STUDIES ON THE SYNTHESIS AND INTRACELLULAR TRANSPORT OF LIPOPROTEIN PARTICLES IN RAT LIVER

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ABSTRACT

Lipoprotein particles ($d < 1.03$ g/ml) were isolated from rough and smooth microsomes and from the Golgi apparatus of rat liver, and were characterized chemically and morphologically. The rough endoplasmic reticulum (ER) particles were rich in protein (50%) and contained phospholipids (PLP) and triglycerides (TG) in smaller amounts, whereas the lipoprotein particles emanating from the smooth ER, and especially the Golgi apparatus, were rich in TG and PLP, resembling very low density lipoproteins (VLDL) of serum. The difference in chemical composition among the particles was associated with change in size both *in situ* and in isolated lipoprotein fractions. The rough ER particles were 200–800 Å in diameter (mean ~420 Å); the smooth ER particles 200–900 Å (mean ~520 Å); the Golgi particles 350–950 Å (mean ~580Å); and serum VLDL 300–800 Å (mean ~450 Å). Generally, lipoprotein particles were rare in the rough ER, frequent but diffusely dispersed in smooth ER, and occurring mainly in clusters in "secretory vesicles" of the Golgi complex. They were seldom observed in the cisternal compartments of the Golgi complex.

At short intervals (<15 min), intravenously injected radioactive glycerol was preferentially channelled into TG, whereas at later time points the majority of the isotope was recovered in the PLP. Three TG pools were distinguished: (*a*) a cytoplasmic pool with a slow turnover rate; (*b*) a membrane-associated TG pool; and (*c*) a pool corresponding to the TG moiety of lipoprotein particles, which showed the highest initial rate of labeling and fastest turnover.

When, after pulse labeling, the appearance of incorporation of radioactive glycerol into TG or PLP of isolated lipoproteins was followed from one subcellular fraction to the other, a sequence of labeling was noted. During the first interval, TG from both rough and smooth microsomal lipoproteins displayed a high rate of labeling with peak value at 6 min, followed by a quick fall-off, while the Golgi lipoproteins reached maximal level at 10–20 min after administration. There was an interval of 10–15 min before the appearance of labeled VLDL in serum.

It is concluded that the assembly of the apoproteins and lipid moieties into lipoprotein particles—presumed to be precursors of liver VLDL—begins in the rough ER and continues in the smooth ER. Also, there is a parallel change in chemical composition and size of the lipoprotein particles as they make their way through the ER and the Golgi apparatus. Some remodeling of the particles may take place in the Golgi apparatus before discharge into the circulation.

It is now well established that the endoplasmic reticulum (ER)[1] is the principal site of lipid synthesis in the liver cell. This is especially true for phospholipids (PLP) and triglycerides (TG). Recently, van Golde et al. (1) demonstrated that enzymes required for synthesis of lecithin and triacylglycerols are localized exclusively in the rough and smooth microsomes. Certain lipids, mainly TG, are thought to be conjugated with an apolipoprotein moiety within the channels of the ER (2–6) although the exact sequence of the assembly of the lipoproteins is not known. It has also been proposed that electron-dense particles observed in the smooth ER and Golgi apparatus constitute precursors of very low density lipoprotein particles (VLDL); after completion, these macromolecules are believed to be released to the circulation (2–6). A comparison of lipoprotein particles isolated from rat liver Golgi apparatus with serum VLDL established that they had similar chemical composition and immunological behavior (5). These findings suggest that the lipoprotein particles are channelled through the ER and at least in part through the Golgi apparatus (3) before release to the circulation at the space of Disse (8). Although the smooth ER (4) has been implicated in the transport and possible assembly of the VLDL in morphological studies, it is not clear whether or not the rough ER is also involved. Whereas Claude (6) concluded that the rough ER does not synthesize TG, in contrast to the smooth counterparts, Stein and Stein (2) and van Golde et al. (1) arrived at an opposite conclusion, namely that both rough and smooth ER take part in TG synthesis. Thus, controversy exists in the literature on the roles that various parts of the ER may play in the lipid synthesis and protein-lipid assembly of the nascent VLDL. The findings of Bungenberg De Jong and Marsh (8) that ribosomes synthesize the protein moiety, and of Lo and Marsh (9) that the Golgi apparatus plays a major role in the attachment of carbohydrate to the lipoproteins are generally accepted.

In the present paper we describe the isolation and characterization of lipoprotein particles from rough and smooth microsomes and compare them with the VLDL particles of the Golgi apparatus and of serum to evaluate more precisely where the lipoprotein particles are assembled before their conspicuous appearance in the Golgi apparatus. Furthermore, the site of synthesis and intracellular pathway of TG and PLP transport in the liver cell in vivo was explored by pulse labeling with [^{3}H]glycerol. In order to accomplish this, the appearance of radioactivity in lipoprotein particles isolated from rough and smooth microsomes and the Golgi apparatus was followed. To evaluate any changes in size of lipoprotein particles as they make their proposed way through the liver cell, isolated particles were also analyzed morphologically and compared to the *in situ* localization and appearance in the liver cell of presumed precursors of VLDL. Since it has been postulated that the liver cell contains various TG pools, an attempt was made also to define these pools in some detail and to evaluate whether [^{3}H]glycerol is preferentially channelled into one or more of these alleged compartments.

MATERIALS AND METHODS

Animals and Fractionation

Male nonstarved rats weighing 100–150 g were used, since the recovery of the Golgi apparatus was higher than for starved animals (10). The livers were thoroughly minced (~0.5 cm pieces) in several changes of ice-cold 0.29 M sucrose. When microsomes were isolated, the livers were homogenized in 0.29 M sucrose (20% homogenate) with a Teflon-glass homogenizer at 250 rpm with four complete strokes. For isolation of the Golgi apparatus, homogenization was performed in 0.5 M sucrose-5 mM $MgCl_2$ at low speed (about 100 rpm) (33% homogenate) with only two complete strokes (11, 12). Total microsomes, and rough and smooth microsomes were prepared as described earlier (13), with certain modifications. A swinging bucket rotor (SW-27) was used instead of an angle head rotor for the Cs^+-containing sucrose gradient (10). 10 ml of the postmitochondrial supernate adjusted to 15 mM CsCl was layered onto 20 ml of 1.30 M sucrose-15 mM CsCl, and centrifuged at 100,000 *g* for 90 min. The use of an SW-rotor effectively counteracted spontaneous aggregation of microsomes due to side wall impaction (10) and also gave higher purity of both smooth and rough microsomes. A Golgi-enriched fraction was isolated according to Morré et al. (11) as modified by Glaumann and Ericsson (12).

Washing Procedures

In order to remove adsorbed proteins and lipids from the isolated cell organelles, the pellets were washed twice in 0.15 M Tris buffer, pH 8.0, and centrifuged at 105,000 *g* for 120 min (13, 14). The first supernate contained a

[1] *Abbreviations used in this paper:* AMP, adenosine monophosphate; ER, endoplasmic reticulum; FFA, free fatty acids; G6P, glucose-6-phosphate; LDL, low density lipoproteins; PLM, plasma membranes; PLP, phospholipids; TG, triglycerides; VLDL, very low density lipoproteins.

floating lipid layer, the second contained only negligible amounts of TG and PLP.

The washed microsomes and Golgi apparatus-enriched fraction were suspended in distilled water (i.e. subjected to osmotic "shock"), and the intravesicular content was released by ultrasonic vibration, performed three times at 1.5 A for 1 min with a Polytron (Kinematica GMBH, Luzern, Switzerland) apparatus under careful cooling (14). The membranes were sedimented at 250,000 *g* for 2 h, and the supernates were used for the isolation of lipoprotein granules. When the supernates from rough and smooth microsomes were tested for NADH-cytochrome *c* reductase and G6Pase activities, no measurable activities were recovered, indicating that no membrane constituents, which might have been released during the sonication procedure, remained in the supernate. Similar findings have been shown by Svensson et al. (15) who demonstrated that the membrane fragments resulting from sonicated rough microsomes all move in a gradient of higher density than that used in this study. The pellet was resuspended in 0.15 M KCl-10 mM EDTA and centrifuged at 105,000 *g* for 90 min to ensure maximum detachment of "nonmembranous" material from the fractions (16). The ensuing pellets were designated "membrane fractions" as an operational term.

Incorporation of [^{3}H]Glycerol

During the incorporation experiments the animals were anesthetized with Nembutal. To minimize surgical stress which is known to increase fatty acid levels, only a small incision (1 cm) was made through the peritoneum, and a small part of the intestine was taken out. For all labeling experiments [2-^{3}H]glycerol (50 mCi/mmol) was injected into a branch of the superior mesenteric vein (20 μCi/100 g in 0.5 ml saline) for exactly 15 s to approach a pulse-labeling type of administration. Attempts to dilute the radioactivity by additional administration of cold glycerol (10 times the isotope dose) did not significantly change the specific labeling of lipids, indicating that the isotope did not alter the glycerol pool(s) of the liver. After decapitation of the animals the livers were rapidly removed and minced immediately in ice-cold 0.29 M sucrose. The entire procedure took 2 min ± 0.5. When indicated, the livers were perfused through the portal vein.

Isolation of Lipoproteins

In agreement with Lasser et al. (17), we found that most serum LDL float between d = 1.040 and 1.05, and that there was little or no lipoprotein present in the d = 1.006–1.04 g/ml fraction in normal rat serum. Isolation of lipoproteins from microsomal fractions at $d < 1.006$ gave little recovery; this was, however, considerably increased if isolation at $d < 1.03$ was performed. Because of the minor risk of overlap between LDL and VLDL at this limit (<1.03), the VLDL fraction of the serum was also isolated in this range from rats starved for 4-8 h to reduce the number of chylomicrons. Preparation of microsomal and Golgi lipoprotein fractions was performed essentially according to Chapman et al. (18, 19).

The sonicated supernates were layered under 2 ml of a NaCl solution of density 1.03 g/ml and centrifuged at 40,000 rpm for 16 h in the no. 60 angle head rotor (34°) of the Christ Omega Ultracentrifuge II set at 10°C. After ultracentrifugation the top layer ($d < 1.03$) was pipetted off, diluted with 0.25 M sucrose to 8 ml, and washed by recentrifugation under identical conditions. The top 1.5 ml constituted the microsomal and Golgi particle fractions. Serum VLDL were separated from 1 ml of blood as described above. The fraction $d < 1.03$ contained almost no detectable albumin as indicated by means of disk electrophoresis (14).

Chemical Analysis

The isolated lipoprotein fractions were delipidized twice with 4 vol of chloroform-ethanol (2:1), or according to Folch et al. (20) with identical results. Butylated hydroxytoluene was used as antioxidant in a nitrogen atmosphere (13). Aliquots were passed through a column containing silicic acid to separate PLP from neutral lipids (21). The chloroform phase contained the neutral lipids and the methanol phase contained the phospholipids. The chloroform extract was used for chromatography on silica gel-loaded papers to separate FFA, TG, cholesterol, and cholesterol esters (22). Identification and extraction from the papers were performed as described previously (13).

Extraction of lipids from the microsomal and Golgi apparatus fractions was done as described earlier (13). Column and paper chromatography were also performed. Protein was determined according to Lowry et al. (23) and RNA was analyzed as previously described (13). Free cholesterol and cholesterol esters were extracted with $FeSO_4$-saturated acetic acid and the amount of cholesterol was determined (24). TG were measured after alkaline hydrolysis (25) with tripalmitate as a standard. FFA were titrated as described earlier (13), with palmitic acid as a standard.

Electron Microscopy

The isolated Golgi fraction was resuspended in 0.25 M sucrose and sedimented for 25 min at 20,000 *g*. Primary fixation was performed in 1.5% cacodylate-buffered glutaraldehyde for 12 h. After a brief buffer rinse, the pellet was postfixed in 2% collidine-buffered OsO_4 (pH 7.4). Isolated rough and smooth microsomes were also fixed in glutaraldehyde and processed for electron microscopy after postfixation in OsO_4. Aliquots of the lipoprotein fractions were fixed in 1% phosphate-buffered OsO_4 (pH 7.2) and centrifuged for 30 min at 20,000 *g*. Pieces of liver were prefixed in glutaraldehyde by immer-

sion and postfixed in OsO_4, or directly fixed in OsO_4. All tissues and pellets were processed and embedded in Maraglas or Epon as described earlier (12).

The diameters of the particles in the different lipoprotein fractions were measured on prints at a magnification of 150,000 with a Zeiss particle-size analyzer (TGZ 3, Carl Zeiss, Inc., New York) set for a linear distribution with intervals of 20 Å between 200 and 1,200 Å. The mean diameters and standard deviations were calculated according to standard procedures.

RESULTS

Chemical and Enzymatic Composition of Membranes

Judging from the apparent specific amount of cytochrome P-450 and AMPase activity, the Golgi preparation contains less than 9% microsomes and less than 6% PLM elements (Table I). By use of succinate cytochrome *c* reductase as a marker for mitochondria, a contamination range of 3–5% may be calculated. Attempts to estimate the possible presence of peroxisomes in the Golgi fraction were not successful because the activity of amino acid oxidase was too low to justify calculation. On the basis of these marker enzymes, the Golgi-enriched fraction can be estimated to consist of at least 70% Golgi apparatus-derived material, which is consistent with the morphological findings. If the Golgi apparatus contains some cytochrome P-450 or AMPase, the actual purity of the fractions can be expected to be somewhat higher than 70%. Because of the equivocalness in the literature (10, 26) as to the extent of glycosyltransferases present in the ER and the Golgi apparatus, we would rather not try to estimate the recovery of the Golgi apparatus, although the distribution of the two glycosyltransferases would indicate a range of 20–25%.

At first glance, the distribution of the glycosyltransferases when used as markers for the Golgi

TABLE I

Distribution of Some Marker Enzymes in Homogenate, Rough and Smooth Microsomal Fraction, Plasma Membrane, and Golgi Apparatus-Enriched Fractions

	Homogenate	Rough Microsomes	Smooth Microsomes	Golgi Fraction	PLM Fraction
Protein mg/g liver	187	11.1	6.3	1.05	1.5
Galactosyl transferase*					
% of homogenate	100	3	13	21	—
per mg protein	5.3	2.6	20.6	198	—
Glc NAc transferase‡					
% of homogenate	100	19.0	15.4	18	—
per mg protein	5.0	16	12.0	160	—
Cytochrome P-450§					
% of homogenate	100	24	16	1	—
per mg protein	0.13	0.53	0.62	0.051	—
AMPase‖					
% of homogenate	100	6.5	7	1	22
per mg protein	0.62	0.68	1.3	1.1	16.8

The glycosyltransferase activities were estimated according to Wagner et al. (1973) with UDP-[*N-acetyl*-^{14}C] glycosamine and UDP-[^{14}C]galactose as substrates by measuring the radioactivity in the TCA precipitate after washing with ethanol and ether. Controls were prepared individually for each fraction by adding the substrate to the TCA precipitate. The time curve was linear for about 5–10 min for microsomes and for about 10–15 mins for the Golgi-enriched fraction. Cytochrome P-450 and AMPase were measured as described earlier (12). The values are the means of four to five experiments.

* Picomoles galactose and ‡*N*-acetyl-glycosamine transferred to TCA-insoluble material ("endogenous acceptor") per 5 min.

§ Nanomoles.

‖ Micromoles P_i per 20 min.

apparatus would indicate that the smooth microsomal fraction contains 6–8% Golgi elements. However, there are several objections to such a calculation. The Golgi apparatus is not homogeneous as regards morphology or enzyme distribution, and at present it is not known whether or not derivatives from its cisternae, or from the associated vesicles, randomly contaminate the smooth microsomal fraction. Second, the assays for the glycosyltransferase activities may be affected by limitation of acceptor and/or by destruction of the added substrate by microsomal or Golgi apparatus-associated pyrophosphatase activities (27). Finally, some hexosamine residues are probably attached to the apoprotein or polypeptide already at the ribosomal site (28) while others are added as the nascent glycoprotein makes it way through the ER system. In this case, one would of course expect a multimodal distribution of certain glycosyltransferases to all parts of the ER. Taking these possibilities into consideration, Wagner et al. (29) estimated that the smooth microsomal fraction (isolated as in this study) contains about 3% Golgi elements. Furthermore, rough and smooth microsomal fractions were regularly checked by electron microscopy and by RNA determination; the purity was in the 90% range or more. The recovery of the total microsomal fraction was estimated to be about 50% as judged from the content of cytochrome P-450 (Table I).

The washing procedure in alkaline Tris buffer was performed to remove adsorbed material, mainly hemoglobin and other adsorbed proteins (13, 14). In addition, cytoplasmic lipids were detached and could be followed visually, since a distinct lipid layer floated at the top of the gradient. Chemical analysis of the supernate disclosed significant amounts of lipids (mainly TG and some PLP, but very little cholesterol), indicating that Tris washing is also effective in detaching adsorbed, nonmembranous lipids as well as in removing loosely attached proteins (13, 14). After Tris washing and an additional KCl-EDTA wash (16), sonication was performed to rupture the vesicles and cisternae and release their contents. The chemical composition of the membranes is illustrated in Table II. The similar PLP/protein ratio for rough and smooth microsomes is a result of the sonication treatment and extensive washing procedures, which remove adsorbed proteins and in addition about 90% of the microsomal ribosomes (13). The rough microsomal membranes contained about 41% lipid, as compared to 51% and 53%, respectively, for smooth and Golgi membranes. When related to PLP, the cholesterol content increased from rough (7%) to smooth microsomal membranes (9%) and to the Golgi membranes (15%). Much higher values have been reported for plasma membranes (30–40%) and for lysosomes (30%) (30). Also, the TG content was higher in the Golgi-enriched fraction than in the microsomal fractions. Consequently, the Golgi complex is an intermediate between the microsomes and plasma-lysosomal membranes with re-

TABLE II

Distribution of Protein, PLP, Triglycerides, and Cholesterol in Membranous Elements of Liver Microsomes and Golgi Apparatus

	Protein	PLP*	Cholesterol‡	TG§	$\frac{\text{Cholesterol}}{\text{PLP}}$	$\frac{\text{TG}}{\text{PLP}}$	$\frac{\text{PLP}}{\text{Protein}}$
			mg/g of liver				
Total microsomes	12.1 ± 1.3	4.8 ± 0.6	0.38 ± 0.04	0.23 ± 0.02	0.079	0.047	0.39
Rough microsomes	6.0 ± 0.7	2.2 ± 0.7	0.15 ± 0.01	0.089 ± 0.01	0.068	0.040	0.36
Smooth microsomes	3.9 ± 0.8	1.7 ± 0.2	0.16 ± 0.01	0.082 ± 0.01	0.094	0.048	0.44
Golgi apparatus	0.50 ± 0.1	0.21 ± 0.02	0.032 ± 0.003	0.023 ± 0.002	0.152	0.11	0.42

Membranous elements denote Tris-washed, sonicated, and KCl-EDTA washed membranes (13–16). The values are the means ± SEM of 12 experiments.

* Phospholipids.

‡ Free and esterified cholesterol.

§ Triglycerides.

gard to its cholesterol per PLP ratio. Similar results have been obtained by Yunghans et al. (31), but in addition to confirming their findings, our data demonstrated that smooth microsomes are intermediates between rough microsomes and the Golgi-enriched fraction, not only in terms of their cholesterol content, but also in regard to their TG content compared on the basis of PLP.

Chemical Composition of Lipoprotein Particles

After Tris washing, sonication, pelleting of the membranous material, and floating-up centrifugation of the resulting supernate, it was possible to obtain lipoprotein particles with a density of <1.03 g/ml, corresponding to about 0.2 mg per gram of liver from total microsomes and almost 0.1 mg from rough and smooth microsomes and from the Golgi-enriched pellet (Table III). Their main components were TG, PLP, cholesterol, and protein. Small but variable amounts of FFA were also noted. This variation is probably the result of various lipases which are known to be present in lysosomes of hepatocytes, and might well be active even at the low temperature used during the isolation procedure. However, the chemical composition of the isolated particles was not identical in the various subcellular fractions. At one extreme were the particles derived from the rough microsomal fraction displaying a high protein content, and at the other were the particles originating from the fraction enriched in the Golgi profiles displaying a high TG content. When the chemical composition is given as percent of weight, a distinct pattern of changes becomes evident (Fig. 1). The protein content decreased from rough (50%) to smooth microsomes (30%) and to the Golgi apparatus (20%), whereas there was a marked increase in TG amount, namely 15%, 24%, and 41%, respectively. The PLP content of the lipoprotein particles constituted an apparent intermediate, since the PLP proportion was enhanced from rough (20%) to smooth microsomes (32%), but then decreased in the particles isolated from the Golgi apparatus (25%). Because of the low concentration of total cholesterol (mainly free and to a lesser extent esterified), any conclusion as to possible differences in percentage distribution does not seem justified. In agreement with Mahley et al. (5) and Chapman et al. (18, 19), the chemical composition of the Golgi lipoproteins and serum VLDL exhibited only minor differences, e.g., mainly in protein and TG composition. These minor differences might be explained by the fact that the serum VLDL originate not only from the liver but also from the intestine (32); furthermore, the serum VLDL are not stable in the circulation, but take part in several exchange reactions, being further transformed into other lipoprotein particles with different densities and chemical make up (33).

Appearance of Labeled TG and PLP in Subcellular Fractions

TG are present in various amounts in the hepatocytes depending upon the nutritional status

TABLE III

Protein and Lipid Composition of Lipoprotein Particles from Subcellular Organelles and of Serum VLDL

	Protein	PLP*	Cholesterol‡	TG§	FFA‖
			μg/g of liver		
Total microsomes	90 ± 12	58 ± 6	15 ± 2	42 ± 3	4 ± 3
Rough microsomes	50 ± 7	19 ± 2	5.3 ± 1	13 ± 1	3 ± 2
Smooth microsomes	25 ± 3	27 ± 3	9.6 ± 1	20 ± 2	2 ± 1
Golgi apparatus	17 ± 1	18 ± 2	5.7 ± 1	29 ± 2	3 ± 2
Serum¶	83 ± 6	109 ± 12	50 ± 4	242 ± 4	16 ± 6

The lipoprotein particles and serum VLDL were isolated as described in Materials and Methods. The values are the means ± SEM of 16 experiments.

* Phospholipids.

‡ Denotes cholesterol (~65%) and cholesterol esters (~35%).

§ Triglycerides.

‖ Free fatty acids.

¶ Micrograms per milliliter.

of the animal (34). They can be changed by starvation and by a number of drugs, and by various noxious compounds such as ethanol (35) or CCl_4 (36). Furthermore, it has been postulated that the liver cell contains two TG pools, one of which has a quick turnover rate and is associated with the microsomal fraction (7, 13), and another which is localized in the cytoplasm and has a slower turnover rate. The presence of TG in both lipoproteins and in membranes would suggest a third compartment. In order to determine if these pools have similar or different metabolic rates, [^{3}H]glycerol was injected into the portal vein and the rate of labeling into TG was followed as a function of time in the 100,000 *g* supernate (cytoplasmic pool), in extensively washed microsomal membranes (membrane pool), and in the lipoprotein-rich fraction (intracisternal pool). In these experiments the livers were perfused via the portal vein with 0.29 M sucrose at 37°C to minimize blood lipids. As illustrated in Fig. 2, it is evident that the TG compartments were not uniformly labeled. The highest rate of incorporation was noted for the lipoproteins, followed by the microsomal membranes and the cytoplasm (supernate) in decreasing order of magnitude. Furthermore, they reached plateau values at different time points: the lipoproteins after 5 min, the membranes somewhat later (~10 min), and the cytoplasm significantly later (~20 min). A similar pattern was noted with separated rough and smooth microsomes and Golgi-enriched fractions, namely a higher and quicker rate of labeling into TG for the lipoprotein fraction than for the membranes (Fig. 3). There was a more rapid fall-off in activity after reaching maximal level in the lipoproteins than there was in the membranes, indicative of different turnover rates between membrane- and nonmembrane-associated TG. Furthermore, the initial rate of glycerol incorporation into TG of Golgi membranes was considerably slower than for rough and smooth microsomal membranes. In all isolated organelles the specific labeling of TG (counts per minute per milligram)

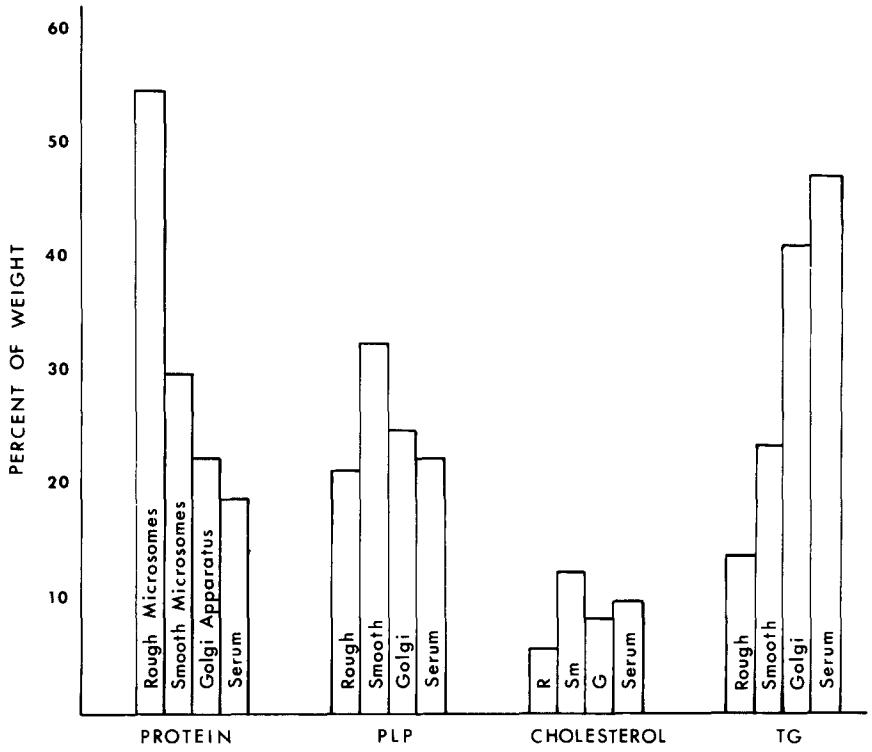


FIGURE 1 Differences in chemical composition of lipoprotein particles from rough and smooth microsomes, the Golgi apparatus, and serum VLDL. The bars represent percent of total weights of measured compounds. The values are the means of 16 experiments. The lipoprotein particles were isolated by floating-up centrifugation as described in Materials and Methods.

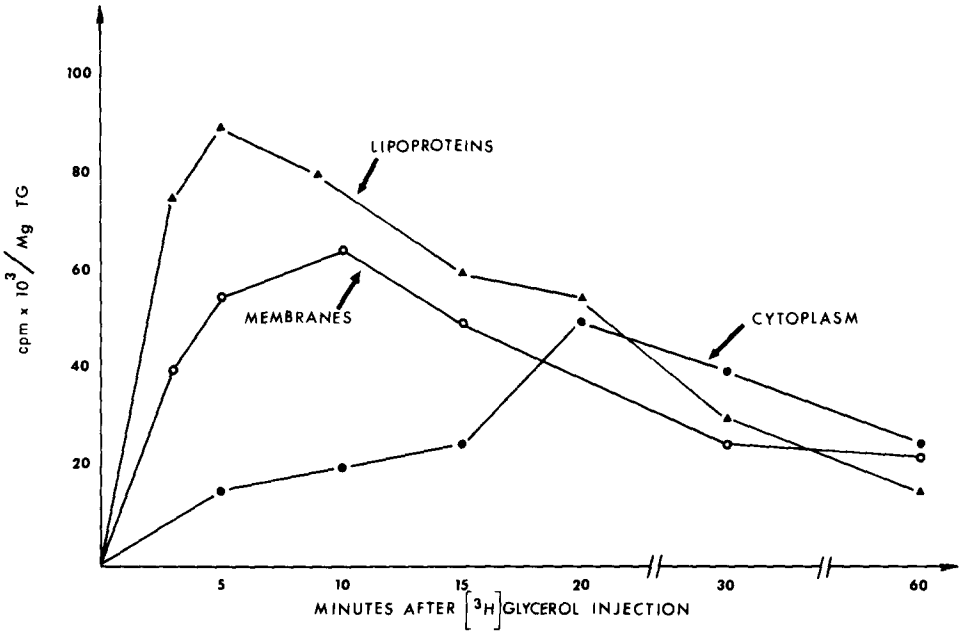


FIGURE 2 Incorporation of [³H]glycerol into TG of microsomal membranes, lipoproteins, and cell cytoplasm at different times after intraportal administration. "Microsomal membranes" signifies Tris-washed, sonicated, and KCl-EDTA-washed membranes. All the time points given in this and the following figures denote the entire period after injection into the portal vein until the livers were placed and minced in ice-cold sucrose. 20 μCi [³H]glycerol/100 g.

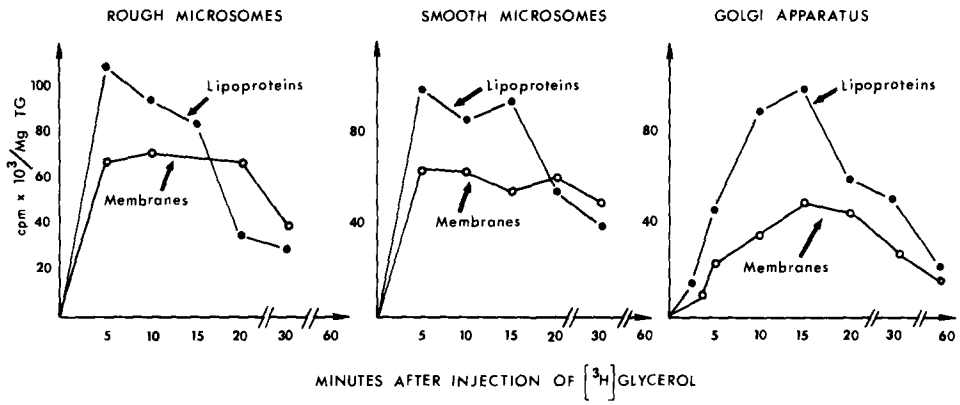


FIGURE 3 Incorporation of [³H]glycerol into TG of lipoprotein particles, rough and smooth microsomal membranes, and Golgi membranes. One typical series of experiments is shown. 20 μCi [³H]glycerol/100 g.

in the lipoprotein particles and in the membranes seemed to equilibrate after ~30–60 min with some tendency for the membranes to remain "hotter" at later intervals in comparison with the lipoprotein particles.

In Fig. 4 the curves for incorporation of [³H]glycerol into the TG moiety of lipoprotein particles isolated from microsomal subfractions, the Golgi apparatus, and in serum VLDL are given with short intervals. During the first interval of 3–5 min, the lipoprotein-associated TG from rough and smooth microsomes displayed a much higher rate of labeling when compared with those from the Golgi fraction. Both rough and smooth mi-

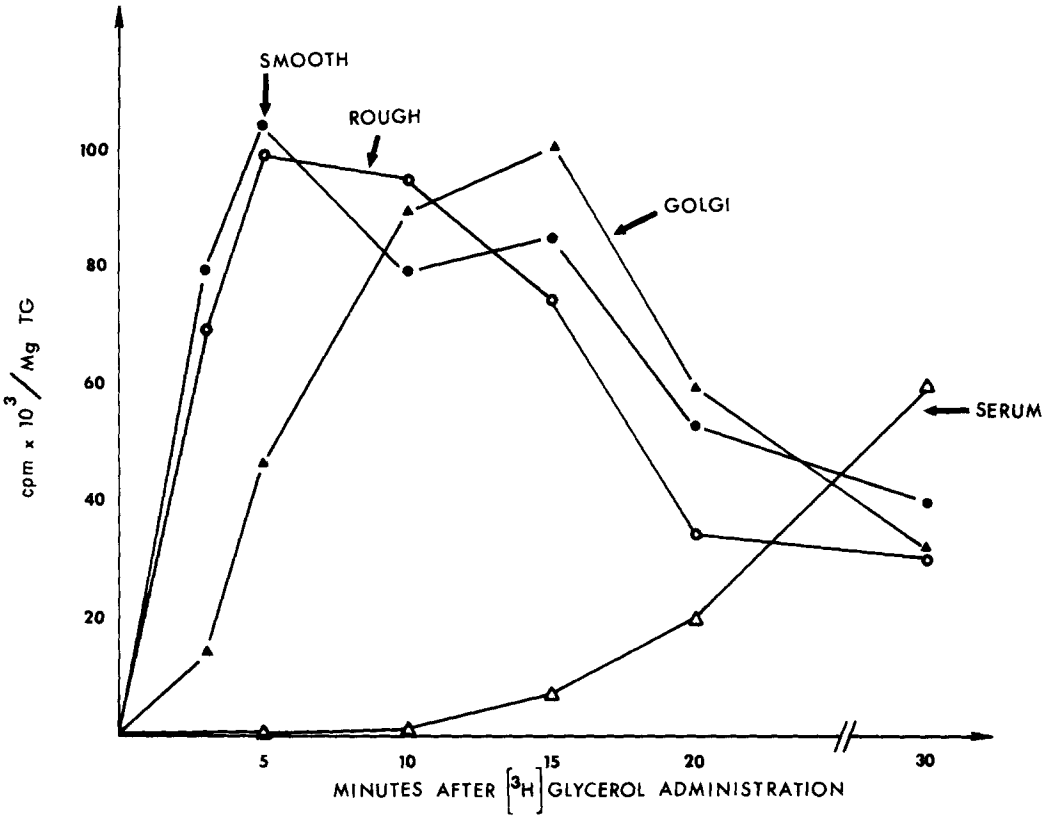


FIGURE 4 [³H]Glycerol incorporation into the TG moiety of lipoproteins from rough and smooth microsomes and from the Golgi apparatus. Each value represents the mean of four experiments out of five or six. Variations from one experiment to the other were in the range of 25%. 20 μCi [³H]glycerol/100 g.

crosomes displayed a similar rate of incorporation with peak values at approximately 5 min followed by a decline, while the radioactivity of the Golgi fraction increased, reaching maximal levels 10–20 min after the administration of the isotope. A small but constant biphasic pattern was noted for the lipoproteins isolated from the smooth microsomes. The equally fast rate of incorporation into rough and smooth microsomes when compared to the Golgi fraction indicates that TG are preferentially, if not exclusively, synthesized in the rough and smooth ER rather than the Golgi apparatus. As regards the serum-VLDL, there was a delay of 15–20 min before measurable labeling of [³H]glycerol into the TG moieties was noted. This was followed by a constant increase up to 30–60 min.

The quantitative role of rough and smooth ER and the Golgi apparatus in the transport of TG is illustrated in Table IV depicting total counts per minute per gram of liver as a function of time after labeling. At 3–5 min, the majority of label was recovered in the rough and smooth fractions, and only 20% in the Golgi fraction. At later time points (15–20 min), the label recovered in the Golgi fraction exceeded the activity of the two microsomal subfractions combined. At all intervals investigated, the smooth microsomes displayed higher total activity in comparison with the amount of label in the rough microsomal fraction.

The appearance of [³H]glycerol-labeled PLP as a function of time in the lipoprotein particles is shown in Fig. 5. It will be noted that there was a similar pattern of PLP labeling in comparison with TG labeling, namely a quick rate of incorporation into microsomal lipoprotein particles with peak values at 5–10 min, whereas the PLP moiety of of the Golgi fraction did not reach maximal level until 15–20 min after administration of the isotope. A lag period of 10–15 min in the appearance of labeled lipid in the serum was noted, followed by a constant increase up to peak values at 30–60

min. No significant difference in "silent" period was noted between serum TG and PLP labeling. Such a difference has been described when the earliest apparent incorporation of radioactivity into the protein moiety after [^{14}C]leucine administration has been compared with [^{3}H]palmitate incorporation into the lipid moiety of serum VLDL (37).

TABLE IV

Distribution of Labeled Lipoprotein TG in Rough and Smooth Microsomes and the Golgi Fraction

Time after injection	Rough microsomes	Smooth microsomes	Golgi fraction
min	*cpm* × 10^3/*g of liver*		
3	910	1,600	435
5	1,310	2,200	1,363
10	1,235	1,600	2,610
15	975	1,740	3,045
20	455	1,040	1,740
30	390	800	928

The lipoprotein particles were isolated as described in Materials and Methods. The values are the means of nine experiments. The rats received 20 μCi [^{3}H]glycerol/100 g in saline into a branch of the portal vein. The time points denote the time after injection until the livers were placed into ice-cold 0.29 M sucrose.

The distribution of radioactivity between the two lipid classes, PLP and TG, of microsomal membranes and of lipoprotein particles, is shown in Table V. After short intervals, the majority of [^{3}H]glycerol was channelled into TG of the membranes and of lipoprotein particles. At later time points (15–60 min), there was a constant increase in the relative percent of label incorporated into the PLP fraction at the expense of the TG labeling. The proportional decrease of label in TG of the microsomal membranes and of the lipoproteins was similar. Table V also demonstrates the remarkable difference in rate of glycerol labeling between PLP and TG, the latter being considerably higher since the TG content in the membranous fraction is only about 5% (Table I) of the PLP content. Thus, the maximal specific activity for the PLP was about 4,000 cpm/mg whereas that of TG extracted from the membranes was about 60,000

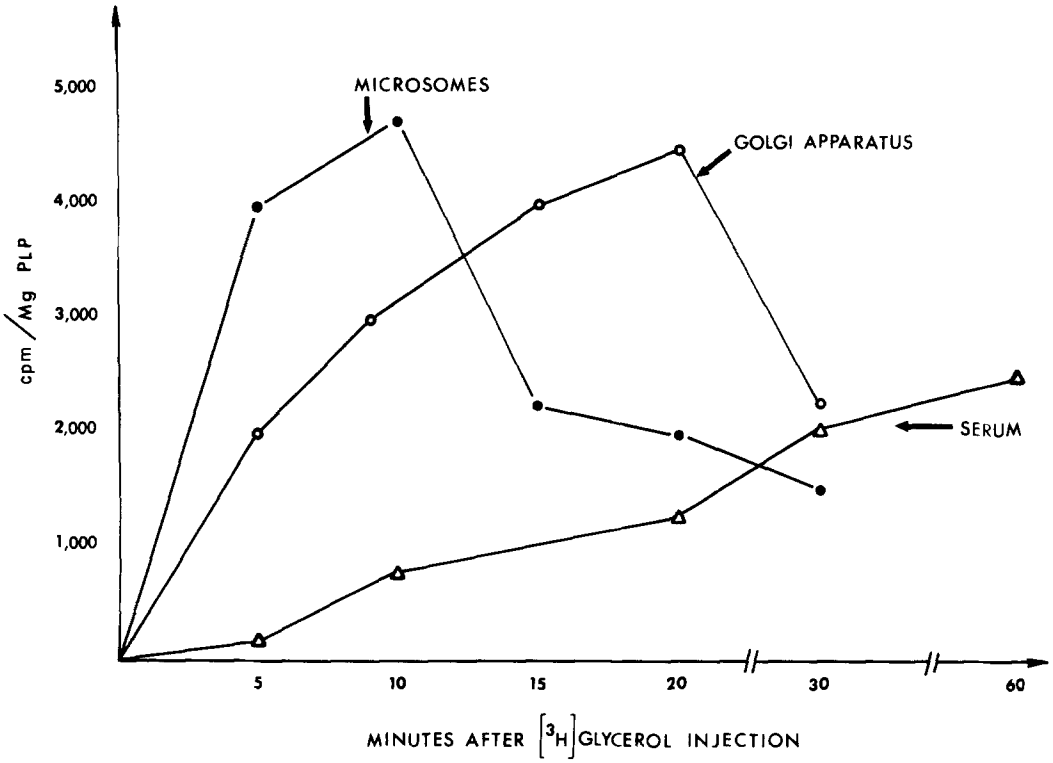


FIGURE 5 [^{3}H]Glycerol incorporation into lipoprotein-PLP of microsomes, the Golgi apparatus, and of serum. Each value represents an average of at least three experiments. Variations from one experiment to another were within 23%. 20 μCi [^{3}H]glycerol/100 g.

TABLE V

Incorporation of [^{3}H]Glycerol into Phospholipids and Triglycerides of Microsomal Membranes and Lipoprotein Particles

Time	Membranes				Lipoprotein Particles		
	Phospholipids		Triglycerides		Phospholipids	Triglycerides	
min	*cpm**	%‡	*cpm**	%‡	*cpm**	*cpm**	%‡
5	9,600	37	12,650	48	82	3,780	14
10	10,800	37	14,950	51	110	3,360	12
15	13,730	49	11,500	41	112	2,520	9
20	15,840	63	6,900	27	174	2,110	8
30	14,400	77	2,900	16	100	1,260	7
60	13,440	82	2,300	14	54	630	4

The values are the means of four experiments. The lipoprotein particles were isolated from total microsomal fractions as described in Materials and Methods. The rats received 20 μCi [^{3}H]glycerol/100 g into a branch of the portal vein.

* cpm denotes per gram of liver.

‡ % denotes percent cpm of total activity in membranes and lipoprotein particles.

cpm/mg, and about 100,000 cpm/mg for the intracisternal content or lipoprotein particles. (Fig. 3).

The rate of [^{3}H]glycerol incorporation into PLP of microsomal membranes and of Golgi membranes also displayed a different pattern (Fig. 6) as was shown for the PLP moiety of lipoprotein particles. The total PLP fraction (separated from NL by silicic acid column chromatography) exhibited a higher rate of labeling in the microsomes in comparison with the Golgi membranes up to 20 min after injection, and the two organelles did not equilibrate until about 30–60 min had elapsed. The low rate of PLP (Fig. 6) and TG labeling (Fig. 3) in the Golgi apparatus is in good agreement with the findings of van Golde et al. (1), who demonstrated that choline phosphotransferase and acyl-CoA:1,2-diacyl-*sn*-glycerol acyltransferases were absent in the Golgi complex, and were almost exclusively localized in the smooth and rough ER.

Electron Microscope Analyses

Fig. 7 shows the *in situ* localization of the electron-dense particles believed to represent VLDL or their precursors. The particles were present, although rare, in the rough ER, while they were comparatively abundant in the smooth ER. The latter site included glycogen areas (nonstarved animals) as well as the transitional elements between rough ER and the Golgi apparatus, and the subplasmalemmal portions of the smooth ER connected with the "distal" ends of rough ER.

In the Golgi apparatus, peripheral dilated portions of the cisternae ("concentrating vesicles") (3–6) and vacuoles located at the "emitting" face were filled with particles, while as a rule the central flattened regions of the cisternae were devoid of particles.

The appearance of the isolated Golgi complex has been described previously (5, 11, 12). Additional features of special interest in the present context are illustrated in Fig. 8. The comparatively high purity of the Golgi fraction has been substantiated by biochemical analysis (11, 12, 18, 19), and was regularly checked in this study. With primary aldehyde fixation the particles were less electron opaque than after fixation in OsO_4 and usually appeared paler than the matrix substance. The particles were present in vesicular and vacuolar structures which as a rule were not in apparent continuity with the cisternae. In general, there were no particles present in the fenestrated plates or the cisternal tubules. On the other hand, some cisternae, or portions of cisternae, contained a highly electron-opaque material which was also observed in some of the vesicles and vacuoles surrounding the cisternal elements. These findings, together with the evidence obtained from the *in situ* observations, suggest that the VLDL particles are associated with special (mainly peripheral) portions of the Golgi system. Whether the highly electron-opaque material seen in the isolated cisternae of the Golgi apparatus but not *in situ* is an artifact resulting from the fractionation procedure, or represents other, more electron-dense macromolecules (presumably other lipoproteins, glyco-

proteins, albumin), also destined for the circulation, which may be channelled through separate (often more centrally located) compartments of the same organelle, is not fully clear at present.

The particles observed in the rough ER in thin sections of liver were 200–400 Å in diameter, those in the smooth ER up to 600 Å, and those in the Golgi apparatus 400–800 Å (Fig. 7). OsO_4 fixation of lipoprotein material released from the subcellular organelles by sonication and isolated by floating-up centrifugation ($d < 1.03$) yielded a pellet which was composed of spherical particles. No identifiable membrane fragments were noted. The particles were, however, of somewhat different sizes depending upon from which organelle they were isolated. Particles isolated from rough microsomes (Fig. 9 *a*), embedded, and studied in thin sections, were 200–800 Å in diameter; corresponding values for particles obtained from smooth microsomes (Fig. 9 *b*) and the Golgi apparatus (Fig. 9 *c*) were 200–900 Å and 350–950 Å, respectively. VLDL isolated from serum varied in diameter from 300 to 800 Å. It will be seen that the lipoprotein particles isolated from rough microsomes have an irregular spherical shape (Fig. 9 *a*). Similar shapes were noted for the lipoprotein particles isolated from smooth (Fig. 9 *b*) microsomes and the Golgi apparatus (Fig. 9 *c*). The size distribution of the isolated particles is shown in Fig. 10. They were evenly distributed in all the fractions, and showed a smaller mean diameter in rough microsomes (420 Å) than in smooth microsomes (520 Å) and the Golgi fraction (580 Å), respectively. As illustrated in Fig. 11, apparent lipoprotein particles were also present in isolated rough and smooth microsomes.

Albumin and Lipoprotein Content in Subcellular Fractions

In earlier studies on albumin synthesis (12, 38), it was shown that at least some of the albumin passes through the Golgi apparatus before being discharged into the sinusoids. In Fig. 12 an attempt is made to correlate the content of albumin and lipoprotein particles to the number of membranes (expressed as milligrams of protein) in the various subcellular fractions. Although larger amounts of albumin, in comparison with lipoproteins, can be recovered from all fractions, including the Golgi-enriched pellet, the packaging mechanism for the presumed VLDL precursors in the Golgi apparatus is much more pronounced than for albumin. This finding is in agreement with

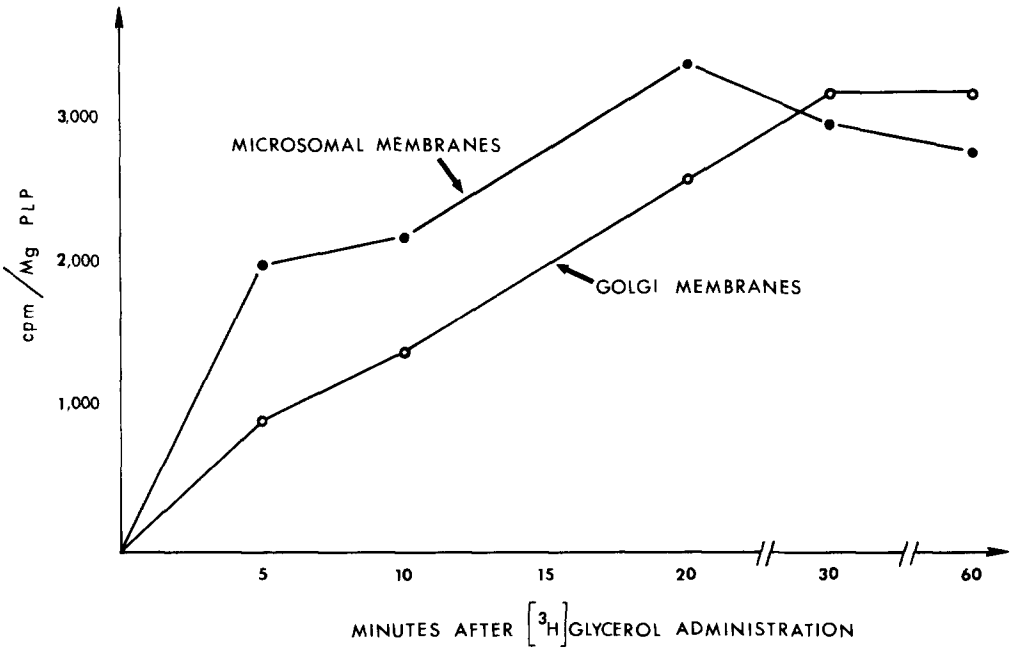
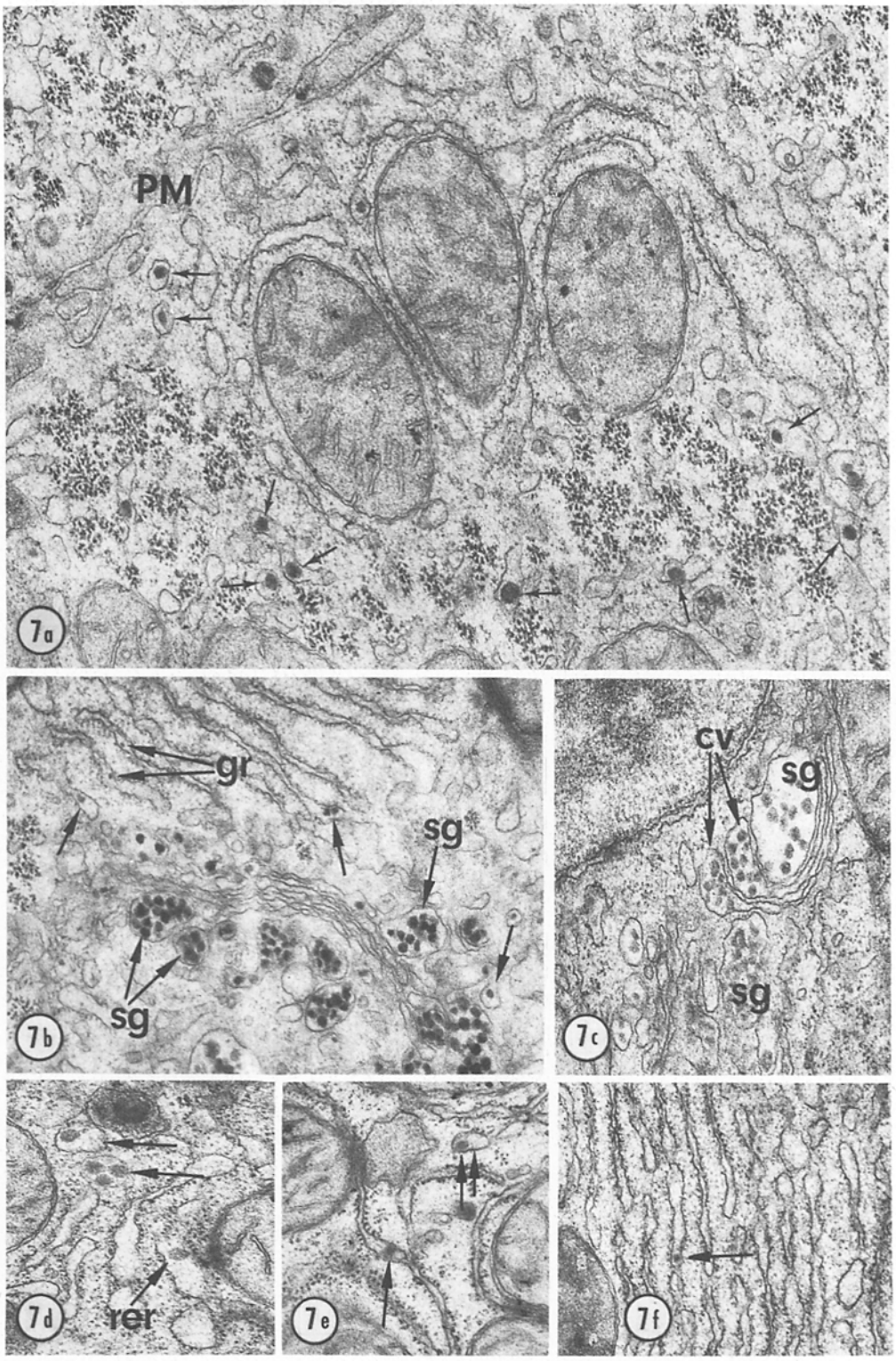


FIGURE 6 Incorporation of [^{3}H]glycerol into PLP of microsomal and Golgi membranes. 20 μCi [^{3}H]glycerol/100 g.


PM
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the morphological observations presented here as well as with previous works (3–9), namely a conspicuous accumulation of characteristic particles in the Golgi-associated secretory vesicles.

DISCUSSION

The site of synthesis of TG and PLP, and the insertion of TG and PLP into lipoprotein particles presumed to be precursors of VLDL, were studied after glycerol labeling. There are several advantages in using glycerol: it penetrates biological membranes rapidly (39); it is present in the cell pool in sufficient amounts to ensure large excess (40); it does not participate in exchange reactions (41) and is not reutilized within hours after administration (40, 42); it is a precursor for TG and also for PLP synthesis (after being phosphorylated); and it is not recovered in the fatty acids of PLP to any significant extent even 1 or 2 days after injection when given in the low dose necessary for isotope experiments (see Eriksson, reference 40, for a more a detailed discussion).

The biosynthesis and intracellular migration of VLDL have been extensively studied and most of the pertinent literature has been recently summarized (6, 43, 44). As with other serum proteins such as albumin (12, 14, 38, 45), transferin (46), HDL, and LDL (8, 43), it seems plausible to assume that the protein moiety of VLDL is also synthesized on ER ribosomes. On the basis mainly of morphological and autoradiographical analyses (2–6), it has been proposed that the ER is the major site for manufacturing those TG and PLP components which when assembled form VLDL. The more exact localization of this conjugation is not known (43, 47). Previous reports were not intended primarily to focus interest on the possibility that TG may be present in both a structural (membrane-associated) and a metabolic compartment. To evaluate such a compartmentalization of lipids, separation of the pools is a prerequisite; this was accomplished in this study. Furthermore, the possibility that lipoprotein particles change their chemical position and morphological appearance as they make their way through the intracisternal apparatus was also investigated.

Our observations are compatible with the existence of three TG pools in the liver cell and thus extend the studies of the Steins and co-workers (2, 7) and of Glaumann and Dallner (13). The first two TG pools include: (*a*) a cytoplasmic (storage?) pool which has a slow turnover and is dependent on the ER for renewal of TG; this pool can apparently be changed by a number of drugs and by variation in nutritional status (34 and 35); (*b*) a membrane pool, or perhaps more accurately, a pool associated with ER membranes which cannot be detached from the membranes by extensive washing procedures or sonication. Re-evaluating the data of Glaumann and Dallner (13), which demonstrated that the majority of both PLP and TG remained in a membrane-attached state after deoxycholate treatment of microsomal membranes, the present finding supports the view that at least a part of TG in the liver cell is tightly bound to ER membranes and is not a consequence of adsorption from the cytoplasm. It is not possible to determine at present whether this TG compartment is only a temporary intermediate one, reflecting the site of synthesis, or whether it also plays a structural and functional role, as do PLP and cholesterol, in the membranes. The third TG pool is localized in the intracisternal space of the ER and Golgi apparatus and displays the highest rate of labeling and the most rapid turnover. The morphological analysis clearly demonstrates that this pool corresponds to lipoprotein particles presumed to be precursors

FIGURE 7 Fine structural distribution of particles in hepatocytes of immersion-fixed rat liver (primary fixation in OsO_4). Thin sections were stained with lead citrate and uranyl acetate. (*a*) Particles (ar.ows) in smooth ER in a glycogen area and near the plasma membrane (*PM*). Maraglas. × 36,000. (*b*) Particles (arrows) in smooth-surfaced terminal ends of rough-surfaced ER near the convex side of the Golgi apparatus and in "secretory granules" (*sg*). Note absence of similar particles within the flattened cisternae of rough ER, which, however, contain smaller granules (forming particles?) in two areas (*gr*). Maraglas. × 24,000. (*c*) Golgi region with particles in "concentrating vesicles" (*cv*) and "secretory granules" (*sg*) (8). Maraglas. × 36,000. (*d*) Particles in vesicular elements of smooth ER (arrow) and in a cisterna of rough ER (*rer*). Maraglas. × 36,000. (*e*) Particles (arrow) in the lumen of rough ER and within an elongated vesicular element of ER (double arrows) covered with ribosomes on one side. Epon. × 25,000. (*f*) Cisternae of rough ER, one of which contains an electron-dense particle (arrow). Epon. × 18,000.

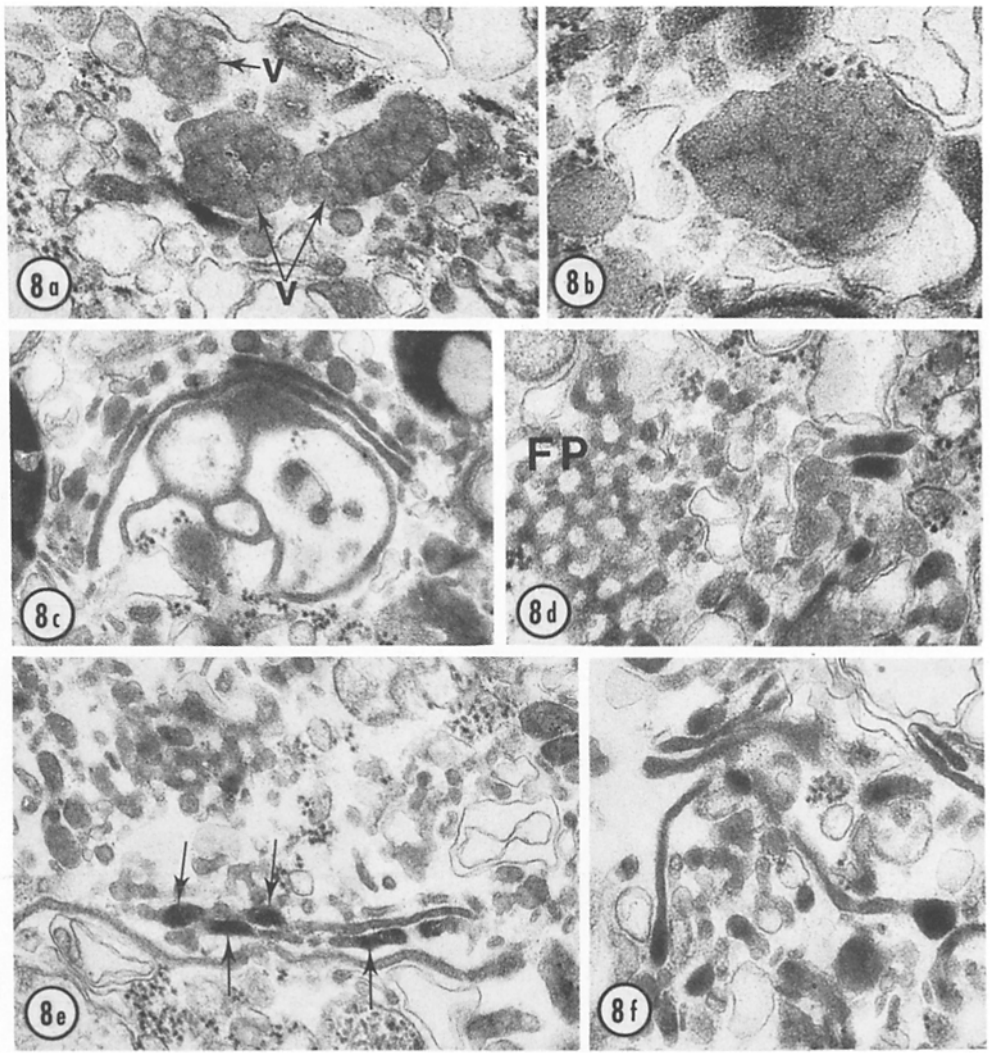

FIGURE 8 Appearance of representative areas of glutaraldehyde-fixed Golgi fraction embedded in Epon. Section staining with lead citrate and uranyl acetate. (*a*) Vesicles (*v*) containing tightly packed particles, 400–800 Å in diameter. × 36,000. (*b*) A higher magnification of particle-containing vesicle. × 66,000. (*c*) Golgi cisternae, one of which forms branching tubular extensions. × 36,000. (*d*) A fenestrated plate (*FP*) lacking particles, surrounded by small vesicles and tubular elements containing homogeneously electron-dense material. × 44,000. (*e*) Small vesicular and tubular elements and cisternal structures with areas containing highly electron-opaque material (arrows). × 45,000. (*f*) Tubular elements with club-shaped ends containing highly electron-opaque material. Note the difference between electron-opaque material in the tubular and cisternal structures (see also [*e*]) and the less dense particles in the secretory vesicles. × 40,000.

of VLDL. It should perhaps be noted in this context that the observed differences between the three TG pools as regards rate of glycerol incorporation and apparent half-lives may be masked to some extent by rapid exchange of triglycerides between the cytoplasm and membranes on the one hand, and between membranes and lipoprotein particles on the other. Such exchange reactions appear to occur freely between various lipoprotein species in the circulation (47).

The rapid appearance and disappearance (35) of labeled VLDL in the circulation is compatible with

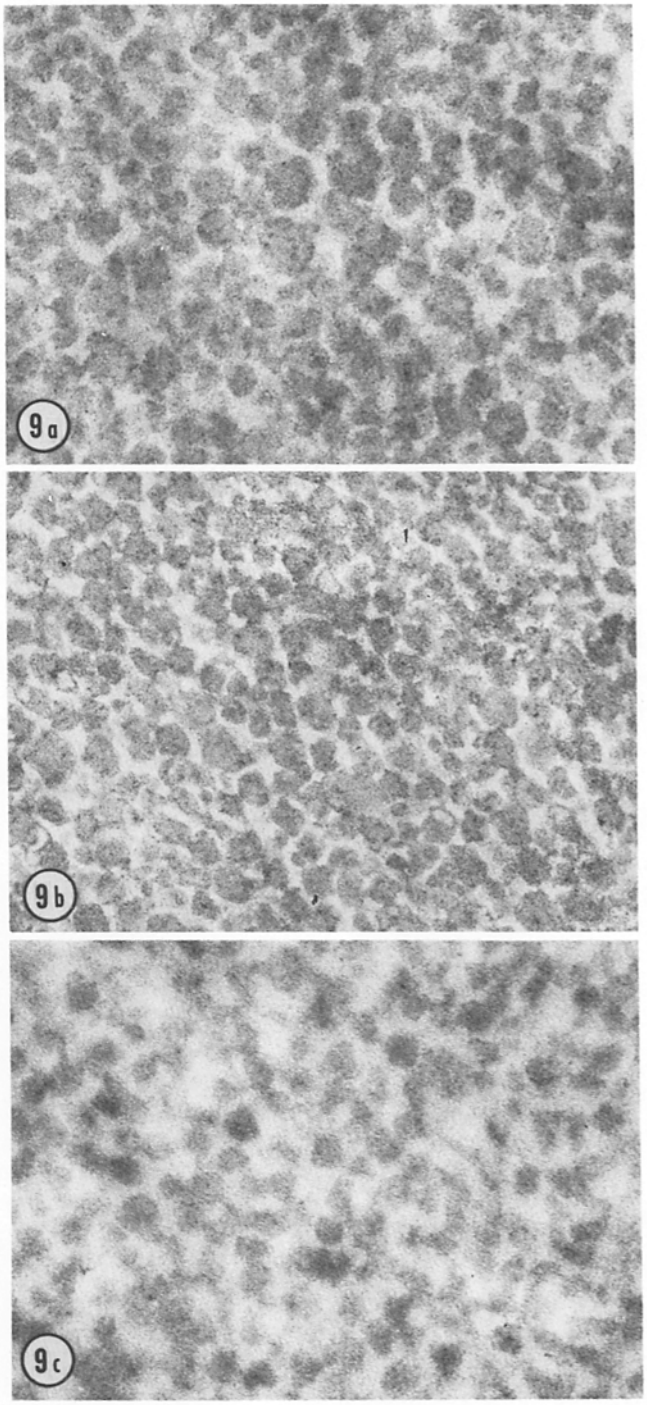

FIGURE 9 Electron microscope appearance of the lipoprotein particles from the rough (*a*) and smooth (*b*) microsomal preparation and from the Golgi-enriched fraction (*c*). The particles are homogeneous, of an irregularly spherical shape, and possibly surrounded by a very thin osmiophilic layer, but not a true membrane. × 150,000.

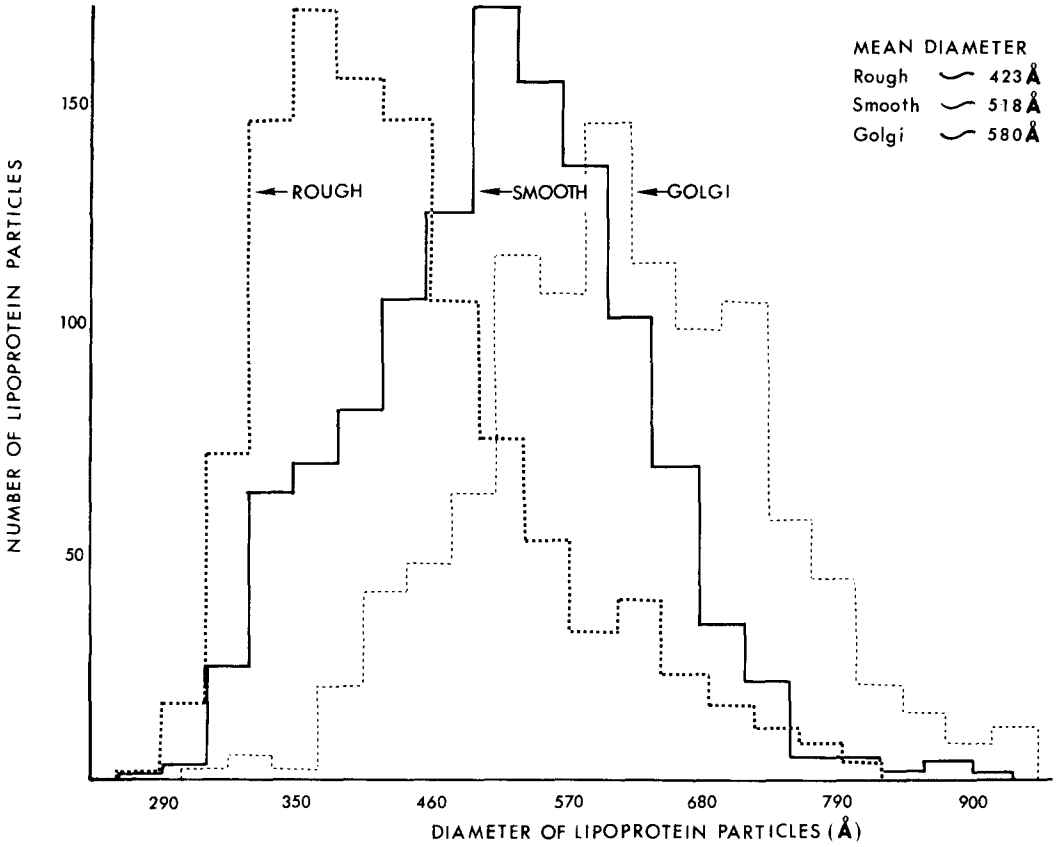

FIGURE 10 Size distribution of liporptoein particles isolated from rough and smooth microsomes and the Golgi apparatus. For all three subcellular fractions and for serum the lipoprotein particles were isolated at d 1.03 g/ml, as described in Materials and Methods. Although there is an overlap between the distribution curves, the mean diameter of the particles increases from rough microsomes (420 Å) to smooth microsomes (520 Å) and to the Golgi apparatus (580 Å). More than 1,000 particles were analyzed for each fraction. Corresponding values for serum from starved rats (18 h) were 200–760 A. Few particles greater than 800 Å (< 1%), interpreted as chylomicrons, were seen among isolated lipoprotein particles from the microsomal fractions, Golgi apparatus, or serum from starved rats.

the findings of a high rate of synthesis and fast intracellular migration for lipoprotein particles. Furthermore, the change in distribution with time between PLP and TG labeling (Table V) also indicates that the TG pool has a very quick turnover rate with a $t_{1/2}$ of less than 1 h both in membranes and in lipoprotein particles, whereas the membrane-associated PLP are more stable, with an estimated half-life in the range of several hours (48, 40). The high specific labeling (~10–20 times higher than that of PLP) and fast turnover of microsomal TG seem to indicate that this lipid is released from the ER in part to the cytoplasmic TG pool (Fig. 2) while the remainder is exported from the liver cell in the form of lipoproteins in less than 1 h.

Efforts to isolate lipoprotein particles from the microsomal fraction at $d < 1.006$ g/ml, which corresponds to the serum VLDL, were unsuccessful. When the density was increased to 1.03 g/ml, which is the lower limit for serum LDL (16) (very little if any rat serum LDL floats at $1.006 < d < 1.04$), substantial amounts were recovered from both rough and smooth microsomes. For the sake of uniformity the Golgi lipoprotein particles were also isolated at $d = 1.03$, although it is recognized that these particles can be isolated at $d = 1.006$ (5, 18, 19). The higher density of the microsomal lipoprotein granules appears to be due to their relatively high content of protein and low content of TG in comparison with both Golgi particles and serum VLDL. Some precaution is required in the

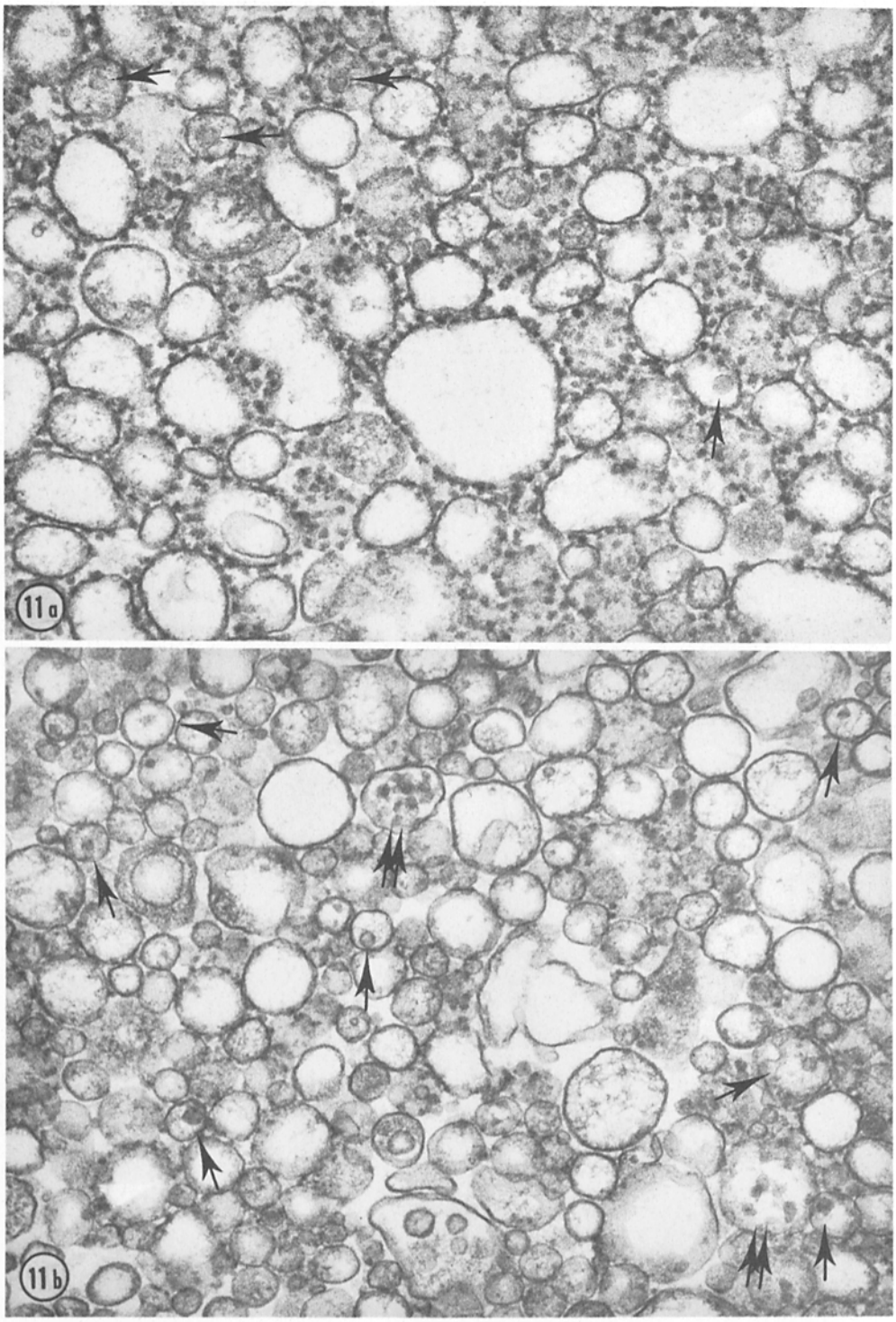

FIGURE 11 *a* In the rough microsomal fraction all vesicles have a large number of attached ribosomes. Most vesicles appear empty or filled with a finely granular material. Within some vesicles (arrows) 200–400 A particles are present. Glutaraldehyde-OsO_4; Epon; lead citrate and uranyl acetate. × 60,000.

FIGURE 11 *b* Smooth microsomal fraction showing only smooth-surfaced vesicles and very few free ribosomes. Many of the vesicles contain one (single arrow) or several (double arrows) spherical particles. Glutaraldehyde-OsO_4; lead citrate and uranyl acetate. × 45,000.

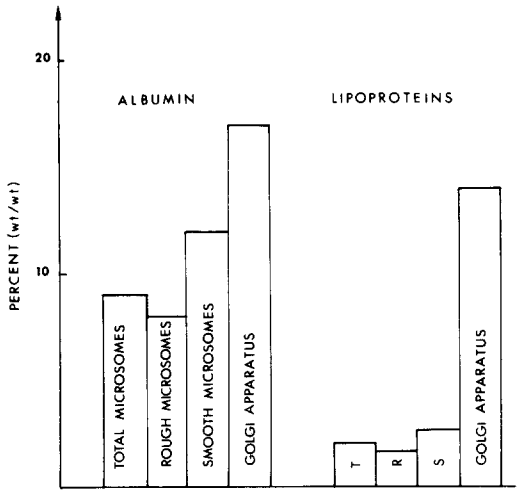

FIGURE 12 Albumin and lipoprotein content as percent (wt/vol) of "membranous" proteins in various subcellular organelles. "Membranous proteins" signifies Tris-washed, KCl-EDTA-washed, and sonicated fractions. Albumin was isolated as described earlier (14).

case of the absolute amounts of protein present in the lipoprotein particles, since protein was calculated with albumin as a standard. The most conspicuous difference between rough and smooth microsomal lipoprotein granules is an increase in relative content of PLP and TG in the smooth variety at the expense of the protein content. These findings add more direct evidence to the earlier hypothesis based on autoradiographic (2) and enzymic analysis (1) that both rough and smooth microsomes are active in the synthesis of TG and PLP for lipoproteins. The increase of TG content of Golgi particles as compared to the corresponding particles in smooth ER is somewhat surprising, since earlier findings (1) as well as the low rate of glycerol labeling into TG after short intervals demonstrated here, are not consistent with participation by Golgi membranes in TG synthesis. Two alternative explanations appear equally plausible: (*a*) the available methods for isolating the Golgi apparatus (11, 12, 49, 50) all give low yields (5–45%) and may therefore not be absolutely representative of the entire organelle, thus leaving open the possibility that some part of the Golgi complex actively synthesizes or adds TG to the lipoprotein macromolecule; (*b*) a reorganization or maturing of lipoprotein particles may occur within the Golgi complex to satisfy the demands of serum. Various apoproteins (HDL, LDL) known to be synthesized by liver ribosomes (8, 43) may travel together in the same lipoprotein package and are later separated into different entities in the Golgi complex or in the circulation. It is interesting to note, in this context, that Chapman et al. (18, 19) recently were able to isolate two types of lipoprotein particles: VLDL ($d < 1.007$) and LDL ($d = 1.007$–1.063). The same authors also suggested that LDL are precursors of VLDL into which they are transferred in the Golgi complex by addition of TG. However, most data at present seem to indicate that LDL are remnants from extrahepatic metabolism of VLDL (33, and see references 43 and 47 for a discussion), although this has not been conclusively demonstrated.

The results of this study are in agreement with the general morphological concepts of transport of VLDL in the liver cell. The sequence of TG labeling of lipoproteins between rough and smooth microsomes on one hand, and the Golgi apparatus on the other, suggests a precursor-product relationship and is compatible with a flow especially of TG and also of PLP from the ER to the Golgi apparatus, although it is recognized that in this study the *insertion* of TG and PLP into lipoproteins was followed rather than the complete *de novo* synthesis of lipoproteins. The biochemical and morphological data presented indicate that the rough ER participates as the first site of protein and lipid assembly and that the formation of lipoproteins presumed to be precursors of VLDL seems to occur in a multistep manner. In this way, TG and PLP (and most probably also cholesterol) are added to the nascent macromolecules or "forming particles" as they travel through the ER. Some modification may occur in the Golgi apparatus as regards the lipid-protein ratio. Concomitant with such a remodeling, the Golgi apparatus is also active in completing the lipoproteins by attachment of at least part of the carbohydrate moiety (9, 43). Parallel to the change in chemical composition and density, the morphological data demonstrated an increase in the size of the particles.

The observation that lipoprotein particles were also present in the rough ER is not in full agreement with the data of Claude (6), who concluded that lipoprotein granules could never be demonstrated within cisternae of the rough ER. It is not possible to determine at present whether or not this difference can be explained by the fact that Claude used rats subjected to partial hepatectomy, whereas we used normal rats, or is due to different fixation and staining procedures. Extraction of

lipids during electron microscope processing is a well-known phenomenon.

At first glance, there seems to be a discrepancy between the biochemical and morphological findings, since few particles believed to represent lipoprotein precursors were observed in the rough ER. However, it seems reasonable to assume that this may be due to the low lipid content (resulting in occurrence of relatively few unsaturated fatty acids) and smaller size in the rough ER, which make the particles less prone to be ultrastructurally demonstrable and perhaps more easily extractable during electron microscope processing.

In conclusion, the present data indicate that the formation of lipoprotein particles presumed to be precursors of VLDL takes place in a multistep manner. After conjugation, at least some of the VLDL is transported preferentially to peripheral portions of the Golgi complex, where some remodeling, concentration, and segregation take place. During the intracellular migration, a gradual increase in size of the lipoprotein particles appears to occur. As to the TG content of the hepatocyte, it seems to be present in three compartments: (*a*) in a cytoplasmic pool; (*b*) in a membrane-associated pool; and (*c*) in a pool corresponding to the TG moiety of lipoprotein particles.

The authors are grateful to Mrs. Helena Jansson, Mrs. Pia Theander, Mrs. Kerstin Brebäck, and Mrs. Britt-Marie Åkerman for skilfull technical assistance, and to Mrs. Brigitte Giertz and Mrs. Christina Sölscher for valuable help in typing the manuscript. Many thanks are also due to Dr. Barry Heatfield for most valuable help in correcting the English.

This work was supported by grants from the Swedish Medical Research Council.

Received for publication 17 May 1974, and in revised form 16 October 1974.

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