Apolipoproteins of the orotic acid fatty liver: implications for the biogenesis of plasma lipoproteins[1]

Rick Hay, Richard Fleming, William O'Connell, Joshua Kirschner, and Wolfgang Oppliger

Department of Pathology and the Specialized Center of Research in Atherosclerosis, The University of Chicago, Chicago, IL 60637

Abstract Rats fed orotic acid develop fatty livers characterized by triglyceride-laden, membrane-bounded vesicles designated "liposomes." We have measured the levels of apolipoproteins in isolated liposomes and other subcellular fractions by SDS-polyacrylamide gel electrophoresis, electrotransfer, and immunodecoration. Apolipoproteins B_h, B_l, E, and C appear to cofractionate; for these proteins, the liposomal pool represents a large portion of their total intracellular mass. However, liposomes are deficient in both variants of apoB relative to apoE and apoC when compared with rat plasma very low density lipoprotein (VLDL). Albumin and apolipoproteins A-I and A-IV are also found in liposomes, but this organelle represents a minor fraction of their total intracellular mass. The liposomal apolipoproteins show varying degrees of association with cisternal lipid and with organelle membranes. ■ Orotic acid may selectively block VLDL production at the level of particle assembly or transorganellar movement. We conclude that liposomal contents probably represent exaggerated accumulations of VLDL assembly intermediates, and that the intracellular partitioning of high density lipoprotein-destined from VLDL-destined components occurs at an early stage in particle biogenesis. Moreover, some unique structural feature of apoB may effect movement of VLDL assembly intermediates through secretory organelles.—**Hay, R., R. Fleming, W. O'Connell, J. Kirschner, and W. Oppliger.** Apolipoproteins of the orotic acid fatty liver: implications for the biogenesis of plasma lipoproteins. *J. Lipid Res.* 1988. **29:** 981–995.

Supplementary key words SDS-polyacrylamide gel electrophoresis • electrotransfer and immunodecoration • plasma lipoprotein assembly and secretion • liposomes • endoplasmic reticulum

Ingestion of orotic acid by rats leads to deficiencies of circulating VLDL and LDL (1–3) and to development of fatty livers (4). These are unusual among experimental fatty livers in that lipid accumulates within and distends the membranes of secretory organelles (5, 6) to form structures designated "liposomes" (7).

The accumulated morphological and biochemical evidence argues that liposomes contain predominantly, if not exclusively, elements of the endoplasmic reticulum (5–12). Liposomes viewed by electron microscopy in situ (5) or as isolated under conditions that do not disrupt ribosomal integrity (9) are studded with membrane-bound ribosomes. The apoC and apoE contents of liposomes lack galactose and sialic acid, which are normally added to glycoproteins by enzymes located in distal parts of the Golgi apparatus (7, 12). The elegant morphological studies of Jatlow, Adams, and Handschumacher (6) and of Novikoff et al. (8) strongly suggest that liposomes develop from previously normal endoplasmic reticulum. Isolated washed liposomes do not contain other morphologically identifiable organelles or nonliposomal membranes, except for rare mitochondria (9); nor do the nonliposomal organelles display any morphology to suggest that they should float under classical fractionation conditions (6, 9). Specifically, the Golgi elements of the orotic acid fatty liver lack particles even as large as plasma VLDL (5, 8, 10).

Liposomes contain triglyceride as the major lipid (11, 13, 14). It has been suggested that the liposomal contents represent intermediates in the assembly pathway for plasma VLDL, prevented from successful secretion by mechanisms still unknown (3, 7–9). The liposome almost certainly does not represent a permanent abnormality of the affected organelle, since both the fatty liver and the dyslipoproteinemia are readily reversed or prevented by adenine or adenosine (2, 11, 15).

It is not clear whether the orotic acid effects are specific or merely selective to inhibition of hepatic VLDL produc-

Abbreviations: apo, apolipoprotein; BAC, N,N'-bisacrylylcystamine; DATD, N,N'-diallyltartardiamide; ETID, electrotransfer and immunodecoration; HDL, high density lipoprotein; LDL, low density lipoprotein; PMSF, phenylmethylsulfonyl fluoride; SDS, sodium dodecyl sulfate; SDS-PAGE, SDS-polyacrylamide gel electrophoresis; TLCK, Na-*p*-tosyl-*L*-lysine chloromethyl ketone; TPCK, N-tosyl-*L*-phenylalanine chloromethyl ketone; VLDL, very low density lipoprotein.

[1]Portions of this study have been presented in preliminary form (Hay, R. V., and R. M. Fleming. 1985. Nascent very low density lipoprotein particles and apoproteins of hepatocytic endoplasmic reticulum. *Circulation.* **72:** III-91).

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tion. Windmueller and Levy (3) observed a reduction in plasma α-lipoprotein (high density lipoprotein [HDL]) in rats fed orotic acid, as well as decreased production of HDL by the perfused orotic acid fatty livers compared to chow-fed controls. Roheim et al. (1) also observed decreased in vivo radiolabeling of HDL, as measured by total activity, in orotic acid-treated rats as compared to controls. On the other hand, Hamilton and his colleagues (10) have detected no quantitative abnormality of nascent HDL or of apolipoprotein A-I production by perfused fatty livers.

We have undertaken a quantitative analysis of apolipoprotein distribution within the orotic acid-induced fatty liver. Our specific goals were *i*) to establish the amounts of precursors to plasma apolipoproteins E and C accumulating in liposomes relative to other subcellular fractions; *ii*) to determine whether apolipoproteins B_h and B_l accumulate within liposomes; *iii*) to determine whether the intracellular distributions of albumin and apolipoproteins classically associated with HDL are influenced by the administration of orotic acid; and *iv*) to examine the state of lipidation of apolipoprotein molecules within the liposomes.

MATERIALS AND METHODS

Chemicals and reagents

Some diet components (sucrose, corn oil) were purchased at a local grocery. Most other diet supplies and glycine were obtained through ICN-Nutritional Biochemicals. Orotic acid monohydrate and partially purified rat serum albumin were purchased from Sigma. ^{125}I-Labeled protein A was purchased from Amersham Radiochemicals or from New England Nuclear. Nitrocellulose was purchased from Millipore or from Schleicher and Schuell; X-ray film (XS-5) was purchased from Kodak. All other chemicals and reagents were of the best commercial grade possible.

Animals and diets

Male rats of the Sprague-Dawley strain weighing 125-150 g at the beginning of feeding were housed in small groups in wire-bottomed cages and exposed to alternating light and dark periods of 12 hr each. They were given water ad libitum and fed the semisynthetic diet described by Pottenger and Getz (7) containing 1% (w/w) orotic acid and 5% (w/w) corn oil for 7-10 days prior to being killed. Donor rats for total plasma, for HDL, and for preparation of normal liver homogenate were maintained on Purina Rat Chow ad libitum. Donor rats for plasma VLDL were fed a modified basal atherogenic diet (ICN-Nutritional Biochemicals) for 1 week prior to being killed.

Purification of apolipoproteins

Plasma obtained from donor rats was adjusted to contain 1 mM phenylmethylsulfonyl fluoride (PMSF), 5-10 mM EDTA, 0.02% NaN_3, and 1 mM butylated hydroxytoluene to minimize proteolysis and oxidation of lipoprotein particles during the subsequent lipoprotein isolation steps. The different lipoprotein classes were prepared from plasma by classical ultracentrifugal flotation (16, 17). Isolated fractions were stored under nitrogen gas at 4°C in the presence of the above preservatives, or frozen in small aliquots at −80°C. Apolipoproteins to be used as immunogens and as standards for electrophoresis and for immunodecoration were prepared from isolated plasma lipoprotein fractions by two cycles of preparative SDS-polyacrylamide gel electrophoresis followed by electroelution from gel slices with minor modifications of the method of Daum, Böhni, and Schatz (18). Apolipoprotein B variants were also purified by preparative electrophoresis on 4% polyacrylamide gels crosslinked with the cleavable reagent N,N'-bisacrylylcystamine (BAC), followed by solubilization of the gels with 2-mercaptoethanol (19) and recovery of the liberated proteins by trichloroacetic acid precipitation. The purity of all preparations was estimated by visual inspection of overloaded, Coomassie Blue-stained analytical electropherograms.

Preparation and testing of antisera

Purified antigens (10-50 μg protein) were emulsified with complete Freund's adjuvant and injected at multiple intradermal sites on the shaved backs of rabbits. After 6 weeks an equivalent aliquot of purified antigen was injected intravenously without adjuvant. The rabbits were bled from peripheral ear veins between 1 and 3 weeks after boosting, and were reboosted and rebled approximately every 6 weeks thereafter. Hyperimmune sera were tested for specificity of reactivity against purified standards, reconstituted lipoprotein mixtures, and whole plasma by electrotransfer and immunodecoration.

Fractionation of fatty livers

Our fractionation scheme, modified from that described by Pottenger and Getz (7) is illustrated in **Fig. 1.** For each preparation the fully developed fatty livers of two animals were combined. Animals were exsanguinated under deep ether anesthesia. The livers were excised, weighed, minced, and homogenized in ice-cold buffer (50 mM HEPES-NaOH, pH 7.4, 100 mM NaCl, 2 mM $MgCl_2$, 0.25 M sucrose) in the presence of freshly added 1 mM PMSF to minimize proteolysis. Homogenization was accomplished with four or five strokes at 1000-1500 rpm of a smooth Teflon pestle within a large glass mortar (Thomas Type C; 40 ml capacity; nominal clearance 0.15-0.23 mm) suspended in an ice-water bath. All subsequent steps were conducted between 0°C and 4°C, and fresh PMSF was added at each resuspension step. The

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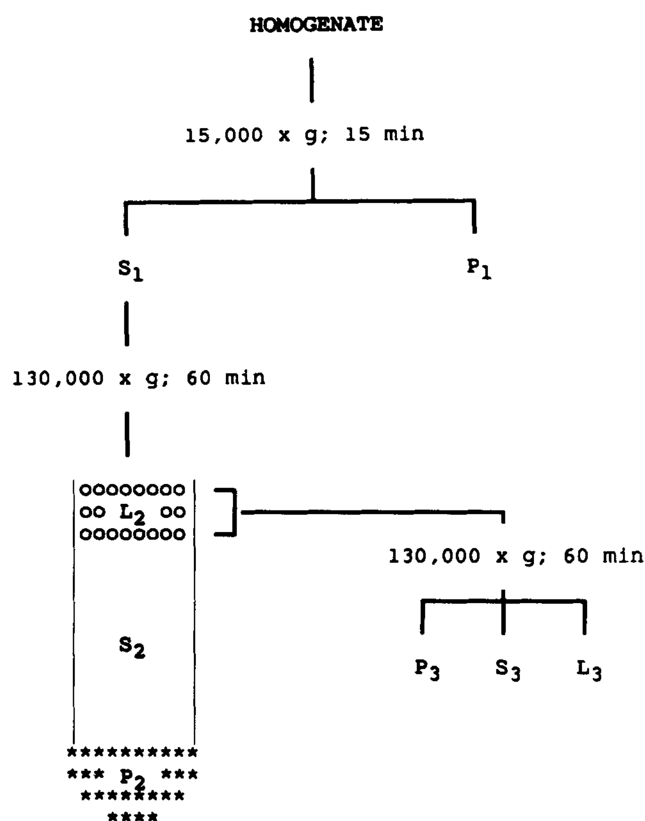

Fig. 1. Fractionation of orotic acid fatty livers. Rats were fed a semisynthetic diet containing orotic acid for 7–10 days prior to being killed. Livers were homogenized in pairs and subjected to subcellular fractionation by the indicated scheme. Fractions are designated as follows: P_1, nuclear-mitochondrial fraction; S_1, postmitochondrial supernatant fraction; P_2, crude microsomal pellet; S_2, cytosol; L_2, crude liposomes; L_3, washed liposomes; P_3, pellet from liposomal wash; S_3, internatant fraction from liposomal wash. Inasmuch as P_3 and S_3 accounted for less than 1% of total recovered protein and for a trivial amount of each apolipoprotein species, they were not analyzed further.

homogenate was subjected to low-speed centrifugation (spin I: 15,000 g_{max}; 15 min) to remove nuclei, mitochondria, and other rapidly sedimenting membranes (P_1). The postmitochondrial supernatant fraction (S_1) was separated by ultracentrifugation (spin II: 130,000 g_{max}; 60 min) into crude liposomes (L_2), microsomes (P_2), and a classical cytosolic fraction (S_2). The liposomal pellicle was resuspended in homogenization buffer with two to three gentle strokes of a loose pestle in a Dounce homogenizer (Wheaton 40 ml with B pestle; nominal clearance 0.15–0.63 mm), and recentrifuged (spin III: 130,000 g_{max}; 60 min) to yield washed liposomes (L_3), washed pellet (P_3), and internatant (S_3) fractions. For the data displayed in Fig. 2 and Table 2, primary fractions P_1, S_2, and P_2 were also recovered from the liver of a normal, chow-fed rat by following the scheme of Fig. 1 through the first ultracentrifugation step. All fractions were analyzed immediately or stored frozen in concentrated suspensions at −80°C until further use.

To resolve isolated liposomes or microsomes into membranes and cisternal contents, the method of Fujiki et al. (20) as adapted by Howell and Palade (21) was used with minor modifications. Freshly isolated organelles were gently resuspended in at least 30 volumes of ice-cold 0.1 M Na_2CO_3, pH 11.3, incubated on ice for 30 min, neutralized with 1/10 volume of 1 N HCl, and then subjected to ultracentrifugation (130,000 g_{max}; 60 min) to pellet the membranes. From washed microsomes (P_5: crude microsomes [P_2] resuspended and resedimented as in Fig. 5) were recovered a membrane pellet (P_6) and a soluble fraction (S_6). From washed liposomes were recovered a lipid-rich pellicle (L_4), a pellet (P_4), and an internatant fraction (S_4). Control experiments (not shown) confirmed the observation by Howell and Palade (21) that this carbonate extraction procedure does not alter the recovery of protein or of lipid from plasma VLDL, and furthermore showed no detectable difference in the protease accessibility of apolipoproteins between carbonate-treated and mock-treated VLDL particles.

Chemical and electrophoretic analysis of subcellular fractions

The protein contents of cells, homogenates, organelles, and isolated lipoprotein and apolipoprotein fractions were estimated by modifications of the method of Lowry et al. (22, 23). All lipid analyses were performed in the Core Chemistry Laboratory of the Specialized Center of Research in Atherosclerosis. Triglyceride and total cholesterol were determined enzymatically using the Auto/Stat Kit and the Rapid Stat Kit, respectively, from Lancer. Cholesteryl ester was determined according to Yamaguchi, Marui, and Hayashi (24). Phospholipids were estimated by the method of Bartlett (25).

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed in most instances using slab gels of acrylamide crosslinked with bisacrylamide and stabilized with linear polyacrylamide as described by Douglas and Butow (26). ApoB variants were better resolved on 3.5–4.0% polyacrylamide gels utilizing the crosslinker N,N'-diallyltartardiamide (DATD) (27) at an acrylamide:DATD ratio of 3.7:1.

Electrotransfer and immunodecoration (ETID)

Electrotransfer of proteins from SDS-PAGE gels to nitrocellulose sheets and immunodecoration of individual antigens with antibody and ^{125}I-labeled protein A followed the basic procedures elaborated by Towbin, Staehelin, and Gordon (28) with modifications suggested by Rott and Nelson (29) and by Johnson et al. (30). Quantitation of antigen-associated radioactivity and conversion of these values to relative antigen concentration followed the procedures described by Suissa (31) as adapted by Hay et al. (32). For each antigen, standard curves of purified

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apolipoproteins, secondary apolipoprotein-containing standards, and of index subcellular fractions were prepared to ensure that the sampling points lay within the linear and proportional range of the assay (31, 32). The specific application of these procedures to the measurement of intracellular apolipoproteins is described in detail in Results.

Statistical analysis

Two-sided *t*-tests for statistical significance and simple and multiple regression analyses for correlations (33) were performed on an IBM personal computer with the program Minitab (Minitab, Inc.).

Enzyme assays

Organelle marker enzymes were assayed for activity as follows. For endoplasmic reticulum, NADPH-cytochrome *c* reductase was assayed according to Tata et al. (34); for Golgi, galactosyltransferase was measured according to Fleischer (35); for plasma membrane, alkaline phosphodiesterase I was measured by the method of Edelson and Gass (36); and for lysosomes, N-acetyl-β-glucosaminidase was determined according to Baggiolini (37).

RESULTS

Characterization of primary subcellular fractions

The scheme illustrated in Fig. 1 yields four primary end fractions. These are the low-speed centrifugation pellet (nuclei, mitochondria, tissue debris, and other rapidly sedimenting membranes, P_1), the high-speed centrifugation pellet (microsomes, P_2), the washed liposomal pellicle (L_3), and the high-speed internatant fraction (presumed to be cytosolic contents, S_2). The secondary fractions obtained by resuspension and recentrifugation of crude liposomes (L_2), i.e., the wash pellet (P_3) and the wash internatant fraction (S_3), are not further evaluated in this report. Each of these wash fractions constitutes less than 1% of total recovered protein and contains a trivial proportion of any of the marker proteins we have examined.

Table 1 summarizes chemical analyses of the primary subcellular fractions. The liposomes constitute only about 3% of the total protein recovered by the fractionation procedure. When the chemical recoveries are normalized to organellar protein, however, liposomes are greatly enriched in all lipid classes. The liposomal fraction has mean concentrations for triglyceride of about 10-fold, for cholesterol of 3- to 4-fold, and for phospholipid of 3- to 15-fold as high as homogenate or the other primary fractions.

There were no significant differences in the ratio of triglyceride to protein among nonliposomal fractions and crude homogenate. The proportion of total cholesterol recovered as cholesteryl ester for homogenate and all four primary fractions was 0.69 ± 0.10 (mean and standard error), with no significant differences among the fractions.

In order to assess whether orotic acid treatment alters the behavior during fractionation of organelles other than the endoplasmic reticulum, we compared the distributions of selected microsomal marker enzymes among primary fractions of fatty liver homogenate to that of liver homogenate from a chow-fed rat. These data are presented in **Fig. 2** and **Table 2.** For this comparison we considered the combination of L_3 + P_2 from the fatty liver as equivalent to the microsomal pellet P_2 from the normal liver. We found no significant differences between the two sets of primary fractions with respect to either the distribution of total protein or the distribution of any of the marker enzymes. As expected, microsomes are enriched in markers for endoplasmic reticulum, Golgi, and plasma membrane; the lysosomal marker enzyme N-acetyl-β-glucosaminidase displays high specific activities in both the low-speed pellets P_1 and the microsomal pellets; and the cytosolic fractions S_2 have the lowest specific activities for all four marker enzymes. Among the primary fractions recovered from fatty liver homogenate, liposomes are most enriched in the endoplasmic reticulum marker NADPH-cytochrome *c* reductase, whereas the nonliposomal microsomes are higher than liposomes with regard to specific activities of the other three markers. Moreover, the total activities of the latter three enzymes in liposomes show no enrichment from the initial homogenate; their activities, therefore, most likely reflect adventitious contamination of "pure" liposomes by other organelles during the fractionation procedure.

Thus, except for stimulating the formation of liposomes, orotic acid treatment does not appreciably perturb the fractionation of microsomal organelles from rat liver. Furthermore, our findings confirm that isolated orotic acid fatty liver liposomes constitute a significantly enriched fraction of endoplasmic reticulum.

Protein components of the primary fractions are displayed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) as illustrated in **Fig. 3.** As the stained electropherograms show, liposomes are similar to microsomes in their overall protein composition. In addition to the proteins evidently shared with microsomes, the liposomes contain stainable protein species of the appropriate electrophoretic mobilities for apolipoproteins E and C (Fig. 3a). Faintly visualized on the electropherograms as well are protein bands having roughly the mobility of the apoB variants (Fig. 3b). However, because these putative apolipoprotein species are accompanied by several other bands in the same region of the gel, it is impractical with this staining technique to determine their relative contributions to different primary fractions.

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TABLE 1. Chemical analysis of orotic acid fatty liver primary fractions

Component	Fraction	Recovery	Overall Component Recovery	Recovered Component Distribution
		mg	%	%
Protein	Hom	5056 ± 318	68.0 ± 5.3	
	P_1	1623 ± 170		47.3 ± 4.4**
	S_2	1294 ± 255		37.4 ± 4.4**
	P_2	422 ± 31		12.4 ± 1.6**
	L_3	99 ± 32		2.9 ± 1.0
Triglyceride	Hom	1583 ± 380	83.7 ± 11.8	
	P_1	446 ± 97		34.2 ± 2.6
	S_1	374 ± 37		29.6 ± 7.1
	P_2	160 ± 41		12.3 ± 2.7
	L_3	331 ± 189		23.9 ± 8.0
Cholesterol	Hom	199 ± 8	55.9 ± 7.4	
	P_1	41 ± 6		36.4 ± 2.6**
	S_2	40 ± 15		35.1 ± 7.9*
	P_2	20 ± 3		18.7 ± 5.9
	L_3	11 ± 3		9.8 ± 3.1
Phospholipid	Hom	505 ± 54	114.7 ± 11.4	
	P_1	245 ± 33		48.9 ± 7.7**
	S_2	77 ± 36		14.8 ± 5.4
	P_2	152 ± 9		30.2 ± 2.1
	L_3	103 ± 35		20.6 ± 8.0

Fraction	TG:Prot	Chol:Prot	PL:Prot
Hom	0.31 ± 0.06*	0.039 ± 0.004*	0.10 ± 0.01**
P_1	0.28 ± 0.06*	0.025 ± 0.005*	0.15 ± 0.02**
S_2	0.30 ± 0.08*	0.032 ± 0.013*	0.06 ± 0.04**
P_2	0.38 ± 0.07*	0.048 ± 0.011*	0.36 ± 0.03**
L_3	3.39 ± 1.53	0.114 ± 0.033	1.05 ± 0.16

Fatty livers from rats fed orotic acid for 7–10 days were fractionated as described in Fig. 1 and the text. Each homogenate (Hom) and primary fraction (P_1, nuclear-mitochondrial pellet; S_2, cytosol; P_2, microsomal pellet; L_3, washed liposomes) was analyzed for content of total protein, triglyceride, cholesterol, and phospholipid. The data from four separate fractionations, each conducted on homogenates pooled from two livers (25–30 g total wet weight), are included; values are presented as mean ± standard error of the sample. Overall recovery of each component is calculated as [mass in (P_1 + S_2 + P_2 + L_3)/mass in Homogenate] × 100%, and recovered component distribution by fraction as [mass in fraction/mass in (P_1 + S_2 + P_2 + L_3)] × 100%. The data shown for cholesterol represent total cholesterol; Chol, total cholesterol; PL, phospholipid; Prot, protein; TG, triglyceride.

For recovery or ratio of components in L_3 versus indicated fraction as calculated by *t*-test: *, $P \leq 0.05$; **, $P \leq 0.005$.

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Measuring intracellular apolipoproteins by electrotransfer and immunodecoration

To accurately estimate the abundance of apolipoproteins in primary subcellular fractions, we have carefully optimized conditions for their immunological detection on nitrocellulose filters. The procedures we have adapted are based on previously published work (23, 28–32); nevertheless, because of the inherent complexity of applying these techniques simultaneously to several protein antigens in multiple subcellular fractions, we offer a detailed protocol here.

We first evaluated the conditions of electrophoresis and electrotransfer. For albumin and apolipoproteins A-I and A-IV, 12% acrylamide gels cast with the crosslinker bisacrylamide and containing linear polyacrylamide were most suitable; for apolipoproteins E and C, we used 15% acrylamide/bisacrylamide gels containing linear polyacrylamide; and for the apolipoprotein B variants, we found 3.5–4.0% acrylamide gels crosslinked with DATD or with BAC superior to the less rigid bisacrylamide-crosslinked gels. All the apolipoproteins save apoB were readily transferred in the buffer recommended by Towbin et al. (28) without additional SDS, for 1 hr at 1.0 amperes with the transfer tank (Hoefer Instruments) suspended in an ice-water bath. These conditions also proved suitable for transfer of liposomal apoE and apoC from isoelectric focusing gels (data not shown). Efficient transfer of the apolipoprotein B variants, on the other hand, required at least 1.5 hr in transfer buffer containing SDS at a final concentration of 0.02% as recommended by Rott and Nelson (29). Finally, while non-B apolipoproteins could

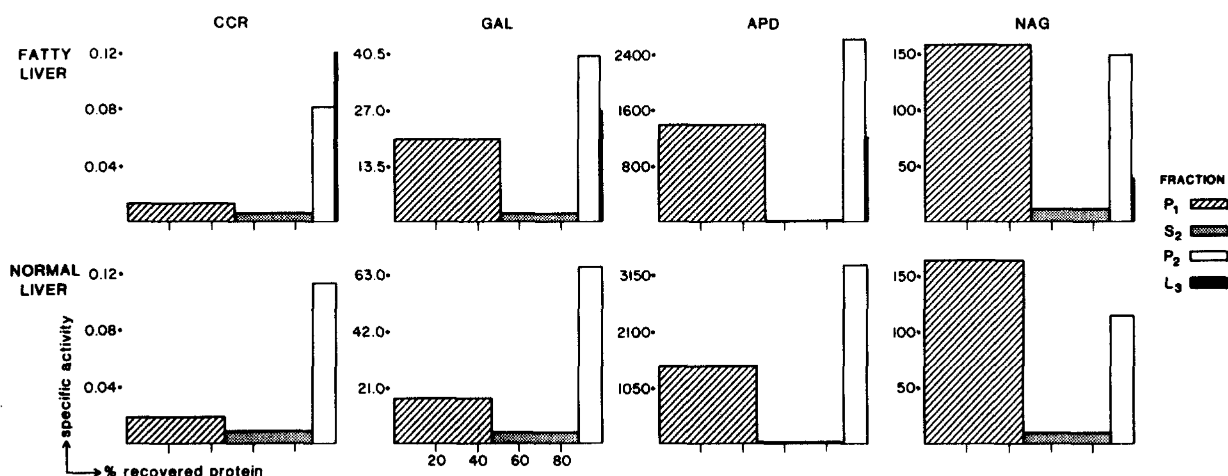

Fig. 2. Marker enzyme activities in primary subcellular fractions. Primary subcellular fractions were prepared from orotic acid fatty liver homogenate (upper panels) and from normal rat liver homogenate (lower panels) by the scheme of Fig. 1 as detailed in the text. Each fraction was assayed for total protein and for the endoplasmic reticulum marker NADPH-cytochrome *c* reductase (CCR), the Golgi marker galactosyltransferase (GAL), the plasma membrane marker alkaline phosphodiesterase I (APD), and the lysosomal marker N-acetyl-β-glucosaminidase (NAG). The data are expressed as de Duve plots. The percent of total protein recovered by fraction is indicated on the abscissa; on the ordinate is displayed the specific enzyme activity for each fraction. The key (right) indicates hatching patterns used for the different fractions. Specific activities respectively reflect the following units: CCR, μmol cytochrome *c* reduced/min per mg protein; GAL, nmol lactosamine generated/hr per mg protein; APD and NAG, nmol substrate hydrolyzed/min per mg protein.

be transferred with previously used buffer, the apolipoprotein B variants transferred very poorly unless freshly prepared electrotransfer buffer was used. These conditions were suitable for purified apolipoproteins, for apolipoproteins present in lipoprotein fractions and in unfractionated plasma, and for apolipoproteins present in tissue homogenate and in subcellular fractions. Control experiments (not shown) confirmed that preimmune sera do not bind appreciably to the filters, that hyperimmune sera are monospecific for the antigen against which they were raised, and that the presence of other serum or homogenate proteins during electrophoresis and electrotransfer does not significantly affect the transfer or detection of individual antigens by this protocol.

Immediately following electrotransfer, all nitrocellulose filters were stained reversibly with Ponceau S (Serva; 0.2% solution [w/v] in 3% trichloroacetic acid). Filters with uneven transfer of indicator protein bands, or with visible evidence of technical irregularities (e.g., air bubbles) interfering with the transfer, were discarded. Ponceau S was removed and nonspecific binding sites on the filters were blocked by incubating the filters for at least 30 min in PBS au lait (PBS, phosphate buffered saline: 10 mM sodium phosphate, pH 7.4, 150 mM NaCl, 0.02% NaN_3; au lait, containing 1% w/v Carnation Nonfat Powdered Milk). Each filter was reacted with 0.1-0.2 ml of the appropriate hyperimmune antisera in 50-100 ml of PBS au lait for at least 12 hr at room temperature with gentle mixing on a gyratory shaker. Nonadsorbed serum components were removed by washing the filters with PBS au lait (three generous washes for a minimum of 15 min each), and immune complexes on the filters were counter-decorated with ^{125}I-labeled protein A (1 μCi per filter) for 1 hr at room temperature. Nonadsorbed radioactivity was removed by washing with PBS au lait as above. Residual

TABLE 2. Distributions of marker enzymes among primary subcellular fractions

Enzyme	Fraction	Percent of Recovered Activity	
		Fatty Liver	Normal Liver
		%	
CCR	P_1	35.2	34.9
	S_2	12.0	14.2
	P_2	43.8	50.9
	L_3	8.9	
GAL	P_1	66.0	46.2
	S_2	4.8	9.8
	P_2	26.8	44.0
	L_3	2.5	
APD	P_1	70.9	63.4
	S_2	0.4	1.2
	P_2	26.9	35.4
	L_3	1.7	
NAG	P_1	80.0	81.7
	S_2	4.4	4.4
	P_2	15.1	13.9
	L_3	0.5	

Fractions were prepared and analyzed as indicated in the legend for Fig. 2. For each enzyme the percentage of the total recovered activity found in each fraction is indicated. Abbreviations: CCR, NADPH-cytochrome *c* reductase; GAL, galactosyltransferase; APD, alkaline phosphodiesterase I; NAG, N-acetyl-β-glucosaminidase.

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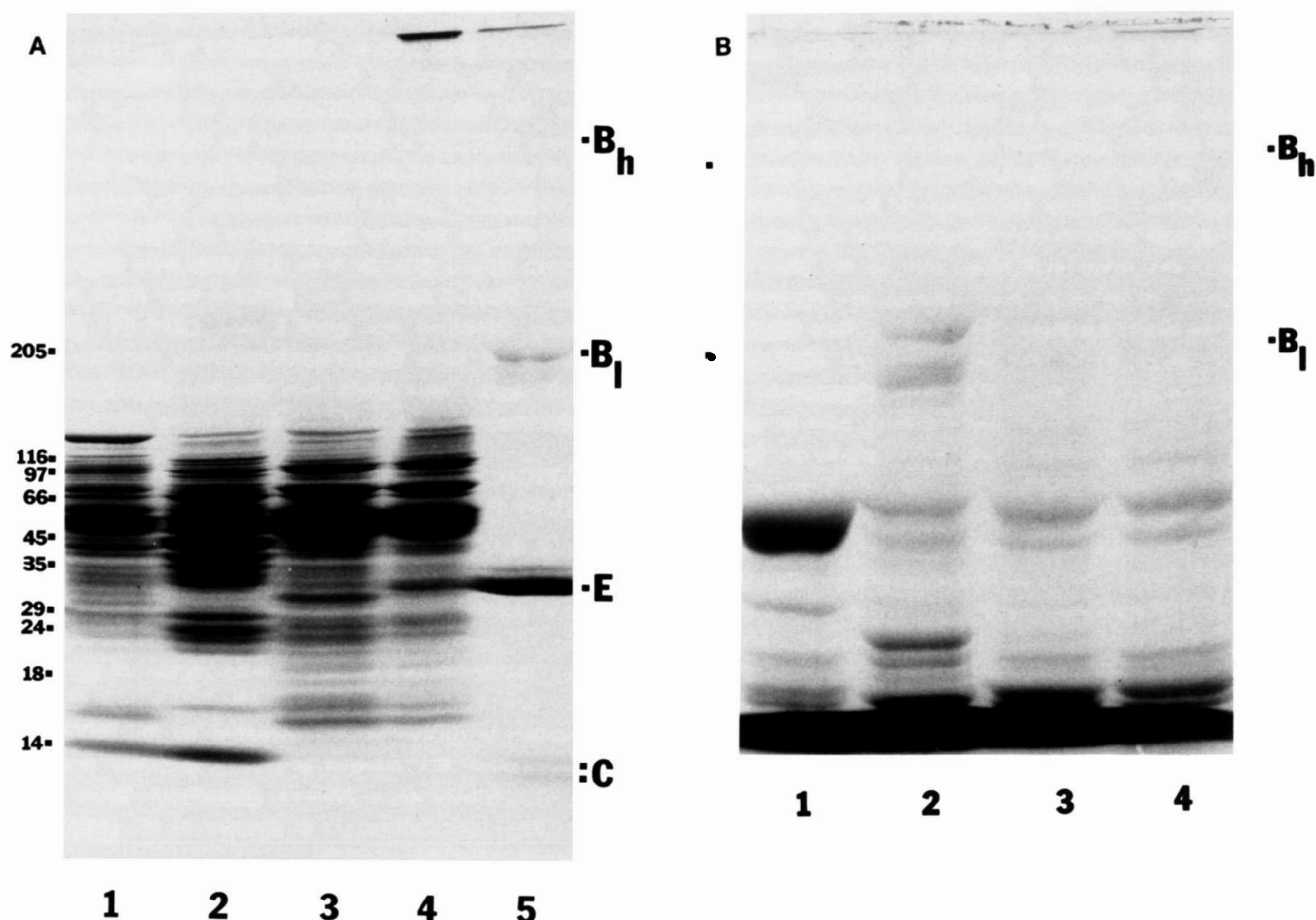

Fig. 3. Protein compositions of primary subcellular fractions. Primary subcellular fractions of orotic acid-induced fatty livers were prepared as outlined in Fig. 1 and aliquots of each fraction were subjected to SDS-polyacrylamide gel electrophoresis. Panel A: Aliquots from each fraction corresponding to 75 μg protein were separated on a tandem (discontinuous gradient) gel consisting of a lower portion of 12.5% acrylamide crosslinked with bisacrylamide and an upper portion of 4% acrylamide crosslinked with DATD. Panel B: Aliquots from each fraction corresponding to 400 μg protein were separated on a 4% acrylamide gel crosslinked with DATD. The gels were stained with Coomassie Brilliant Blue R-250. Photographs of the stained gels are shown. For panel A, the mobilities of standard marker proteins are indicated on the left (according to apparent relative molecular mass in kilodaltons). The mobilities of marker apolipoproteins are indicated on the right of each panel. Fractions shown are as follows: lanes 1, P_1; lanes 2, S_2; lanes 3, P_2; lanes 4, L_3; lane 5 of panel A, rat plasma VLDL (25 μg protein).

milk components, which rendered the filters brittle when dried, were removed by a final wash with PBS alone. A penultimate wash with PBS containing 1% Triton X-100 was sometimes necessary to reduce the filter background for transfers involving the apolipoprotein B variants.

The washed filters were air-dried, mounted, wrapped in cellophane, and exposed to X-ray film with intensifying screens at −80°C. Autoradiograms were passed through an automated film processor to ensure a high uniformity of development. Exposed regions of the film (corresponding to counterdecorated immune complexes of the filter) were excised, the emulsions were eluted therefrom with NaOH, and the $A_{500\ nm}$ of the film emulsion suspensions were determined spectrophotometrically as detailed by Suissa (31). Within the limits discussed below, the $A_{500\ nm}$ reading is proportional to the filter-bound radioactivity, which in turn is proportional to the quantity of antigen bound to the nitrocellulose (31, 32).

The $A_{500\ nm}$ readings obtained from autoradiograms are influenced by the surface concentration of antigen presented to the nitrocellulose filter (28), the antiserum titer, the specific activity of ^{125}I-labeled protein A, the time of autoradiographic exposure (31), the volume of NaOH used for film elution, and the capacity of the spectrophotometer (31). Therefore, it is more realistic to consider the "useful" range of antigen detection than either the "sensitivity" or "limits of detection" for the overall electrotransfer and immunodecoration procedure. Quantifiable autoradiographic bands were routinely obtained within the ranges of 0.5–20 μg of purified antigen, 10–100 μg of normal rat plasma protein, 2.5–50 μg of VLDL protein, and up to 500 μg of subcellular fraction protein. Auto-

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radiographic exposure times ranged from 1 day for major antigenic components to 1 month for very minor antigen components of individual subcellular fractions.

To ensure that the $A_{500\ nm}$ readings reliably reflect the amount of antigen subjected to electrophoresis and electrotransfer, we routinely titrated index fractions and standards to generate a linear regression of $A_{500\ nm}$ versus μg of loaded sample protein (32). Saturation of the assay may result from saturation of binding sites on the filter, from overexposure of the film, from eluting the excised film bands in too small a volume of NaOH, or from exceeding the linear range of the spectrophotometer. Fortunately, saturation for any of these reasons is manifested by a decrease in apparent slope and linearity and by an increase in the apparent y-intercept generated by the regression equation. Data are therefore entered stepwise into the regression program beginning with the lowest antigen load and proceeding to the highest. When antigen loads are reached whose corresponding absorptance values lead to a fall in the correlation coefficient and/or to conversion of the y-intercept to a value that is statistically greater than zero, they are excluded from the regression analysis. Within these limits, the relative abundance of a given antigen in two different protein mixtures is proportional to the ratio of the slopes given by the regression formulae for the respective mixtures (32).

For reasons of economy, we usually express the abundance of a given antigen in arbitrary units (1 unit = 0.001 $A_{500\ nm}$) (see Tables 3, 5, and 6). When we wish to convert arbitrary units to absolute units (μg), as in Fig. 5, the abundance of antigen in the index mixture is compared by cotitration with that in a secondary or primary apolipoprotein standard of known antigen concentration; likewise, the absolute concentration of antigen in the secondary standard is determined by cotitration of that standard with a purified primary apolipoprotein standard of known absolute protein concentration. For each cotitration, the relative antigen abundances are determined by comparing slopes generated by the respective regression formulae. Pooled normal rat plasma was used as the secondary standard for apoA-I and apoA-IV; pooled plasma VLDL from hypercholesterolemic rats was used for apoE, apoC, and apoB; and albumin concentrations were determined directly from the primary standard.

Distribution of apolipoproteins among primary subcellular fractions

Fig. 4 shows a composite photograph of autoradiograms from immunodecorated nitrocellulose replicas for one set of fractions. Even as estimated visually, the distributions of apolipoproteins B, E, and C among the primary fractions clearly differ from those of apolipoproteins A-I, A-IV, and albumin. The first group of apolipoproteins is preferentially enriched in the liposomal fractions, while the second group of proteins is more evenly distributed on a relative abundance basis, with notably high concentrations of antigen in the presumed cytosolic fraction (see below).

In **Fig. 5** are displayed as de Duve plots (18) the results of quantifying the different protein antigens from one

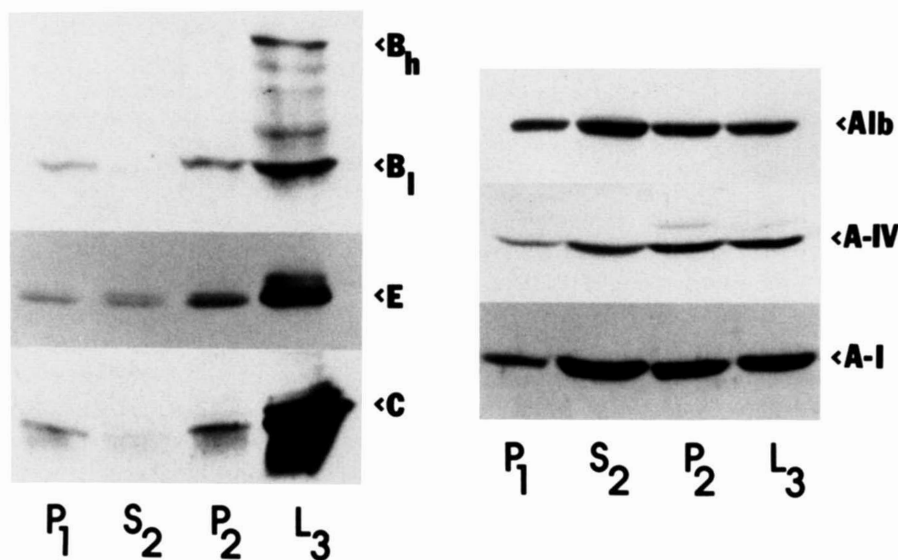

Fig. 4. Apolipoprotein antigens of primary subcellular fractions. Aliquots of each primary fraction from one orotic acid fatty liver subcellular fractionation were subjected to SDS-PAGE followed by electrotransfer to nitrocellulose filters and immunodecoration with specific anti-apolipoprotein antibodies. Immune complexes were counter-decorated with iodinated protein A, and the filters were exposed to X-ray film. A composite photograph of the resulting autoradiograms is shown. For each antibody used, equal amounts (by mass) of estimated protein from each fraction were loaded onto the gel. The lanes are designated by the primary fraction loaded, and the reactive apolipoprotein species are indicated on the right side of each panel; Alb, albumin.

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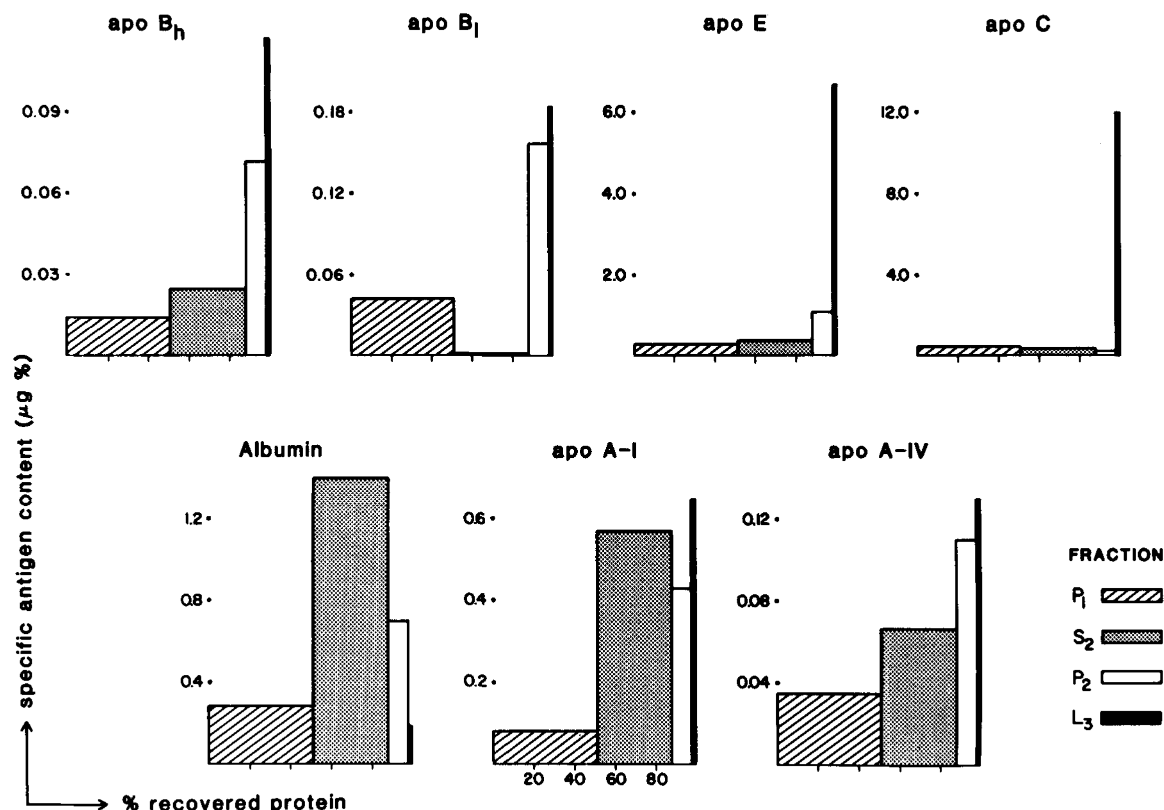

Fig. 5. Quantitation of apolipoprotein antigen concentration in primary subcellular fractions. Nitrocellulose filter replicas of electropherograms were prepared for one orotic acid fatty liver subcellular fractionation and subjected to immunodecoration and counterdecoration as in Fig. 4. The bands visualized on the resulting autoradiograms were quantified spectrophotometrically for relative antigen concentration, and the absolute antigen concentrations were derived by comparison with standard immunodecoration curves obtained for purified apolipoprotein standards. The data are expressed as de Duve plots. A separate panel is shown for each antigen quantified. On the abscissa is indicated the percent of total protein recovered by fraction; on the ordinate is displayed the specific antigen concentration for each fraction (in μg antigen per 100 μg of fraction protein). The key (lower right) indicates hatching patterns used for the different fractions.

fractionation by ETID as outlined above. The abscissa indicates the relative proportions of total protein recovered in each primary fraction; the ordinate expresses the specific concentration of apolipoprotein in each fraction; and the area under the bar for each fraction is proportional to the mass of antigen in the entire fraction. Presentation of the data in this form confirms the visual impression from Fig. 4 that apolipoproteins E and C are highly enriched on the basis of specific antigen concentration in the liposomal fraction relative to other primary fractions, whereas apolipoproteins A-I and A-IV and albumin are not. Both apolipoprotein B variants are also enriched in liposomes relative to other fractions.

The distributions of apolipoproteins among the primary fractions from all four fractionations as determined by ETID are summarized in **Table 3.** Not only are apoE and apoC enriched in liposomes on the basis of specific antigen concentration, but the liposomes comprise a large intra-

TABLE 3. Apolipoprotein distribution in orotic acid fatty livers

Fraction	ApoE	ApoC	$ApoB_h$	$ApoB_l$	ApoA-I	ApoA-IV	Albumin
	%	%	%	%	%	%	%
P_1	33.0 ± 7.6	41.9 ± 10.5	21.1 ± 14.8	41.4 ± 7.4*	14.6 ± 2.9**	31.8 ± 6.8**	15.1 ± 4.7*
S_2	23.0 ± 8.3	27.7 ± 14.0	33.6 ± 18.6	9.6 ± 8.5	62.6 ± 5.3**	48.7 ± 5.9**	75.4 ± 6.0**
P_2	15.4 ± 5.5*	3.4 ± 2.8**	20.0 ± 4.9	32.8 ± 7.8*	17.4 ± 3.9**	15.6 ± 5.6*	8.1 ± 1.6**
L_3	28.6 ± 6.3	27.0 ± 5.7	25.3 ± 7.8	16.3 ± 8.2	5.3 ± 1.6	3.9 ± 0.8	1.3 ± 0.2

The orotic acid fatty liver primary fractions prepared for Table 1 were analyzed for apolipoprotein content by electrotransfer and immunodecoration. For each apolipoprotein, the percentage of the total recovered mass (in arbitrary units) found in each fraction is indicated (mean ± standard error of the sample).

For recovery of apolipoprotein in L_3 versus indicated fraction: *, $P \leq 0.05$; **, $P \leq 0.005$.

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cellular pool of these apoproteins, even though this organelle constitutes only a minor portion of the total cellular protein mass. As Fig. 5 illustrates, the absolute mass of the apolipoprotein B variants recovered in all the fractions is small compared to other apolipoprotein species; nonetheless, Table 3 reveals that a substantial proportion of the total intracellular mass of apoB is also liposomal. The dominant variant of apoB by mass in liposomes is $apoB_l$, with little variability in the ratio of these variants among preparations (B_h:B_l = 0.52 ± 0.07 for the series).

The sequential and overall recoveries of apolipoproteins as determined by ETID were carefully monitored for one complete fractionation. The mass of each apolipoprotein (in arbitrary units) recovered in the new fractions generated at each of the three centrifugation steps of the fractionation scheme (Fig. 1) was compared with the mass of apolipoprotein present in the starting fraction for that spin. There was no significant difference (at α = 0.05) between the sequential recovery of total protein and that of any individual apolipoprotein species. Mean and standard errors for recovery of all apolipoproteins were 78.3 ± 30.6% for spin I, 88.1 ± 32.7% for spin II, and 90.6 ± 14.3% for spin III. These pooled data give a value of 86.1 ± 28.3% per spin for the apolipoproteins, compared to 83.0 ± 8.2% per spin for total protein. In this fractionation, the overall apolipoprotein recovery as judged by ETID (all end fractions compared to homogenate) ranged from 70.3% for apoC to 101.2% for apoE. The overall measured total protein recovery was 64% for this experiment, and 68.0 ± 5.3% for the series (Table 1). We conclude that the losses of apolipoprotein mass are not disproportionate to the total protein loss during the fractionation protocol.

Our finding such high levels of apoA-I, apoA-IV, and albumin within the classically defined cytosolic fraction (Table 3, data for fraction S_2) was not expected. We have entertained three possibilities to explain this phenomenon. First, that these proteins are derived from plasma not flushed from the liver, but cofractionating with the soluble cytosol; second, that they are released from their surrounding secretory pathway organelle membranes by the mechanical stress of the fractionation procedure; and third, that the conditions of centrifugation were not sufficient to pellet all the nonliposomal microsomal membranes from the fatty liver homogenate. Control experiments were conducted to test each possibility, with the following results.

1. Perfusion of the livers with phosphate-buffered saline prior to fractionation did not appreciably alter either the protein mass distribution or the specific concentration of the antigens in each fraction.

2. When the homogenization and centrifugation conditions used here were applied to fractionation of liver from a normal, chow-fed rat, 70–90% of the total cellular apoA-I, apoA-IV, and albumin were recovered in the pelleted fractions (P_1 and P_2), with the highest specific concentration of each antigen in the microsomal pellet (P_2).

3. Recentrifugation of the cytosolic fraction of homogenate from an orotic acid-induced fatty liver in excess of the force required to pellet microsomal membranes from normal rat liver (360,000 g_{max}; 90 min) resulted in pelleting of only an additional 5% of the total "cytosolic" protein, and of 5% of the apoA-I, apoA-IV, and albumin.

4. The albumin, apoA-I, and apoA-IV present in the cytosolic fraction of the orotic acid fatty livers were more resistant to proteolysis in the absence of detergent than in its presence (23), indicating that the proteins exist in the "cytosolic" fraction in some protected form.

We conclude that administration of orotic acid most likely results in sequestration of intracellular precursors to at least albumin, apoA-I, and apoA-IV in forms as yet undefined but not present within the normal liver. We suspect that these proteins occupy membranous organelles having modified density characteristics due to the altered lipid composition of membranes in this lesion (14). Perhaps these unidentified organelles are too light to pellet under our buffer and centrifugation conditions, yet too dense to cofractionate with the liposomes.

In order to assign levels of significance to the apolipoprotein and lipid associations suggested by Fig. 5 and by Tables 1 and 3, we performed simple and multiple regression analyses for the distribution among fractions of each component against all others. These analyses are summarized in **Table 4.**

The highest overall correlations by simple two-component regression analysis were seen for the set {albumin,

TABLE 4. Regression analysis of fatty liver primary fraction components

Regression	r^2
Albumin vs. apoA-I	0.94**
Albumin vs. apoA-IV	0.74**
ApoA-I vs. apoA-IV	0.71**
Albumin vs. apoA-I + A-IV	0.95
Prot vs. Chol	0.81**
Prot vs. apoA-IV	0.61**
Chol vs. apoA-IV	0.70**
Prot vs. Chol + apoA-IV	0.81
TG vs. apoC	0.70**
TG vs. apoE	0.44*
TG vs. apoC + E	0.70
ApoE vs. apoC	0.54*
ApoE vs. apoC + B_h	0.73[a]
ApoE vs. apoC + B_h + B_l	0.82[a]
PL vs. $apoB_l$	0.60**

Simple and multiple regression analyses of chemical and apolipoprotein components with respect to their distributions among the primary orotic acid fatty liver fractions were performed. Abbreviations are as given in Table 1; *, $P \leq 0.01$; **, $P \leq 0.001$.
[a] $P \leq 0.05$ for all covariates; the other multiple regressions listed contain covariates for which $P \geq 0.05$.

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TABLE 5. Protease resistance of liposomal apolipoprotein E

Trypsin	Trypsin-Resistant ApoE	
	− Triton X-100	+ Triton X-100
mg/ml	*% of control*	
0	100	100
0.01	56	65
0.05	43	39
0.20	53	6

Washed liposomes (L_3) were prepared as outlined in Fig. 1 and described in the text, and then adjusted to an estimated protein concentration of 5 mg/ml in homogenization buffer (without PMSF). Equal-volume aliquots were incubated with the indicated final trypsin concentrations for 30 min in an ice-water bath. One of each pair of aliquots was first adjusted to contain 1% (v/v) Triton X-100. At the end of incubation, the reactions were quenched with a mixture of PMSF, TLCK, and TPCK at a final concentration of 1 mM each. Samples were dissociated with electrophoresis sample buffer and heat (95°C, 3 min), and bulk lipid was extracted from the samples with diethyl ether prior to SDS-PAGE. Apolipoprotein E was quantified by ETID of a volume of sample equivalent to 0.4 mg of initial L_3 protein.

apoA-I, apoA-IV} and for the set {total protein, cholesterol, apoA-IV}. However, multiple regression failed to suggest a significant association of these components in higher level groupings than pairs. Another potentially cofractionating set of {triglyceride, apoC, apoE} was suggested by simple regression but not supported by multiple regression analysis. However, the association of apoE with apoC (r^2 = 0.54) was substantially strengthened by including the distributions of $apoB_h$ and $apoB_l$ in the regression formula (r^2 = 0.82). We conclude that the association of these four apolipoproteins within the cell is unlikely to reflect a chance event, and that they can be considered to cofractionate within the fatty liver as early in the secretory pathway as the liposome. Finally, the most significant association found by simple correlation for $apoB_l$ was with phospholipid.

Distribution of apolipoproteins within the liposomal fraction

We next considered how liposomal apolipoproteins are associated with the membranes and the lipid contents of the organelle. For the experiment summarized in **Table 5**, aliquots of freshly isolated washed liposomes were incubated with a range of trypsin concentrations. Half the liposomal aliquots were treated beforehand with Triton X-100 to solubilize the organelle membranes, thereby deliberately exposing sequestered apoE to the protease solution. The difference in apoE degradation between the detergent-treated and detergent-untreated aliquots at a given protease concentration indicates the extent to which apoE is protected by the surrounding membranes. The data of Table 5 suggest two distinct populations of apoE molecules within the liposomal fraction. One, an "exposed" population amounting to somewhat less than half the total apoE, is readily degraded at the lowest concentration of trypsin without a measurable detergent effect. The remaining, "sequestered" population, is resistant to even a high concentration of trypsin and requires dissolution of the organelle membranes for maximal exposure to solution. We infer that the sequestered population represents apoE within sealed liposomal vesicles, on the cisternal side of intact organelle membranes. The exposed population could represent either apoE within liposomes whose membranes have been damaged despite our care in fractionation, or apoE bound to the cytosolic face of the membranes. Although we cannot presently exclude either of these possibilities, we note the recent report by Cole and Stockhausen (38). They concluded that most of the intracellular apoE in normal rat liver is not associated with microsomal membranes, and that some fraction of apoE associated with microsomes may not be involved directly in plasma lipoprotein secretion. Perhaps the exposed population of liposomal apoE molecules represents this latter fraction.

What is the state of lipidation of the liposomal apolipoproteins? The organelle as isolated with intact membranes must float as a unit; however, some of its apolipoprotein contents may be more tightly associated than others with cisternal lipid. To address this question, we have separated the cisternal contents of isolated liposomes from the organelle membranes by the carbonate extraction procedure used by Howell and Palade (21) to investigate intracellular lipoprotein particles, as diagrammed in **Fig. 6.** Three secondary fractions were obtained from washed

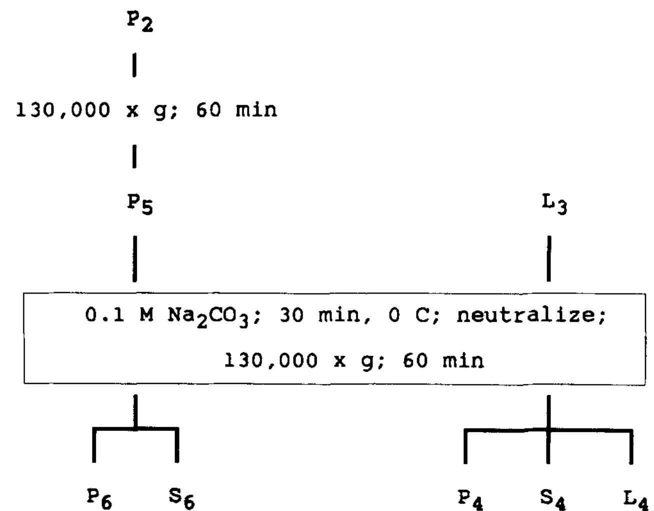

Fig. 6. Preparation of secondary fractions from fatty liver microsomes and liposomes. Crude microsomes (P_2) were resuspended in homogenization buffer and recentrifuged to yield washed microsomes (P_5). Washed liposomes (L_3) and washed microsomes were subjected to sodium carbonate extraction followed by ultracentrifugation. From washed liposomes were recovered a lipid-rich pellicle (L_4), a pellet (P_4), and an internatant fraction (S_4). From washed microsomes were recovered a pellet (P_6) and a supernatant fraction (S_6).

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liposomes: the pellet (P_4), containing the organelle membranes; a pellicle (L_4), more fragile than that formed by the unbroken organelles; and an internatant fraction (S_4). Two secondary fractions, a membrane pellet (P_6) and a supernatant fraction (S_6), were prepared from washed nonliposomal microsomes for purpsoes of comparison. We have operationally designated the respective liposomal secondary fractions as membrane-associated (pellet), poorly/non-lipidated (internatant), and well-lipidated (pellicle). We have quantified the relative mass distributions for the apolipoproteins among these secondary fractions (**Table 6**). Due to restrictions on the availability of anti-apoB, we have not quantified the distribution of apolipoprotein B variants within the microsomal secondary fractions.

The results of the carbonate extraction permitted several conclusions. First, the partitioning of bulk organellar protein between the membrane-associated and -nonassociated fractions is different. The nonliposomal microsomes have roughly twice as much protein in the membrane pellet as in the soluble fraction, whereas liposomes release more than half of the organellar protein into soluble or lipidated form upon carbonate treatment. Second, of the apolipoproteins examined in both kinds of organelles, each shows a much greater proportion to be membrane-associated in nonliposomal microsomes than in liposomes. Third, with the possible exception of apoB_l, very little of the liposomal complement for any apolipoprotein was membrane-associated. Finally, partitioning of carbonate-solubilized apolipoproteins between the poorly/non- and highly lipidated fractions varied considerably. Apolipoproteins E, C, and A-I were found predominantly in highly lipidated form, but apoA-IV was preferentially solubilized to the internatant fraction. As we expected, the presumably soluble protein marker albumin was found largely in the poorly/non-lipidated fraction. However, despite the apparently constant ratio of intraliposomal apoB variants among different fractionations, apoB_h and apoB_l are not identically distributed among the carbonate subfractions of the liposome. ApoB_h appears more highly lipidated than apoB_l in this situation, whereas about twice as much apoB_l sediments with the organelle membranes.

TABLE 6. Apolipoprotein distribution among secondary fractions

	From Liposomes (L_3)			From Microsomes (P_5)	
Component	L_4	S_4	P_4	S_6	P_6
Total protein	45	11	44	30	70
ApoB_h	59	36	5	ND	ND
ApoB_l	35	55	10	ND	ND
ApoE	65	33	2	48	52
ApoC	89	10	1	37	63
Albumin	9	87	4	(100)	(0)
ApoA-I	75	23	2	36	64
ApoA-IV	21	77	2	63	37

Secondary fractions were prepared from washed microsomes (P_5) and from washed liposomes (L_3) by sodium carbonate extraction as diagrammed in Fig. 6. Apolipoproteins were quantified in each fraction by ETID. For each protein the percentage of the total recovered mass found in each fraction is indicated. Albumin was below the limits of detection in P_6. ND, not determined.

DISCUSSION

This study provides the first comparison of intracellular distributions for individual apolipoprotein species within the orotic acid fatty liver. We have demonstrated that the orotic acid fatty liver liposomes selectively concentrate intracellular lipids and the apolipoprotein classes normally associated with rat plasma VLDL. Our findings extend the earlier work of Pottenger and Getz (7) by showing that liposomes contain apolipoproteins B_h and B_l in addition to apolipoproteins E and C, and that these liposomal apolipoproteins constitute a significant proportion of their respective intrahepatic pools. In contrast, although orotic acid fatty liver liposomes contain apoA-I, apoA-IV, and albumin, the contribution of these liposomal proteins to their overall intracellular pools is small.

It is well appreciated that production of VLDL and production of HDL by the mammalian liver are regulated differently (39). Both Banerjee and Redman (40) and Morré and Ovtracht (41) have provided evidence that the pathways for movement of VLDL-destined and HDL-destined components through the Golgi apparatus may be topologically distinct. Our data argue that the rat hepatocyte may begin sorting apolipoproteins normally destined to become VLDL components from other protein species even proximal to the step in lipoprotein secretion perturbed by orotic acid.

Where does that step occur? According to our data, the only conspicuous effect of the orotic acid-induced state, as far as subcellular fractionation of the liver is concerned, is to transform a portion of normally pelleting microsomes into floating liposomes. These liposomes have features characteristic of rough endoplasmic reticulum, including membrane-bound ribosomes. Moreover, the liposomal pellicle is enriched in the endoplasmic reticulum marker NADPH-cytochrome *c* reductase, but deficient in marker enzymes for Golgi, plasma membrane, and lysosomes relative to the pelleted nonliposomal microsomes. Other data also support assignment of liposomes to a pre-Golgi position in the secretory pathway. First, despite the gross accumulation of triglyceride in hepatocytes, the Golgi apparatus in fully developed orotic acid fatty livers is devoid of particles identifiable as VLDL by electron-microscopy. Second, even though orotic acid fatty livers have abundant galactosyltransferase activity, the apoE and apoC molecules occupying the liposomes lack galactose and sialic acid. Based on these considerations and our

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current model of VLDL secretion (39), we suggest that liposomes may represent the junctional complex between smooth and rough endoplasmic reticulum proposed by Alexander, Hamilton, and Havel (42) as the site for initial assembly of triglyceride and VLDL-destined apolipoproteins.

Whether the liposomal contents represent exaggerated amounts of normal intermediates unable to accomplish the next step in their metabolic pathway, much as the LDL and intermediate density lipoprotein (IDL) particles that accumulate in the plasma of individuals lacking the LDL receptor (43), or nonfunctional components trapped in an organelle monstrosity, such as the Berkeley body found in certain secretory mutants of yeast (44), is not established. We favor the former interpretation both because of the selectivity of the orotic acid lesion for VLDL production and because of its ready and complete reversibility. During reversal of the lesion with adenine or adenosine, the liposomal lipid contents are seen to demulsify, and the Golgi apparatus gains particles of typical VLDL morphology (11).

Even though VLDL-destined apolipoproteins appear to cofractionate in the orotic acid fatty liver, our data argue that extensive remodelling of VLDL particle precursors must happen during their exodus from the liposome or its counterpart in normal liver. It has been observed (21, 45) that VLDL-like particles isolated from hepatic Golgi apparatus closely resemble both newly secreted particles and circulating VLDL particles in overall structure. As determined by our immunodecoration procedure, the stoichiometry of apolipoproteins in the liposomes differs substantially from our plasma VLDL standard (B_h:B_l:E:C = 0.6:0.9:35:63 by mass for liposomes vs. 12:12:49:27 for plasma VLDL). The most striking difference is that liposomal contents are profoundly deficient in the amounts of apolipoprotein B variants relative to apoE and apoC. We predict that movement of the putative VLDL assembly intermediates out of the liposome should entail a net acquisition of apoB by the particles. Preliminary reconstitution experiments with model peptides (46) and carbonate-released liposomal contents support this prediction (J. Krause, S. Meredith, and R. Hay, unpublished observations). We do not know how the relative deficiency of apoB is brought about, or how this deficiency is related to selective accumulation of triglyceride at a particular point in the secretory pathway. However, it is generally thought that apoB secretion and triglyceride secretion are obligatorily coupled (39). It may be that entry of apoE- or apoC-containing triglyceride-rich particles (but perhaps not of apoE- or apoC-containing phospholipid-rich particles, such as precursors to nascent HDL) into the secretory organelles distal to the liposome is critically dependent upon some unique structural feature of the apoB molecule, such as its high potential for β-strand and β-sheet secondary structure (46, 47).

Despite the highly significant statistical association of apoB, apoE, and apoC within the orotic acid fatty liver, the biological relationship of $apoB_l$ to the other components concentrated in the liposome may not prove so straightforward. Of the cofractionation set {$apoB_h$, $apoB_l$, apoE, apoC}, $apoB_l$ is statistically the weakest component of the regression formula; on the basis of simple regression, $apoB_l$ is more significantly associated with phospholipid than with any other apolipoprotein species. Their proposed structural relatedness notwithstanding (48), the two apoB variants as expressed by rat liver are subject to different kinds of metabolic regulation (49–51) and they may perform similar but not identical functions in lipoprotein biogenesis. Perhaps some unusual feature of the $apoB_l$ variant, such as phosphorylation (52), enables it to interact in preference to $apoB_h$ with a subset of nascent hepatic lipoprotein particles. Consistent with this possibility are observations that $apoB_l$ may be found in the HDL fraction (49, 50) even when no $apoB_h$ and triglyceride are secreted (10); that the kinetics of $apoB_h$ and $apoB_l$ secretion from rat liver differ (48); and that the $apoB_l$-containing particles secreted by rat liver have a different overall density profile from those containing $apoB_h$ (R. Padley and G. S. Getz, unpublished observations). In recent experiments with cultured rat hepatoma cells capable of producing VLDL (53), we have found $apoB_h$ to be much more sensitive than $apoB_l$ to modulation of its secretion by orotic acid (S. Tanabe, R. Fleming, and R. Hay, unpublished observations).

There are apparently major differences in the partitioning of non-B apolipoproteins between liposomes and nonliposomal microsomes. Without exception, the proportion of apolipoprotein antigen mass associated with membranes (as defined by carbonate extraction of the organelles) is much higher in the microsomes. The significance of this finding is not immediately obvious. However, Borchardt and Davis (54) have recently proposed that the apolipoprotein B molecules of the endoplasmic reticulum may remain in a membrane-associated configuration until a suitable lipid acceptor appears on the lumenal surface of the membrane. In the nonliposomal microsomes with relatively low concentrations of cisternal lipid, apolipoproteins tend to remain membrane-associated; in the triglyceride-laden liposomes with an apparent surfeit of acceptor lipid, apolipoproteins could be preferentially adsorbed onto the particle surface.

Through immunoadsorption analysis of the pellicle released from liposomes by carbonate extraction (L_4), we have tentatively identified a particle containing both $apoB_h$ and apoE, but no detectable apoC or $apoB_l$ (55). Preparative scale isolation of this particle has not yet been achieved.

By what mechanisms does orotic acid block VLDL secretion at the level of the liposome? What factors permit the intraliposomal particles to become so much larger

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than plasma or Golgi VLDL? What is the molecular basis for the apparent deficiency of apoB in this lesion, and how is apoB metabolism related to the morphologic lesion? What happens biochemically to the liposomal particles during reversal of the fatty liver? Such issues now await elucidation.

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