C for 4 hr, the bottles were centrifuged at 1000 *g* in a Sorvall refrigerated centrifuge for 10 min. The solvent was removed by aspiration, fresh alcohol–ether solution was added, and the bottles were rotated for another 4 hr. After another centrifugation at 1000 *g* for 10 min, the bottles were filled with pure ether and rotated for 12 hr. The precipitates were then washed once with ether and dried under nitrogen in an ice bath. 3 ml of solubilization mixture containing 0.01 M Tris–HCl, 0.27 mM EDTA, and 8 M urea, pH 8.6, was added to each bottle; the bottles then remained at room temperature for 3 hr with occasional agitation. Acrylamide gel electrophoresis in 8 M urea at pH 8.9 (18, 19) was performed on the samples, using acrylamide concentrations of 15, 7.5, 5, and 4%. The low ionic strength of the solubilization medium made it possible to obtain good results both with and without a stacking gel. Gels were stained with Coomassie brilliant blue R250 (20).

Isolation of VLDL from Normal Rat Serum

EDTA, neutralized to pH 7.0 with NaOH, was added to normal rat serum from nonfasted, chow-fed rats so that the final EDTA concentration was 1.34 mM. Chylomicrons were removed by centrifugation for 30 min at 10,000 rpm in a 30.2 Spinco rotor. The subnatant was then centrifuged for 22 hr at 30,000 rpm. The top 2 ml in each tube was removed by slicing the tube. The collected material was restored to its original volume with NaCl solution, of d 1.006 g/ml, 1.34 mM with respect to EDTA, and washed twice more by centrifugation at 30,000 rpm for 22 hr. The top 2 ml that was recovered (by tube slicing) from the final centrifugal wash was then dialyzed overnight against 0.05 M Tris–HCl, 1.34 mM EDTA, pH 8.6. The purity of this VLDL solution was tested by reacting it in gel diffusion against rabbit antirat serum. Only one precipitation arc was formed; this showed a complete identity with an arc formed by a reaction between the sample and rabbit antirat VLDL. SDS was added to the VLDL, and it was incubated in a manner similar to that used for the liposomes. All of the procedures performed on the liposomes were performed concurrently and identically on the VLDL, and on microsomes from livers of OA-treated animals.

Isolation of HDL

HDL was isolated from OA-treated rats and from rats fed the identical diet lacking orotic acid. The animals were fed the diets for 7 and 12 days. Serum containing EDTA (as described above) was adjusted to a density of 1.063 with KBr. After centifuging for 24 hr at 30,000 rpm in a Spinco 30.2 rotor, the top 3 ml was removed

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with a tube slicer and the remaining contents of the tube were collected. The density was adjusted to d 1.20 g/ml, and the samples were centrifuged at 40,000 rpm for 24 hr in a Spinco 40.3 rotor. The top 1.5 ml of each tube was collected after the tubes were sliced, and it was recentrifuged twice more in an identical manner. Aliquots from OA and control serum were totally delipidated and electrophoresed in 8 M urea by the procedure described for the liposomes.

Agarose–Acrylamide Electrophoresis

The procedure for lipoprotein electrophoresis was the same as that of Narayan, Creinin, and Kummerow (21) except for the following modifications. The components of the separating gel, except the agarose, were made in one solution with a concentration twice that of the final gel. This solution was heated to 60°C. A solution of equivalent volume containing 2% agarose was autoclaved for 7 min and allowed to cool to 60°C. The two solutions were mixed and allowed to cool in the dark. A special cutter was designed to cut the gel into columns with a diameter equal to that of the inside of the electrophoresis tubes. The gels were cut to the required length, pushed into the tube, 0.2 ml of stacking gel solution was pipetted into each tube above the separating gel, and distilled water was carefully layered on top. The stacking and separating gels were simultaneously photopolymerized. After electrophoresis the gels were stained with Oil Red O according to the method of Smithies (22). We have found that, for gels of large pore size, the use of agarose shortens the preparation times and makes the gels easier to handle without affecting resolution.

Zonal Centrifugation

Zonal centrifugation was performed according to the method of Wilcox and Heimberg (23) in a Spinco 15 Ti rotor. A sucrose gradient with d 1.00–1.21 g/ml was employed. Samples were centrifuged for 24 hr at 35,000 rpm. At the end of the centrifugation 15-ml fractions were collected in an UltroRac fraction collector (LKB Instruments Inc., Rockville, Md.). The optical densities of the fractions were measured at 280 nm with a Gilford model 2400 spectrophotometer (Gilford Instrument Laboratories Inc., Oberlin, Ohio).

Other Methods

Gel diffusion was performed in gels containing 1% agarose and 0.05 M Tris–HCl, pH 8.6. Samples were allowed to diffuse for 3 days before rinsing for 3 days, drying, and staining with amido black (24).

Serum LDL was isolated from nonfasted chow-fed rats by ultracentrifugation (25). Anti-LDL antiserum was made by the following procedure. A solution containing 3 mg of LDL was mixed with Freund's adjuvant and injected intramuscularly into the back of a rabbit. An equal quantity of LDL, without adjuvant, was injected intradermally and intravenously 7 and 14 days after the original injection. On the 23rd day the animals were exsanguinated. The purity of the antiserum was shown by the fact that it formed only one arc on gel diffusion against whole rat blood; this arc showed a total identity with isolated serum LDL.

Protein determinations were performed according to the method of Lowry, Rosebrough, Farr, and Randall (26) using human serum as standard.

For lipid analysis, 1 ml of sample was extracted with 18 ml of chloroform–methanol 2:1 (27). Lipid extracts were washed twice with 2 M KCl (28); cholesterol (29, 30), phospholipid (31), and neutral glyceride (32) determinations were performed on the extracts.

RESULTS

Perfusate Analysis

It has been shown that perfused livers of OA-treated animals are incapable of secreting either LDL or VLDL (6). We have confirmed this. In Fig. 1 is shown Oil Red O-stained electrophoresis patterns of the perfusates from an OA (tubes *4* and *5*) and a control liver (tubes *2* and *3*), before and after 3 hr of perfusion. A normal rat serum sample is included for comparison (tube *1*). The control liver appears to have secreted lipoprotein (mainly as VLDL), while the orotic acid liver did not. After even a fourfold concentration of the low density fraction of the perfusates of OA livers, by ultracentrifugation at 1.006

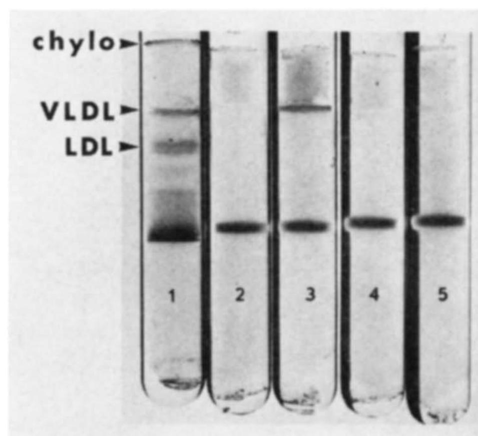

Fig. 1. Acrylamide–agarose electrophoresis of perfusates stained with Oil Red O. Gel *1* represents normal rat serum. Gels *2* and *3* are the control perfusates before and after 3 hr of perfusion, respectively. Gels *4* and *5* are the OA perfusates before and after 3 hr of perfusion. Bands not labeled represent serum proteins which have been fixed but do not stain with Oil Red O. HDL migrates with the protein and is discernible only when isolated from the remaining plasma proteins.

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g/ml, electrophoresis failed to reveal the presence of stainable lipid, while the control perfusate, after similar concentration, could be diluted 16-fold before stainable lipid could not be detected on the electropherograms. After concentration, control perfusates formed precipitation lines when reacted against anti-LDL antiserum on gel diffusion plates, while similarly concentrated OA perfusates formed no precipitation lines. Thus, neither the lipid nor the protein part of LDL or VLDL could be demonstrated in the perfusates of OA livers. Supplementation of the perfusates with amino acids and sodium linoleate did not change these findings.

Liver Analysis

If orotic acid treatment interfered with the secretion of LDL and VLDL rather than with the synthesis of their constituent apoproteins, these latter should be demonstrable in OA livers. Liposomes, microsomes, and cell sap were examined for the presence of LDL apoprotein. Table 1 gives the composition of OA liposomes as isolated by our procedure. When liposomes were isolated from the perfused OA livers and partially delipidated with ether, they showed a precipitation line when reacted against anti-LDL on gel diffusion (Fig. 2, wells *3* and *5*). The line formed a complete identity with partially delipidated serum VLDL (well *4*) and a partial identity with nondelipidated serum LDL (not shown here) or VLDL (well *6*). No line formed when normal rabbit serum was used instead of anti-LDL or when anti-LDL was incubated with purified LDL prior to placing it in the well. Liposomes either totally delipidated (i.e., with alcohol and ether) or treated only with SDS showed reactivity with anti-LDL antiserum.

If microsomes and cell sap from OA and control livers were similarly partially delipidated, they showed no precipitation line when diffused against anti-LDL antiserum. However, the high amount of protein in the microsomes made it impossible to concentrate them after incubation with SDS without making the extract so viscous that it would not diffuse in agarose.

TABLE 1 Chemical Composition of Isolated Liposomes and Fraction III*

	Liposomes	Relative Concentration: Liposomes	Relative Concentration: Fraction III*
	mg/g wet wt of liver		
Protein	0.37	1.00	1.00
Cholesterol	1.93	5.27	0.30
Phospholipid	0.98	2.67	3.34
Total neutral glycerides	53.4	146.1	1.87

* Fraction III is the peak with density between 1.020 and 1.060 g/ml on zonal centrifugation of partially delipidated (SDS treatment) liposomes from livers of OA-treated rats.

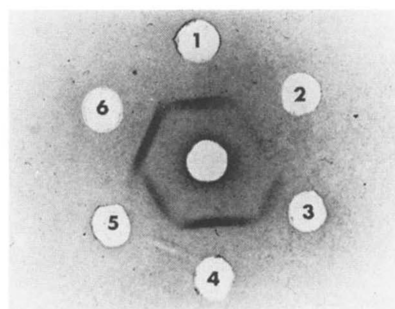

Fig. 2. Gel diffusion of treated VLDL and liposomes against anti-LDL antiserum. The center well contains anti-LDL antiserum. Wells *1* and *4* contain VLDL partially delipidated with ether. Wells *3* and *5* contain liposomes partially delipidated with ether. Well *2* contains totally delipidated VLDL, and well *6* contains nondelipidated VLDL. The gel was stained for protein with amido black.

Liposomes Partially Delipidated with SDS

It is not clear whether the LDL or VLDL proteins present in OA livers exist as free apoproteins or as lipoproteins. If the liposomes were partially delipidated with SDS only, 74% of the protein recovered in the subnatant after the low speed centrifugation floated upon subsequent ultracentrifugation at d 1.063 g/ml (24 hr at 30,000 rpm in a Spinco 30.2 rotor, using sucrose to adjust the density). When purified plasma VLDL was treated in a similar manner, 38% of the protein floated at d 1.063 g/ml; with similarly treated microsomes only 2% floated.

Fig. 3 shows the results of zonal centrifugation of SDS-delipidated liposomes. On the ordinate is represented the absorbance of each fraction at 280 nm. Fractions with density of 1.00 g/ml or less were noticeably milky, and their absorbances are in part due to light scattering. There appears to be a discrete fraction whose peak falls at d 1.025 g/ml. Tubes with density between d 1.020 and 1.060 g/ml (designated fraction III) were pooled and analyzed for lipid and protein. 27% of the total liposomal protein was contained in this fraction. Table 1 shows the relative abundance of its constituents. Prolonged treatment with SDS caused a net movement of protein to the bottom of the gradient, suggesting that the protein, in the density range of fraction III, is associated with lipid as a lipoprotein (neither lipid nor protein alone will band at this density). The SDS apparently removes the neutral lipid much more easily than the phospholipid, suggesting that the relation of neutral lipid to the protein is more tenuous. The possibility that fraction III might represent previously unassociated lipid and protein which have associated in the presence of SDS cannot be completely excluded.

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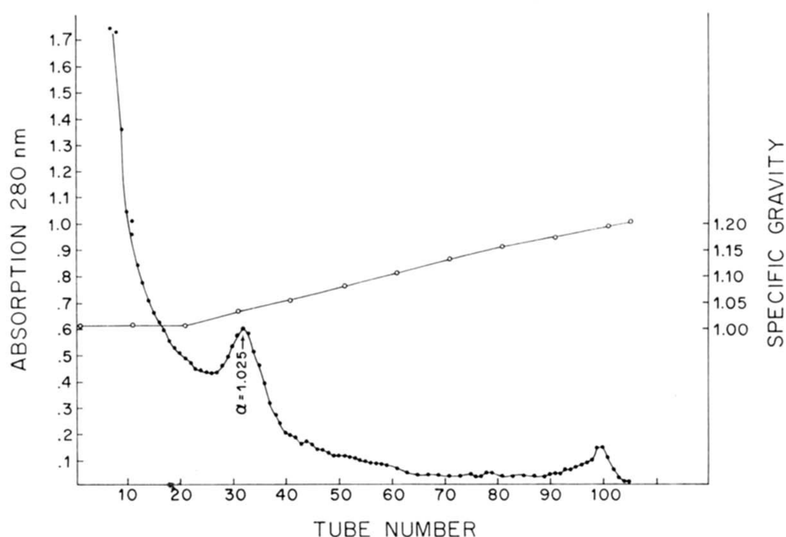

FIG. 3. Liposomes were made 0.2 M with respect to SDS and incubated for 90 min at 37°C. After low speed centrifugation (2000 g for 5 min) to remove most of the neutral lipid and treatment with Dowex 1 to remove the SDS, the sample was introduced to the bottom of the sucrose gradient. The sample was centrifuged for 24 hr in a Spinco 15 Ti rotor at 35,000 rpm. The gradient was collected (15 ml per tube) by displacement with a denser sucrose solution.

Apoprotein Analysis of VLDL and Liposomes

If the liposomes were in fact a site of accumulation of VLDL precursors, unable to be secreted, the VLDL apoproteins should be isolatable from the liposomes. Fig. 4 shows the results of acrylamide electrophoresis in 8 M urea at pH 8.9 of totally delipidated liposomal and VLDL samples. The middle gel is from a sample in which liposomes and VLDL were mixed prior to delipidation. Two major bands (*2* and *3*) and one minor band (*1*) of liposomes and VLDL comigrate. The one remaining major VLDL band (*4*) has no equivalent in the liposomal sample. Gels containing 4%, 5%, 7.5%, and 15% acrylamide showed similar relative results, although the absolute distance of migration varied with the density of the gel. Other investigators have obtained similar electrophoretic patterns for delipidated VLDL (25, 33). Their studies suggest that bands *3* and *4* are also present in HDL, while band *2* is unique to VLDL. Antiserum made against liposomal band *3* (purified by preparative acrylamide electrophoresis method to be presented later) reacted with totally delipidated VLDL.[2] The LDL apoprotein does not enter the gel under these conditions, so the bands seen in Fig. 4 represent the components of VLDL but do not include the LDL apoprotein.

[2] We have recently been able to demonstrate that bands 2 and 3 of the liposome are immunologically identical to their corresponding VLDL bands when reacted against antiserum made to purified VLDL apoproteins.

When liposomes or VLDL were totally delipidated without prior incubation with SDS, electrophoresis of the apoproteins gave the same results as shown in Fig. 4. Delipidation and electrophoresis of the liposomal and VLDL fractions from the zonal centrifugation separations indicated that all the apoproteins were also present in all these fractions, suggesting that the fractions were

FIG. 4. Acrylamide electrophoresis in 8 M urea at pH 8.9 of totally delipidated liposomes and VLDL. Gels *a* and *c* are totally delipidated liposomes and VLDL, respectively. In gel *b*, liposomes and VLDL were mixed prior to delipidation. Direction of migration is from top to bottom. Concentration of acrylamide was 4%. Gels were stained for protein with Coomassie brilliant blue. The amount of liposomal material used is greater than the amount of VLDL in order to emphasize the points of comigration.

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separated on the basis of the degree of delipidation, rather than by protein structure.

When microsomes at protein concentrations equivalent to those of the liposome preparations were totally delipidated and electrophoresed, no protein entered the gel. This suggests that the liposomal apoprotein bands seen on electrophoresis are probably derived from the contents of the liposome rather than from its surrounding envelope, which appears on electron microscopic examination to be a derivative of the endoplasmic reticulum.

HDL Apoproteins

Since some of the apoproteins found in VLDL, including that missing from OA liposomes, are common to HDL, we examined the apoproteins of HDL from the serum of both OA-treated animals and animals on the same diet but lacking OA. Fig. 5 shows the results of acrylamide electrophoresis in 8 M urea of totally delipidated control and OA serum HDL from equivalent amounts of serum. Components *3* and *4* of VLDL are immunologically and electrophoretically similar to components *5* and *6* of HDL (25, 33). We have found that these bands comigrate on electrophoresis at concentrations of acrylamide ranging from 4 to 15%. In control HDL, band *5* was always more intense than *6*; the reverse was true of OA HDL. This is of particular interest, since band *6* comigrates with band *4* of VLDL, which is entirely missing from the liposomal proteins. Band *7* is prominent in OA HDL but very faint in control HDL. Similar results were found for HDL derived from animals on the diet for either 7 or 12 days.

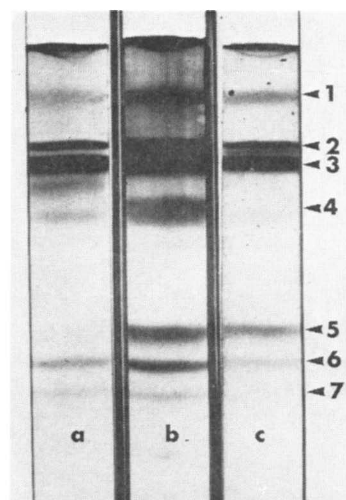

FIG. 5. Acrylamide electrophoresis of totally delipidated serum HDL. Gels *a* and *c* are samples of HDL isolated from equivalent amounts of sera of OA-treated animals and control animals, respectively. In gel *b*, the OA and control HDL were mixed prior to electrophoresis. Conditions of electrophoresis were similar to Fig. 4, except the gel was 5% with respect to acrylamide.

DISCUSSION

Our studies of the orotic acid fatty liver have led to the following conclusions: (*a*) in agreement with Windmueller and Levy (6), upon perfusion of livers from rats fed OA, no detectable amounts of LDL or VLDL were secreted into the perfusate, while normal livers under similar circumstances secreted significant quantities of VLDL; (*b*) liposomes isolated from the liver cells of OA-treated rats contain a protein which reacts with anti-LDL antiserum; (*c*) upon acrylamide gel electrophoresis, the major apoproteins of totally delipidated liposomes, solubilized in 8 M urea, comigrate with similarly treated VLDL except for one fast migrating band which is present in delipidated VLDL but missing from the extracts of delipidated liposomes; and (*d*) HDL apoproteins from the sera of both control and OA-treated animals contain apoproteins in common with VLDL, including that missing from the liposome; however, these are in different quantitative relationship to one another in OA HDL compared to normal HDL.

The fact that LDL and VLDL[2] antigens were isolated from those OA livers, shown, upon perfusion, to be incapable of secreting LDL or VLDL, suggests that OA treatment interferes with the secretion of these lipoproteins rather than with the synthesis of their protein constituents. Earlier investigations suggesting that OA does not inhibit apoprotein synthesis have been reported (34); however, this is the first demonstration of the presence of apoprotein within the liver during OA treatment. Our results indicated that the OA livers were incapable of secreting more than 7% of the VLDL or LDL secreted by normal livers during perfusion; we were never able to demonstrate LDL in the perfusate of OA livers, either immunologically or electrophoretically, even with supplementation of the perfusate with fatty acids and amino acids. Furthermore, perfusion of OA livers prior to liposome isolation eliminated the possibility that the isolated lipoproteins were derived from the vascular space of the liver. Since the vascular space contained no detectable LDL and VLDL, the isolated proteins must have come from the liver cells.

The liposome appears to be the main site of storage or accumulation of VLDL proteins resulting from OA treatment. Our inability to recover LDL or VLDL proteins from OA microsomes suggests that if these proteins are present in this subcellular fraction, they must be in much lower concentration than in the liposomal fraction. That these proteins are merely endoplasmic reticulum membrane proteins, which after detergent or lipophilic solvent treatment have the same general properties and behavior as VLDL protein, is also rendered very unlikely by our inability to isolate them, either electro-

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phoretically or immunologically, from the microsome fraction.

It has been difficult to gain a clear assessment of the relationship between the lipid and the apoprotein within the liposome. The relative amounts of protein and lipid in the liposome suggest that they contain proportionately much more neutral lipid than would be expected of serum VLD-type protein. Thus, the liposome probably contains a substantial amount of "free" triglyceride. On the other hand, however, much of the protein seems to be associated with lipid, as evidenced by the fact that after partial delipidation with SDS, most of the protein of the liposome floats at d 1.063 g/ml, under conditions used for the isolation of normal serum LDL, a protein normally associated with a substantial amount of neutral and phospholipid. This conclusion is further supported by the appearance of much of the liposomal protein, upon zonal centrifugation in a region of the density gradient expected of such a lipoprotein. Compositional analysis of this density fraction reveals not only the presence of substantial amounts of phospholipid but also a relationship of phospholipid to protein which is very similar to that of serum VLDL (35). To prove more securely that lipid and protein are being stored in the liposome as lipoprotein rather than as separate lipid and apoprotein, it would be valuable to demonstrate the existence of discrete particles within the liposome containing both lipid and protein. We have been unable to provide electrophoretic evidence, by either acrylamide, agarose, or paper electrophoresis, for discrete lipoprotein in the liposomal extracts before or after partial SDS delipidation. The application to the liposomes of the procedures used by Mahley et al. (36) for the isolation from rat-liver Golgi apparatus of particles which behaved electrophoretically like serum lipoprotein did not meet with success. This is apparently due to the large "excess" of neutral lipid which could not be differentially removed (either by sonication or SDS treatment). The products of these procedures merely yielded smears of Oil Red O-staining material on electrophoresis. The presence of SDS in the isolation procedure for partially delipidated products might be dramatically altering the association of lipid and protein that exists within the liposome, so that the adventitious association of lipid and protein during their isolation cannot be completely excluded. The difference in size between OA liposomes (0.2–0.5 μ) (5) and rat VLDL (0.03–0.08 μ in diameter) (37) suggests that the liposome cannot represent a single trapped lipoprotein particle. If, however, liposomes were sacs of discrete lipoproteins, electron microscopic examination would be expected to reveal grape-like clusters of lipoproteins similar to those seen in Golgi vesicles (37). The fact that the contents of liposomes generally appear as large single droplets suggests that these bodies are not simply the sites of storage of unsecreted VLDL particles.

The major difference between the apoproteins found in the liposome and those of serum VLDL is the absence of the fastest-migrating major polypeptide (band 4) from the acrylamide electrophoretic pattern of the former. Band 4 has been shown to be immunologically indistinguishable from the slightly slower migrating band 3 (33). The two polypeptides apparently differ in their carbohydrate content.[3] If this is accepted to be the only difference between bands 3 and 4, it may be suggested that all of the major VLDL polypeptides are synthesized in the liver of the OA-fed rat, but that this dietary treatment results in an incomplete addition of one or more carbohydrate moieties to at least one of these polypeptides, or that band 4 is rapidly secreted by the liver while band 3 is retained. There appear to be three possible explanations for the possibility that extra carbohydrate is not added. OA could regulate the synthesis of the appropriate glycosylating enzyme. Alternatively, this hypothetical enzyme could be inhibited by the accumulation of a metabolic product such as UDP-*N*-acetyl glucosamine, which has been shown to double in concentration in the liver of OA-treated rats (38). Consistent with this explanation is the observation that dietary adenine administration prevents the rise in UDP-*N*-acetyl glucosamine as well as the appearance of a fatty liver. The precedent for such a regulatory mechanism has been described elsewhere (39). Thirdly, it is possible that OA treatment interferes with the access of the substrate for the particular glycosylation reaction to the site on the enzyme. Recently, Lo and Marsh (40) demonstrated that the major incorporation of *N*-acetyl glucosamine into serum lipoproteins occurs in the Golgi vesicles, which are known to contain all the major VLDL apoproteins, apparently with their complete complement of carbohydrate (36). It is possible that the Golgi apparatus is most active in the formation of glycolipoprotein, because this is the site of highest concentration of acceptor apolipoprotein; and perhaps OA treatment prevents the transport of the latter to the Golgi apparatus.

In view of the fact that the VLDL apoprotein which migrates as band 4 on gel electrophoresis is among the peptides which are apparently common to VLDL and HDL, we examined the serum HDL of OA-treated rats. It was surprising to find that HDL from OA-treated animals contained significant quantities of an apoprotein which migrates as band 6, and which corresponds to band 4 of VLDL. A number of possible explanations can be suggested for this finding, but the observations reported in this paper do not allow a distinction between these possibilities. It is possible that under these circumstances all or some of the peptides found in the serum are made

[3] Personal communication from Dr. Virgil S. LeQuire.

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extrahepatically, such as in the intestine; the intestinal production of LDL is not inhibited by OA treatment (41). Perhaps the mechanism of glycosylation or secretion of HDL and VLDL by the liver, or both, are different, or perhaps band 4 of VLDL and band 6 of HDL are not truly identical, at least with respect to their carbohydrate composition. Only further work can distinguish these possibilities.

The results reported in this paper indicate that OA treatment does not interfere with the synthesis of the polypeptide backbone of the VLDL apoproteins, but rather with their secretion or the final stages of assembly. Irrespective of the ultimate detailed explanation for these findings, OA treatment clearly presents a useful model for the study of the stages and regulation of lipoprotein assembly and secretion.

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References

1. Standerfer, S. B., and P. Handler. 1955. Fatty liver induced by orotic acid feeding. *Proc. Soc. Exp. Biol. Med.* **90:** 270–271.
2. Baglio, C. M., and E. Farber. 1965. Reversal by adenine of the ethionine-induced lipid accumulation in the endoplasmic reticulum of the rat liver. *J. Cell Biol.* **27:** 591–601.
3. Lombardi, B. 1966. Considerations on the pathogenesis of fatty liver. *Lab. Invest.* **15:** 1–20.
4. Schlunk, F. F., and B. Lombardi. 1967. Liver liposomes. I. Isolation and chemical characterization. *Lab. Invest.* **17:** 30–38.
5. Novikoff, A. B., P. S. Roheim, and N. Quintana. 1966. Changes in rat liver cells induced by orotic acid feeding. *Lab. Invest.* **15:** 27–49.
6. Windmueller, H. G., and R. I. Levy. 1967. Total inhibition of hepatic β-lipoprotein production in the rat by orotic acid. *J. Biol. Chem.* **242:** 2246–2254.
7. Windmueller, H. G. 1964. An orotic acid-induced, adenine-reversed inhibition of hepatic lipoprotein secretion in the rat. *J. Biol. Chem.* **239:** 530–537.
8. Creasey, W. A., L. Hankin, and R. E. Handschumacher. 1961. Fatty livers induced by orotic acid. I. Accumulation and metabolism of lipids. *J. Biol. Chem.* **236:** 2064–2070.
9. Deamer, D. W., F. A. Kruger, and D. G. Cornwall. 1965. Total liver protein and amino acid incorporation into liver protein in orotic acid-induced fatty liver. *Biochim. Biophys. Acta.* **97:** 147–149.
10. Roheim, P. S., S. Switzer, A. Girard, and H. A. Eder. 1966. Alterations of lipoprotein metabolism in orotic acid-induced fatty liver. *Lab. Invest.* **15:** 21–26.
11. Mortimore, G. E. 1963. Effect of insulin on release of glucose and urea by isolated rat liver. *Amer. J. Physiol.* **204:** 699–704.
12. John, D. W., and L. L. Miller. 1969. Regulation of net biosynthesis of serum albumin and acute phase plasma proteins. *J. Biol. Chem.* **244:** 6134–6142.
13. Rajalakshmi, S., W. R. Adams, and R. E. Handschumacher. 1969. Isolation and characterization of low density structures from orotic acid-induced fatty livers. *J. Cell Biol.* **41:** 625–636.
14. Granda, J. L., and A. Scanu. 1966. Solubilization and properties of the apoproteins of the very low- and low-density lipoproteins of human serum. *Biochemistry.* **5:** 3301–3308.
15. Karush, F., and M. Sonnenberg. 1950. Long-chain alkyl sulfates. Colorimetric determination of dilute solutions. *Anal. Chem.* **22:** 175–177.
16. Gotto, A. M., R. I. Levy, F. T. Lindgren, and D. S. Fredrickson. 1969. Partial delipidation of human serum β-lipoprotein by sodium decyl sulfate. *Biochim. Biophys. Acta.* **176:** 667–669.
17. Scanu, A., H. Pollard, R. Hirz, and K. Kothary. 1969. On the conformational instability of human serum low-density lipoprotein: effect of temperature. *Proc. Nat. Acad. Sci. U.S.A.* **62:** 171–178.
18. Reisfeld, R. A., and P. A. Small, Jr. 1966. Electrophoretic heterogeneity of polypeptide chains of specific antibodies. *Science (Washington).* **152:** 1253–1255.
19. Potts, J. T., R. A. Reisfeld, P. F. Hirsch, A. B. Wasthed, E. F. Voelkel, and P. L. Munson. 1967. Purification of porcine thyrocalcitonin. *Proc. Nat. Acad. Sci. U.S.A.* **58:** 328–335.
20. Chrambach, A., R. A. Reisfeld, M. Wyckoff, and J. Zaccari. 1967. A procedure for rapid and sensitive staining of protein fractionated by polyacrylamide gel electrophoresis. *Anal. Biochem.* **20:** 150–154.
21. Narayan, K. A., H. L. Creinin, and F. A. Kummerow. 1966. Disc electrophoresis of rat plasma lipoproteins. *J. Lipid Res.* **7:** 150–157.
22. Smithies, O. 1959. Zone electrophoresis in starch gels and its application to studies of serum proteins. *Advan. Protein Chem.* **14:** 65–114.
23. Wilcox, H. G., and M. Heimberg. 1970. Isolation of plasma lipoproteins by zonal ultracentrifugation in the B14 and B15 titanium rotors. *J. Lipid Res.* **11:** 7–22.
24. Williams, C. A., and M. W. Chase. 1968. Methods in Immunology and Immunochemistry. Academic Press Inc., New York. 30.
25. Koga, S., D. L. Horwitz, and A. M. Scanu. 1969. Isolation and properties of lipoproteins from normal rat serum. J. *Lipid Res.* **10:** 577–588.
26. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193:** 265–275.
27. 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.
28. Tarlov, A. R., and E. P. Kennedy. 1965. The β-galactoside permease system and the metabolism of phospholipids in *Escherichia coli*. *J. Biol. Chem.* **240:** 49–53.
29. Abell, L. L., B. B. Levy, B. B. Brodie, and F. E. Kendall. 1952. A simplified method for the estimation of total cholesterol in serum and demonstration of its specificity. *J. Biol. Chem.* **195:** 357–366.

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30. Huang, T. C., C. P. Chen, V. Wefler, and A. Raftery. 1961. A stable reagent for the Liebermann-Burchard reaction. *Anal. Chem.* **33:** 1405–1407.
31. Bartlett, G. R. 1959. Phosphorus assay in column chromatography. *J. Biol. Chem.* **234:** 466–468.
32. Pinter, J. K., J. A. Hayashi, and J. A. Watson. 1967. Enzymatic assay of glycerol, dihydroxyacetone, and glyceraldehyde. *Arch. Biochem. Biophys.* **121:** 404–414.
33. Bersot, T. P., W. V. Brown, R. I. Levy, H. G. Windmueller, D. S. Fredrickson, and V. S. LeQuire. 1970. Further characterization of the apolipoproteins of rat plasma lipoproteins. *Biochemistry.* **9:** 3427–3433.
34. Roheim, P. S., S. Switzer, A. Girard, and H. A. Eder. 1965. The mechanism of inhibition of lipoprotein synthesis by orotic acid. *Biochem. Biophys. Res. Commun.* **20:** 416–421.
35. Mahley, R. W., R. L. Hamilton, and V. S. LeQuire. 1969. Characterization of lipoprotein particles isolated from the Golgi apparatus of rat liver. *J. Lipid Res.* **10:** 433–439.
36. Mahley, R. W., T. P. Bersot, V. S. LeQuire, R. I. Levy, H. G. Windmueller, and W. V. Brown. 1970. Identity of very low density lipoprotein apoproteins of plasma and liver Golgi apparatus. *Science (Washington).* **168:** 380–382.
37. Jones, A. L., N. B. Ruderman, and M. G. Herrera. 1967. Electron microscopic and biochemical study of lipoprotein synthesis in the isolated perfused rat liver. *J. Lipid Res.* **8:** 429–446.
38. von Euler, L. H., R. J. Rubin, and R. E. Handschumacher. 1963. Fatty livers induced by orotic acid. II. Changes in nucleotide metabolism. *J. Biol. Chem.* **238:** 2464–2469.
39. Kornfeld, S., R. Kornfeld, E. F. Neufeld, and P. J. O'Brian. 1964. The feedback control of sugar nucleotide biosynthesis in liver. *Proc. Nat. Acad. Sci. U.S.A.* **52:** 371–379.
40. Lo, C., and J. B. Marsh. 1970. Biosynthesis of plasma lipoproteins. *J. Biol. Chem.* **245:** 5001–5006.
41. Windmueller, H. G., and R. I. Levy. 1968. Production of β-lipoprotein by intestine in the rat. *J. Biol. Chem.* **243:** 4878–4884.

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