Nascent high density lipoproteins from liver perfusates of orotic acid-fed rats

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Abstract Uniformly fatty livers from orotic acid-fed rats secreted almost no very low density lipoproteins (VLDL) but normal amounts of nascent high density lipoproteins (HDL) accumulated in perfusates. When lecithin:cholesterol acyltransferase (LCAT) was inhibited, nascent HDL were uniformly discoidal and lacked cholesteryl esters. Lipid and apoprotein compositions of nascent HDL from normal and fatty livers were similar whether LCAT was inhibited or not. Apolipoprotein B-100 was not detected in perfusates of uniformly fatty livers, but small amounts of apolipoprotein B-48 were present in HDL_2 fractions. Nascent lipoproteins were not seen in Golgi compartments, but lipid-rich particles were clearly evident in endoplasmic reticulum cisternae adjacent to the *cis* face of the Golgi complex, suggesting that orotic acid blocks VLDL secretion by preventing translocation of nascent particles from the endoplasmic reticulum to the *cis* Golgi compartment. The accumulation of normal amounts of discoidal HDL in liver perfusates despite virtual absence of triglyceride-rich lipoproteins in Golgi secretory compartments, the space of Disse, and the perfusate is inconsistent with the concept that nascent HDL are exclusively a product of surface remnants cast off during lipolysis of chylomicrons and VLDL.—**Hamilton, R. L., L. S. S. Guo, T. E. Felker, Y-s. Chao, and R. J. Havel.** Nascent high density lipoproteins from liver perfusates of orotic acid-fed rats. *J. Lipid Res.* 1986. **27:** 967-978.

Supplementary key words apoproteins • discoidal particles • endoplasmic reticulum • Golgi apparatus • lecithin:cholesterol acyltransferase

Windmueller and Levy (1) provided the first evidence that high density lipoproteins (HDL) are distinct secretory products of rat livers. They showed that alpha lipoproteins accumulate in recirculating perfusates of livers from rats fed orotic acid, despite apparent total inhibition of the secretion of beta lipoproteins. Their observations were extended by Marsh (2) who showed that apoprotein patterns of nascent HDL from single pass perfusates of livers from normal and orotic acid-fed rats were similar.

We have shown that nascent HDL which accumulate in perfusates of rat livers are discoidal when lecithin:cholesterol acyltransferase (LCAT) is inhibited (3). Our continuing studies of these particles have supported the concept that they have an origin distinct from that of nascent very low density lipoproteins (VLDL) which are secreted from the liver (4, 5). It is well established, however, that surface components leave triglyceride-rich lipoproteins during lipolytic processing and appear in HDL. Recent reviews (6, 7) have focused attention on the possibility that these surface components may exist, at least transiently, as lamellar structures, including discs resembling nascent hepatogenous HDL. Lamellar structures can be produced during lipolysis of chylomicrons and VLDL under certain circumstances (8, 9), but convincing evidence that vesicles or discs are produced in this way under physiological conditions is lacking.

Nascent discoidal HDL have now been found in perfusates of livers from rats (3), guinea pigs (10), monkeys (11), rabbits (12), and in medium of human Hep G2 cells (Erickson, S., P. E. Fielding, and R. L. Hamilton, unpublished observations). Although we have shown that discoidal particles are present in uncentrifuged perfusates of rat and guinea pig livers (3, 10), the possibility that these particles are derived from the triglyceride-rich particles present in these systems has not been excluded. We have therefore reinvestigated this question by studying the properties of nascent HDL in perfusates of fatty livers of rats fed orotic acid. We have found that discoidal particles accumulate in these perfusates at normal rates and that their properties closely resemble those in perfusates of normal livers, despite the virtual absence of nascent triglyceride-rich particles in either the perfusate or Golgi compartments of hepatocytes.

Abbreviations: VLDL, very low density lipoproteins; LDL, low density lipoproteins; HDL, high density lipoproteins; LCAT, lecithin:cholesterol acyltransferase; MVBs, multivesicular bodies.

METHODS

Animals

Male rats (300–400 g) were fed standard Purina chow (Ralston Purina Co., St. Louis, MO) to which was added 1%, 2%, or 4% orotic acid by weight. The chow containing orotic acid was fed for 7, 14, or 21 days in an attempt to eliminate apoprotein B from liver perfusate lipoproteins completely (see Results). Control rats were fed the standard Purina chow with no additions. For each experiment a group of five or six rats was fed the orotic acid diet and livers were perfused only if they had no visible pink to tan areas (i.e., were uniformly white after brief flushing with perfusion medium). Most of these livers were from animals fed 1% or 2% orotic acid for 14 days.

Liver perfusions

The techniques of liver perfusion were the same as described previously in detail (3). Pairs of liver were perfused for 5 hr with 40–50 ml of medium containing washed rat erythrocytes at 20–25% hematocrit in Krebs-Hensleit buffer containing 150 mg/dl glucose and equilibrated with 95% O_2 and 5% CO_2, pH 7.4. To inhibit LCAT secreted by the liver, DTNB (5,5' dithiobis nitrobenzoic acid) (0.2–0.3 ml of a 0.02 M solution in 0.075 M phosphate buffer, pH 7.4) was added to the perfusate of one liver at 30-min intervals to obtain a final concentration in the perfusion medium of about 1.0 mM (3). Control rats were fed ad libitum or were fasted for 16 hr. To minimize the presence of triglyceride-rich lipoproteins within the space of Disse, rats fed orotic acid were usually fasted for 16 hr before liver perfusions. To reduce further the content of such lipoproteins, livers were flushed in one of two ways: *1*) after 30 ml of perfusate was passed through the liver during its connection to the perfusion apparatus, an additional 30–40 ml was recirculated for 10 min; this was then discarded and replaced by 40–50 ml of fresh medium; *2*) 100 ml of perfusate was passed through the liver at 10 ml/min for 10 min prior to establishing recirculation-perfusion with 40–50 ml of fresh medium. Erythrocytes were removed by centrifugation before perfusate plasma was concentrated to about 50 ml by ultrafiltration (Xm 50 membrane, Amicon, Danvers, MA).

Separation of lipoproteins

Lipoproteins were isolated from the perfusate by centrifugation for 1×10^8 *g-min* at 4°C in a 40.3 rotor of a Beckman ultracentrifuge (Beckman Instruments, Inc., Palo Alto, CA). Perfusate plasma was first obtained by sedimenting erythrocytes at 15,000 *g* for 20 min in a Sorvall refrigerated centrifuge at 4°C. Perfusate lipoproteins were obtained by flotation after adjusting the density of perfusate plasma by the addition of anhydrous KBr or KBr solutions containing 0.04% disodium EDTA and 0.01% sodium azide. Lipoprotein fractions were purified by recentrifugation at the upper density limit. All lipoproteins were exhaustively dialyzed against 0.15 M sodium chloride containing 0.04% disodium EDTA and 0.01% sodium azide. Three fractions were isolated: *1*) $d < 1.075$ g/ml (VLDL + LDL); *2*) $1.075 < d < 1.175$ g/ml (HDL_2); and *3*) $1.175 < d < 1.21$ g/ml (HDL_3).

Electron microscopy

Perfusate fractions were negatively stained on special coated grids (13) and photographed at 60,000 diameters at 80 KV in a Siemens 101 electron microscope. Discoidal particles are best illustrated when they are induced to form rouleaux structures as described previously (3, 4). All fatty livers were fixed at the termination of perfusion for two purposes: *1*) to examine the fixed tissue in slices to determine whether or not any detectable normal tissue, not visible from the surface, was present; and *2*) to prepare the tissue for ultrastructural study. None of the fatty livers that were selected for perfusions were found to have normal tissue by this approach. When normal tissue was visible, it was usually in a small region adjacent to the right side of the portal vein; such livers were not used. Fifteen ml of buffer was pumped from the reservoir through the liver to clear most erythrocytes from the system and fixative (2% freshly made paraformaldehyde and 1% glutaraldehyde in 0.1 M phosphate buffer) was then pumped directly into the portal vein. Cubes about 1 mm^3 were cut from slices from different lobes and post-fixed in 4% osmium tetroxide in veronal acetate buffer at 4°C for 2–4 days. The tissue was block-stained in uranyl acetate, dehydrated in acetone, and embedded in Epon. Thin sections were doubly stained with uranyl acetate for 30–45 min and lead citrate for 5–15 min. The prolonged fixation in osmium tetroxide was used because we found it imparted a pronounced electron density to the hepatic triglyceride droplets which otherwise appear completely electron lucent (14, 15).

Analyses

Cholesterol and cholesteryl esters, phospholipids, and triglycerides were measured in lipoprotein fractions by previously described methods (16–18). Proteins were measured by the method of Peterson (19). Apoproteins were separated by SDS polyacrylamide electrophoresis in gels of 6% for standard runs or on gels of 3% to separate B apoproteins (20) and by isoelectric focusing electrophoresis (21). To measure synthesis of apoproteins, [4,5-^{3}H]lysine (Amersham) was added at 30-min intervals to the perfusate reservoir so that a total of about 1.0 mCi was used. SDS gels were sliced into 2-mm sections, dissolved in 30% H_2O_2, and assayed for ^{3}H as described previously (22). Perfusate apoproteins E and A-I were quantified by radioimmunoassay (23, 24).

TABLE 1. Content of lipoprotein-protein in perfusates

Source	Body Weight	VLDL + LDL (d < 1.075 g/ml)	HDL (1.075 < d < 1.21 g/ml)
	g	*mg protein*	
Normal			
Fed (6)[a]	390 ± 60	1.87 ± 0.58	0.67 ± 0.25
+ DTNB (6)	376 ± 45	1.79 ± 0.31	0.61 ± 0.07
Normal			
Fasted (4)	371 ± 55	0.55 ± 0.21	0.29 ± 0.03
+ DNTB (4)	376 ± 60	0.57 ± 0.36	0.39 ± 0.10
Orotic acid-fed + DTNB (6)	390 ± 62	0.15 ± 0.11	0.67 ± 0.29
Orotic acid-fasted (6)	338 ± 60	0.07 ± 0.06	0.70 ± 0.17
Orotic acid-fasted + DTNB (7)	366 ± 63	0.08 ± 0.04	0.61 ± 0.14

Values given as mean ± SD.
[a]Number of experiments; all perfusions were for 5 hr.

RESULTS

Lipoprotein recoveries

Our preliminary perfusions were from animals that had access to food until the experiments began. The recovery of HDL (mg of protein) was the same (**Table 1**) as we found previously for normally fed animals not fed orotic acid (3). Although the VLDL + LDL fractions were greatly reduced (by 90–95%), we had hoped to completely eliminate these lipoproteins. In all subsequent studies, orotic acid-fed rats were fasted overnight in an attempt to reduce the amount of chylomicrons or chylomicron remnants in the space of Disse. The overnight fast did not reduce the amount of HDL protein recovered from the perfusate (Table 1), but the combination of fasting and more thorough flushing reduced the amount of lipoprotein protein in the VLDL + LDL density fraction to less than 0.1 mg (too little to characterize). As in perfusates from normal rats, little material was recovered in the HDL_3 fraction. Fasting overnight substantially reduced the recovery of lipoproteins from perfused livers of normally fed rats (Table 1). Addition of DTNB to the perfusion medium had little effect on the amount of HDL protein recovered from livers of normally fed rats (as reported previously) and of the fasted animals that had been fed orotic acid.

Composition of nascent HDL_2

The composition of the HDL_2 fraction in liver perfusates from orotic acid-fed rats resembled that of HDL_2 from livers of normally fed rats (**Table 2**). The only significant difference was that the unesterified cholesterol content was about twofold higher in the HDL_2 from the fatty livers. When DTNB was added to perfusates of fatty livers, cholesteryl esters were not detectable (Table 2). Because we had found earlier that an apoB-containing particle is present in the discoidal HDL_2 from perfused livers of normally fed rats (25), we compared the composition of the unretained fraction, which is composed almost entirely of discoidal particles, after removal of the apoB-containing particles by anti-apoB affinity chromatography. The composition of this fraction very closely resembled that of the discoidal HDL_2 from perfusates of orotic acid-fed animals (which contained very little apoB, see below). The composition of the retained particles, recovered in three experiments, was as follows: 18.5% ± 0.5% triglyceride, 17.2% ± 2.7% cholesteryl ester, 7.1% ± 1.5% unesterified cholesterol, 22.3% ± 1.5% phospholipids, and 35.0% ± 3.7% protein (mean values ± SD).[1] HDL_2 fractions from blood plasma of orotic acid-fed rats resembled those of normally fed rats except for an increased content of unesterified cholesterol (Table 2).

Apoproteins of nascent HDL

SDS polyacrylamide gel electropherograms showed that apoE, apoA-I, and C apoproteins were the predominant apoproteins of perfusate HDL_2 from fatty livers obtained in the absence of DTNB, as in normals (**Fig. 1**). However, apoA-IV appeared to be absent, and much less apoB was present in HDL_2 from perfusates of orotic acid-fed animals. In 3% SDS gels, the apoB in HDL_2 fractions from both normally fed and orotic acid-fed animals was a single band of mobility corresponding to apoB-48 (**Fig. 2**). In no instance were we able to eliminate apoB-48 from perfusate HDL_2 fractions from orotic acid-fed animals. As shown in Fig. 1, much less apoB-48 was present in HDL_2 from fatty livers.

[1]In one experiment, no proteins other than apoB were seen when the proteins of the particles retained on an anti-apoB affinity column were separated on a 6% SDS polyacrylamide gel.

TABLE 2. Composition of nascent HDL_2

Source	Protein	Phospholipids	Unesterified Cholesterol	Cholesteryl Esters	Triglycerides
			%		
Orotic acid perfusate control (4)[a]	46.1 ± 2.4	23.8 ± 2.5	14.0 ± 2.3	14.0 ± 1.1	4.1 ± 3.0
Normal perfusate control (5)[b]	41.5 ± 2.7	31.8 ± 1.4	6.3 ± 1.0	16.8 ± 2.3	3.6 ± 0.5
Orotic acid perfusate + DTNB (4)	41.1 ± 2.4	41.8 ± 2.0	14.7 ± 1.6	ND	2.4 ± 0.3
Normal perfusate + DTNB (2)[c]	37.8 ± 0.4	44.3 ± 0.3	14.1 ± 0.2	0.6 ± 0.1	3.2 ± 0.2
Orotic acid blood plasma (2)	43.7 ± 0.5	22.0 ± 2.2	9.2 ± 0.1	25.1 ± 1.7	ND
Normal blood plasma (5)[b]	44.3 ± 0.9	25.9 ± 1.7	5.1 ± 0.3	23.6 ± 1.2	1.2 ± 0.9

Values given as mean ± SD; ND, not detectable.
[a]Number of experiments.
[b]From previously published studies (3).
[c]Unretained fraction from an anti-apoB affinity column, which contains virtually pure discoidal particles as judged by electron microscopy.

In isoelectric focusing gels, the pattern of the isoforms of the apoproteins of HDL_2 from livers of normally fed rats was similar to that of orotic acid-fed rats, except that the more acidic isoforms of apoE were more prominent in samples from rats given orotic acid (**Fig. 3**).

Distribution of newly synthesized apoproteins

In those liver perfusions in which [^{3}H]lysine was added to the perfusion medium to label newly synthesized apoproteins, the bulk of the ^{3}H was in VLDL + LDL (ca. 70%) from livers of normally fed rats; HDL_2 contained about 21–24% of the total (**Table 3**). Orotic acid feeding caused a marked reversal in this distribution with the bulk of the label (81–91%) in HDL_2 and small amounts (3–10%) in VLDL + LDL (Table 3). HDL_3 fractions were not altered by orotic acid feeding and DTNB additions had little effect. However, orotic acid feeding caused substantial changes in the distribution of radiolabeled apoproteins in HDL_2. ApoB-48 contained 32–49% of the total ^{3}H in HDL_2 from normal livers and only 8–10% in HDL_2 from livers of orotic acid-fed animals (**Table 4**). In addition, no ^{3}H could be detected in the apoA-IV region of gels from HDL_2 of orotic acid-fed animals, and ^{3}H in apoE, apoA-I, and C apoproteins was increased.

Accumulation of apoE and A-I in perfusates

As shown in **Fig. 4**, orotic acid feeding had little effect on the rate of accumulation of apoA-I in perfusates, but the rate of accumulation of apoE was about 75% lower than in perfusates of livers from normally fed rats.

Because the concentration of apoproteins and lipoproteins in a recirculating liver perfusion system is a reflection of the combined effects of secretion and re-uptake by the liver, we compared the uptake of endogenously labeled triglycerides and cholesteryl esters of perfusate VLDL from livers of normal and orotic acid-fed rats. About one-half of each of the labeled VLDL lipids was taken up in 60 min by livers in both conditions (data not shown).

Structure of nascent HDL_2 from livers of orotic acid-fed rats

Perfusate HDL_2 from fatty livers of orotic acid-fed rats were mainly round particles with diameters between 75–125 Å (mean ~100 Å), closely resembling those found previously for HDL_2 from livers of normally fed rats (3). As in HDL_2 fractions from livers of normally fed rats, there were also a few particles of 150–250 Å diameter, some of which were shown to be discs when seen of edge (**Fig. 5**, top). When DTNB was added to perfusates of fatty livers, most particles were discs (Fig. 5, bottom) of the same dimensions (44 ± 4 Å on edge by 175–225 ± 25 Å, mean diameter ~200 Å) as those from livers of normally fed animals (3).

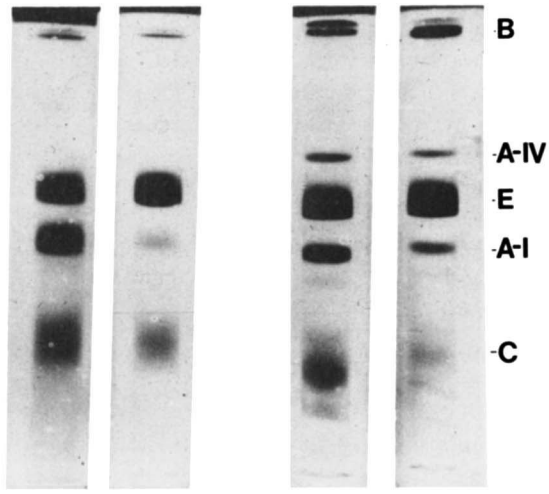

Fig. 1. Polyacrylamide gel (6%) electrophoretograms of nascent HDL_2 of liver perfusates from orotic acid-fed rats (left pair) and normally fed rats (right pair). Proteins of control HDL_2 are on the left and those from perfusates treated with DTNB are on the right of each pair. Much less apoB is present and no detectable apoA-IV is seen in HDL_2 from livers of orotic acid-fed animals.

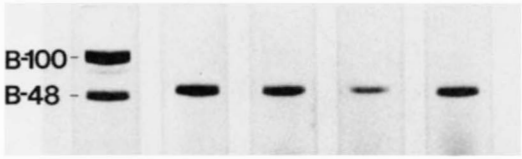

Fig. 2. SDS polyacrylamide gel (3%) electrophoretograms (top portions of gels) of apoB components of nascent HDL_2. The gel on the left is normal rat serum VLDL + LDL showing both apoB-100 and apoB-48; the next two gels are HDL_2 from normally fed rats (with or without DTNB); the last two gels are from orotic acid fed-rats (with or without DTNB). All HDL_2 fractions contain apoB-48 and lack apoB-100.

Measurements of the distribution of particle diameters showed that less than 5% of perfusate discoidal particles (when LCAT was blocked) overlapped plasma HDL_2, whereas the diameter of 15–20% of control perfusate HDL_2 overlapped the discs (**Fig. 6**). This is consistent with the presence of discoidal particles in control perfusate HDL_2 fractions (Fig. 5 and ref. 3) and the apparent absence of discs from normal plasma. About 40% of control perfusate HDL_2 was 75–100 Å in diameter whereas fewer than 10% of plasma HDL_2 were this small.

Structure of hepatocytes of orotic acid-fed rats

As reported by others (14), the triglycerides accumulating in fatty hepatocytes of orotic acid-fed rats occurred in two different forms: large cytoplasmic lipid droplets not enclosed by a membrane and smaller droplets within swollen cisternae of the endoplasmic reticulum (**Fig. 7**). The proportions of these two forms of lipid droplets varied considerably in adjacent hepatocytes. The lipid droplets in the cisternae of the ER were often 2,000–3,000 Å in diameter. They occurred singly or in groups of two to four, were often asymmetrical, and were usually surrounded by granular material (**Fig. 8**, bottom). Some of this material was closely associated with the droplets. A few intensely electron dense particles were also present. Morphologic evidence of nascent VLDL transport within Golgi cisternae was absent in all hepatocytes, as indicated by the complete lack of lipid-staining particles (Figs. 7 and 8 top). Multivesicular bodies (MVBs) adjacent to empty Golgi cisternae, usually on the side opposite the swollen ER cisternae, were prominent. They appeared to contain few if any endocytosed lipoproteins, but numerous bilayer vesicles were seen (Fig. 8, top).

DISCUSSION

Orotic acid and VLDL secretion

Windmueller and Levy (1) reported that perfusates of "uniformly fatty livers" from rats fed orotic acid (1 or 2%) for 8–11 days contained no detectable newly synthesized triglycerides and no beta lipoproteins. However, small amounts of triglycerides and beta lipoproteins were present in perfusates of livers that had small areas or lobes that appeared nearly normal. Novikoff and Edelstein (14) found that plasma apoB levels were undetectable 7 days after feeding diets containing 1% orotic acid, but gradually increased after 2 and 3 weeks on the diet. Because our first efforts to eliminate VLDL and LDL completely from perfusates of fatty liver were not successful, we fed diets containing different amounts of orotic acid (1, 2, and 4%) for different lengths of time (1, 2, and 3 weeks). Uniformly fatty livers were not consistently produced, however, and small and variable amounts of VLDL + LDL protein were often recovered from the perfusion medium. We therefore fasted the orotic acid-fed rats overnight and flushed the livers more thoroughly to reduce the amount of trapped chylomicron remnants. This approach virtually eliminated VLDL and LDL from the perfusates of uniformly fatty livers (Table 1).

Examination of fatty livers with the electron microscope showed that Golgi cisternae in all hepatocytes appeared to be devoid of nascent VLDL. We intensely fixed the tissue in osmium tetroxide to increase the electron density of intracellular triglycerides, because others reported that they were unable to visualize triglycerides in these fatty livers (14, 15). Unless triglycerides are satisfactorily stained, it is very difficult to identify nascent triglyceride-rich particles. One group has reported their presence in Golgi cisternae, suggesting that the block in

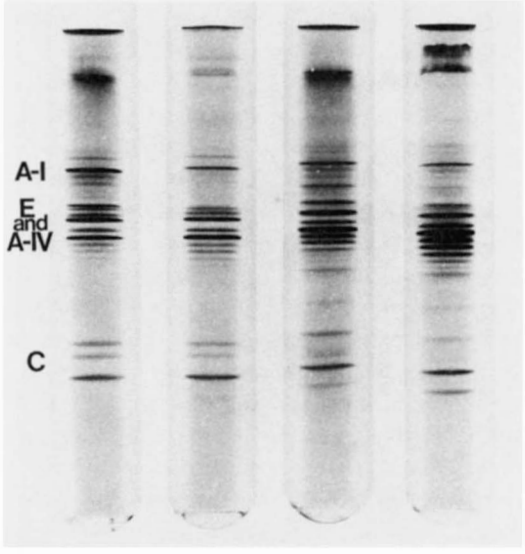

Fig. 3. Isoelectric focusing gel electrophoretograms of proteins of HDL_2 from livers of normally fed rats (left pair) and from fatty livers of orotic acid-fed rats (right pair). Samples from two control perfusions are on the right and samples from perfusions containing DTNB are on the left of each pair.

TABLE 3. Percent distribution of ^3H in lipoprotein proteins from liver perfusates

Source	VLDL + LDL (d < 1.075 g/ml)	HDL_2 (1.075 < d < 1.175 g/ml)	HDL_3 (1.175 < d < 1.21 g/ml)
		% of ^3H	
Normal			
Control (3)[a]	71.7 ± 6.4	21.3 ± 2.1	7.0 ± 4.4
+ DTNB (3)	70.0 ± 1.7	23.7 ± 3.8	6.3 ± 3.1
Orotic acid-fed			
Control (4)	10.5 ± 4.1	81.0 ± 5.2	8.5 ± 6.0
+ DNTB (4)	2.8 ± 2.2	91.0 ± 6.5	6.5 ± 4.4

[^3H]lysine was added to perfusates at 30-min intervals as described in Methods. Values given as mean ± SD.
[a]Number of experiments.

TABLE 4. Percent distribution of ^3H in apoproteins of nascent HDL_2 from liver perfusates

Source	B-100[a]	B-48	A-IV	E	A-I	C's
				% of ^3H		
Normal						
Control (2)[b]	ND	32.0 ± 2.8	6.0 ± 1.4	11.5 ± 3.5	30.5 ± 4.9	20.0 ± 2.8
+ DTNB (2)	ND	49.0 ± 7.1	1.5 ± 0.7	27.0 ± 9.9	7.0 ± 0.0	15.5 ± 3.5
Orotic acid						
Control (4)	ND	9.5 ± 3.0	ND	25.3 ± 6.8	32.5 ± 7.7	32.8 ± 3.3
+ DTNB (4)	ND	8.0 ± 1.4	ND	44.0 ± 5.3	19.0 ± 4.6	29.0 ± 6.1

[^3H]lysine was added to perfusates at 30-min intervals as described in Methods. Values are given as mean ± SD; ND, not detectable.
[a]B-100 region of the gel; no stainable protein was evident.
[b]Number of experiments.

VLDL secretion produced by orotic acid led to accumulation of nascent VLDL in Golgi secretory vesicles (15). In that study, a "Golgi" fraction was isolated which contained triglyceride-rich particles in vesicular structures, as determined by negative staining. However, we believe that the images interpreted as Golgi secretory vesicles (Figs. 4, 6, and 9 in ref. 15) are in fact MVBs containing endocytosed remnants and numerous bilayer vesicles (see structure of isolated MVBs in Figs. 4 and 6, ref. 26). The method used by these investigators (15) to obtain "Golgi" fractions was shown subsequently to co-isolate large amounts of contaminating MVBs (27).

Our observation that Golgi cisternae in hepatocytes of these fatty livers lack VLDL confirms the observations of Novikoff and Edelstein (14). Lipid-rich particles accumulate in apparently discrete vesicles derived from the endoplasmic reticulum (ER). In addition, typical flattened rough ER (RER) cisternae are absent (14). It is of considerable interest, therefore, that isolated triglyceride-rich particles from these fatty livers reportedly contain both apoB-100 and apoE (28). These particles appear to accumulate within ER cisternae immediately adjacent to the *cis* side of Golgi stacks, which has been shown to receive nascent secretory proteins from the RER (29, 30). The *trans* side of the Golgi stacks, from which secretory proteins exit en route to the cell surface (29, 30), is often identifiable in these hepatocytes by the presence of adjacent MVBs (Figs. 7 and 8 top). Thus, one may postulate that orotic acid blocks translocation of nascent VLDL from the ER to the *cis* Golgi compartment.

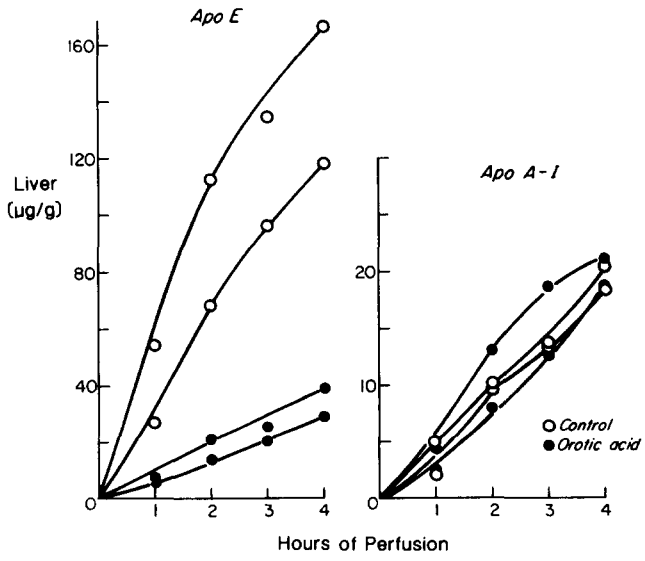

Fig. 4. Accumulation of apoE and apoA-I in liver perfusates of normal and orotic acid-fed rats. Note that much less apoE is secreted by the fatty livers.

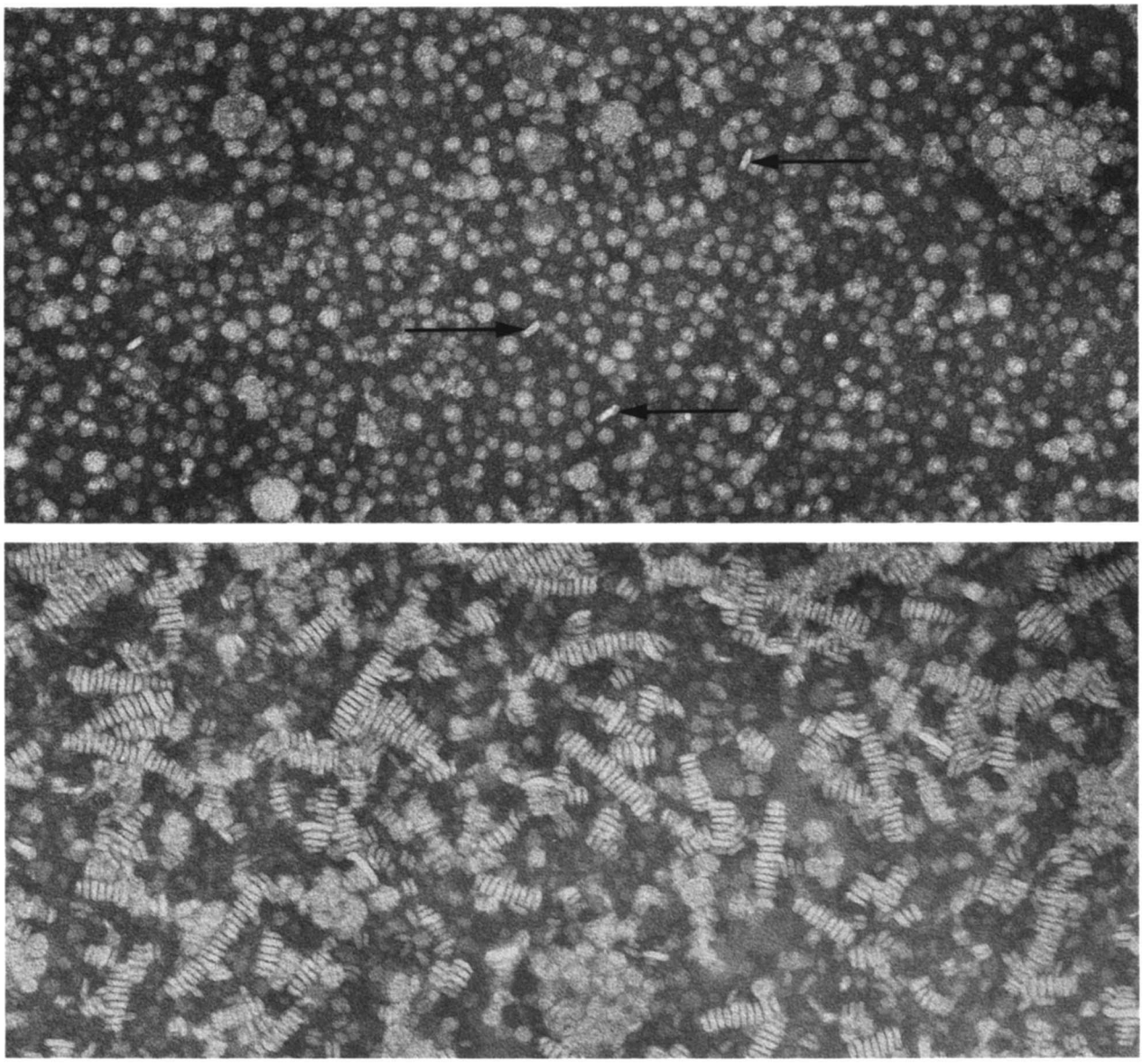

Fig. 5. Negative stains of nascent HDL_2 from perfusates of fatty livers of orotic acid-fed rats. Top: control HDL_2 is mostly particles 75–125 Å, but a few larger particles of about 200 Å are present which appear as discs (arrows) when on edge. Bottom: HDL_2 from perfusate with LCAT inhibition by DTNB appear mostly as discs with the same dimensions (44 by 200 Å) as those reported from normal livers with LCAT inhibition (3). × 180,000.

Is the defect in rat liver produced by orotic acid analogous to human abetalipoproteinemia? In classical abetalipoproteinemia, all apoB-containing lipoproteins are completely absent from plasma, suggesting that apoB is required for biosynthesis or secretion of VLDL and chylomicrons (31). In normotriglyceridemic abetalipoproteinemia, chylomicrons containing apoB-48 are secreted normally but VLDL containing apoB-100 are not (32), a situation resembling that of the orotic acid-fed rat. By contrast, in Anderson's disease, chylomicrons are not secreted (33). Enterocytes isolated from subjects with this disorder become fatty after a fat-rich meal, but no chylomicrons appear in plasma. However, apoB-48 has been identified with peroxidase-labeled monoclonal antibodies within enterocytes from these subjects (33). The defect in patients with abetalipoproteinemia may not be the inability to synthesize apoB. A recent report states that livers of subjects with abetalipoproteinemia contain apoB-100 mRNA of apparently normal size (34). Thus, the defect may be in post-translational processing or transport of apoB-100, possibly similar to that produced by orotic acid.

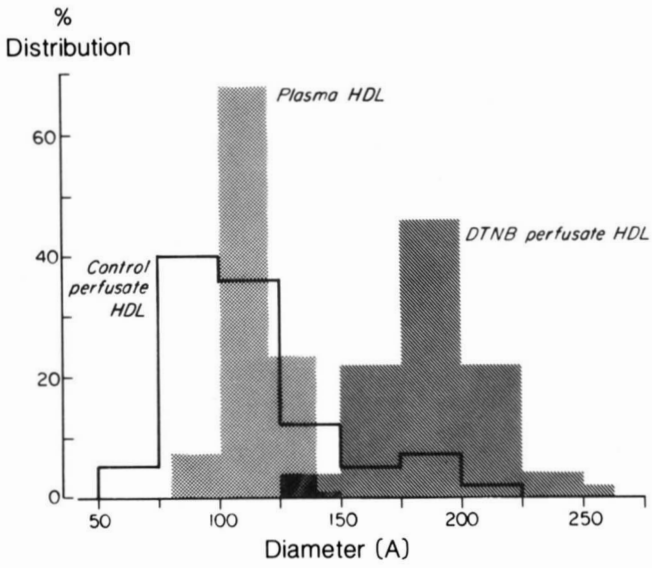

Fig. 6. Histogram of the distribution of diameters of HDL_2 fractions obtained from perfused livers of normally fed rats, with or without inhibition of LCAT by DTNB, and from normal rat plasma.

Orotic acid and nascent HDL secretion

Although we were able to block virtually all secretion of VLDL and LDL in fatty acid livers which contained no normal hepatocytes, HDL accumulated in the perfusate at a normal rate. Fasting the orotic acid-fed rats overnight did not appear to reduce the amount of newly synthesized HDL. In contrast, fasting the control rats substantially reduced the recoveries of both HDL and VLDL. The basis for this is unclear. The lipid and apoprotein contents of nascent HDL from normal and fatty livers were similar, whether or not LCAT in the perfusates was inhibited. Moreover, the structure and size of nascent HDL particles from both kinds of livers were essentially the same. The major difference between HDL_2 fractions from normal and fatty livers was the mass of newly synthesized apoB present. In both cases, the only apoB in HDL_2 fractions was the lower molecular weight form (apoB-48). Perfused livers of normals and cholestatic rats secrete a distinct apoB-containing particle which is isolated in the HDL_2 fraction (22, 25). Although much less apoB-48 accumulated in HDL_2 fractions from fatty livers than those from normal livers, a small amount was always present and it contained newly incorporated [^{3}H]lysine. When apoB-containing particles are immunosorbed from the HDL_2 fraction obtained from normal perfused livers in the presence of DTNB, the unretained discoidal particles are virtually indistinguishable from those obtained from the perfused fatty livers. The immunosorbed apoB particles from normal livers contain only trace amonts of proteins other than apoB-48 and about equal amounts of triglycerides and cholesteryl esters. Because much less apoB (too little to analyze) was obtained from the HDL_2 fraction from fatty livers than from the normal livers, the fat-laden livers of orotic acid-fed rats presumably secreted more "true" nascent HDL_2 than the normal livers.

Origin of HDL

Our previous studies of the mechanism of formation of discoidal HDL particles in liver perfusates have discounted the possibilities that they are ultracentrifugal artifacts or arise by interaction of apoproteins with erythrocytes present in the perfusates (3–5, 10). Although it has been evident for many years that the origin of HDL is likely to be complex (5), with contributions from direct secretion of nascent HDL particles and from the polar lipids and proteins that leave the surface of chylomicrons and VLDL during lipolysis (8), most recent discussions have emphasized the latter process. In several reviews, Eisenberg (7, 35–37) has proposed that discoidal HDL in liver perfusates are artifacts of this particular system, and he has suggested that "constituents originating from the surface coat of lipolyzed triglyceride-rich lipoproteins constitute the major, if not the only, source of HDL precursors" (7). We believe that our current observations indicate clearly that discoidal HDL in liver perfusates are not derived from the surface of nascent VLDL, either in Golgi compartments or in the perfusate; therefore, the possibility that these particles represent physiologically significant precursors of plasma HDL must be taken seriously.

HDL are found in patients with classical abetalipoproteinemia, in whom neither chylomicrons nor VLDL are secreted into the blood. In this circumstance HDL must originate as distinct particles (5). Whether they are formed intracellularly or extracellularly remains to be established. Both apoE and apoA-I can form discoidal complexes in vitro upon mixing with phospholipids. Therefore, newly secreted apoE and apoA-I could form discs by associating with phospholipids derived from cell membranes to which they are exposed in the perfusates.

Alternately, discoidal HDL may be secreted as such, much like nascent VLDL (3, 4). However, neither we nor others have obtained convincing evidence to support this concept. Some disc-like particles are seen in HDL fractions following rupture of isolated "Golgi" fractions, but they are few, are mixed with other membrane fragments, and may represent artifacts brought about by rupture of the Golgi membrane or by centrifugal forces (38). Banerjee and Redman (39) found that apoA-I from "Golgi" fractions of chicken liver floated at HDL density whereas apoA-I from RER fractions did not. Human apoA-I has been shown to form lipid complexes that float at HDL density when incubated with ruptured rat liver microsomes, but not after it was incubated with ruptured red cell ghosts (40). These findings may be consistent with those in the

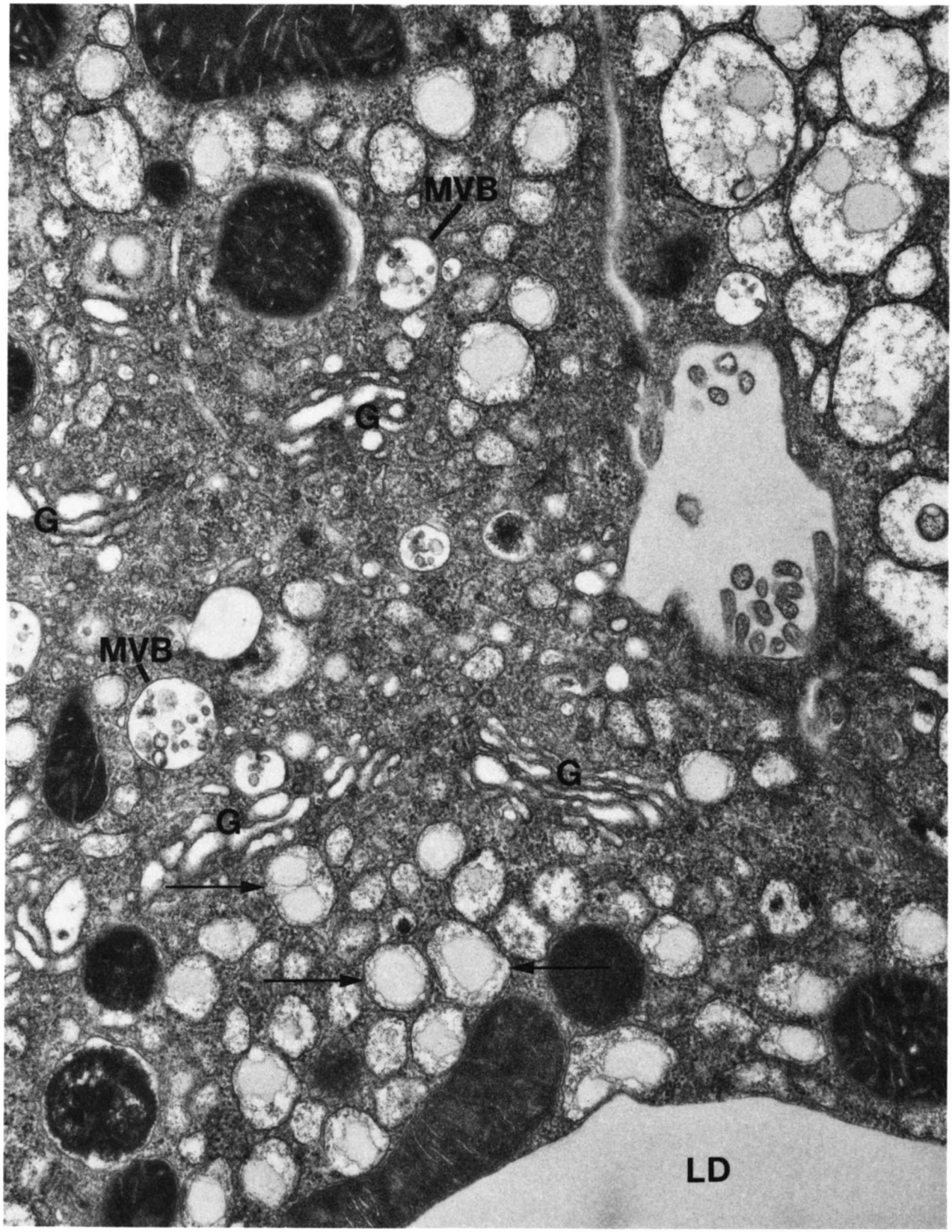

Fig. 7. Portions of hepatocytes (near biliary pole) of orotic acid-fed rats. Triglyderides accumulate in two forms: large cytoplasmic lipid droplets (LD) lacking a cell membrane, and much smaller particles occurring singly or in small groups within the cisternal spaces of dilated endoplasmic reticulum (arrows). Note the prominent Golgi (G) cisternae which lack stainable lipoprotein particles and adjacent multivesicular bodies (MVB) which also lack lipoprotein particles. × 20,000.

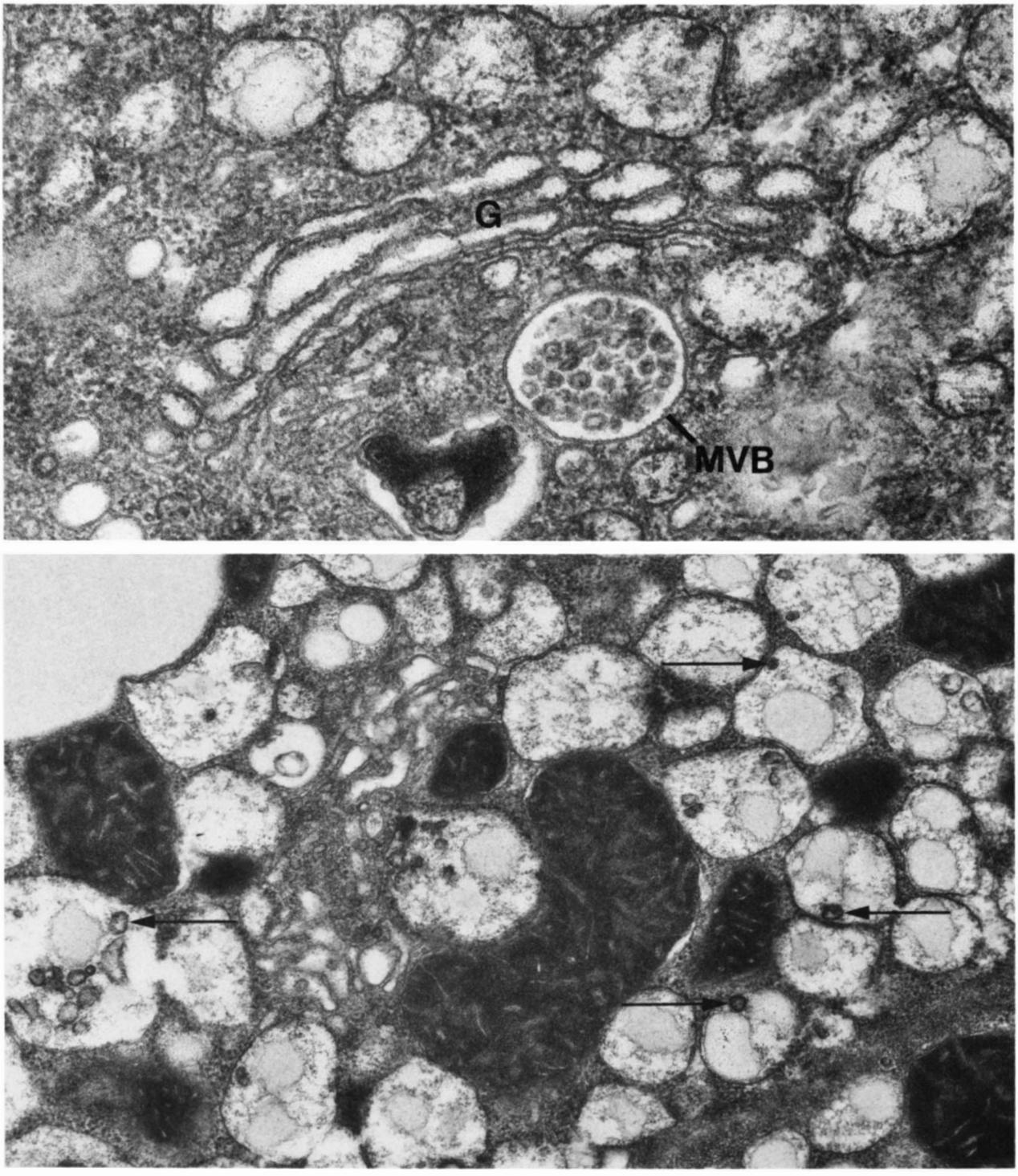

Fig. 8. Higher magnification illustrates typical empty Golgi (G) cisternae (lacking nascent VLDL), lipoprotein particles within cisternae of endoplasmic reticulum (top) identifying the *cis* side of the Golgi complex, and a multivesicular body (MVB) on the *trans* side of the Golgi complex containing many bilayer vesicles and no endocytosed lipoproteins. × 60,000. Bottom: This image clearly illustrates lipid droplets with associated granular material (precipitated proteins?) and other electron dense particles (arrows) within cisternae of the endoplasmic reticulum. × 40,000.

chicken because the total microsome fraction contains Golgi membranes as well as ER (41). Although apoE is associated with nascent VLDL released from Golgi membranes (42), little is known about its lipidation sequence from the endoplasmic reticulum to the extracellular space. Our finding that orotic acid-feeding reduced the mass of apoE secreted from fatty livers to about 25% of normal is consistent with the observation that about one-half of nascent apoE is nor-

mally found in VLDL of liver perfusates (43). Because apoE and apoA-I tend to dissociate from lipoproteins during ultracentrifugation (5), non-dissociating techniques may be required to address the process by which nascent HDL are assembled.

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