Characterization of lipoprotein particles isolated from the Golgi apparatus of rat liver

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ABSTRACT It has been proposed that particles within tubules and vesicles of the Golgi apparatus of liver cells are precursors of very low density lipoproteins in blood plasma. To characterize these particles we isolated a cell fraction rich in Golgi apparatus and associated particles from rat liver in quantities sufficient for analysis. Particles freed from the membranes of the Golgi apparatus and floated at d = 1.006 were studied by chemical analysis, immunodiffusion, and paper electrophoresis.

The lipid composition of the Golgi particles was similar to that of very low density lipoproteins from the same rats. The protein content was about 10% of dry weight for both the Golgi particles and plasma very low density lipoproteins. The Golgi particles formed lines of identity with plasma very low density lipoproteins during immunodiffusion against antiserum to plasma very low density lipoproteins. On paper electrophoresis, however, many Golgi particles remained near the origin, with only a few migrating to the pre-β position.

It was concluded that the lipoproteins in the Golgi apparatus are the precursors of plasma very low density lipoproteins.

SUPPLEMENTARY KEY WORDS chemical composition · immunochemistry · electrophoresis · plasma very low density lipoproteins · Golgi apparatus · liver

ELECTRON MICROSCOPIC studies have correlated the appearance of 300–1000 A particles within the hepatocyte Golgi apparatus with synthesis and secretion of plasma very low density lipoproteins (VLDL) (1–4). It has been shown that similarly located particles contain lipid (5–8). The development of a procedure for the isolation of the Golgi apparatus from plant cells (9) led to the isolation of this organelle from rat livers, making possible the study of the hepatic Golgi apparatus and its products of secretion. The isolated Golgi apparatus either from plant cells (10) or from rat liver (11, 12) is composed chiefly of flattened plates, anastomotic tubules, and large secretory vesicles. The vesicles and tubules of the hepatocyte Golgi apparatus contain 300–1000 A particles tentatively identified as VLDL.

This paper describes a method based on the procedure used by Morré and others (to be published)[1] for the isolation of a cell fraction from rat liver composed primarily of Golgi apparatus and lesser components of other cell organelles. The purity of this cell fraction from rat liver has been reported to be 80% or greater, based on morphologic and enzymatic analysis (13). The physical and chemical properties of the 300–1000 A particles associated with the Golgi apparatus are reported.

METHODS

Isolation of Golgi Apparatus

White male rats (Holtzman Co., Madison, Wis.), 175–250 g in weight, were fasted for 3 to 4 hr before use. 6–12 animals were used in each experiment. The rats were anesthetized with pentobarbital (6 mg/100 g of body wt) injected intraperitoneally and then exsanguinated: as much blood as possible was drawn from the terminal aorta into a syringe containing a solution of disodium EDTA (3 mg of Endrate Disodium [Abbott] per ml of blood). The blood obtained was used for the isolation of plasma VLDL.

In some experiments, blood was removed from livers by perfusion with 50 ml of 0.15 M sodium chloride. However, it was found that most of the blood could be drained from the liver if the exsanguination were followed by clamping the hepatic artery and portal vein and

Abbreviation: VLDL, very low density lipoproteins.

[1] D. J. Morré, R. L. Hamilton, R. W. Mahley, V. S. LeQuire, H. H. Mollenhauer, and W. P. Cunningham. Manuscript in preparation.

cutting the inferior vena cava below the diaphragm. The livers were removed, minced with scalpels, placed in 20 ml of chilled Tris–malate homogenizing medium (37.5 mM Tris–malate, pH 6.4; 0.5 M sucrose; 1% dextran; and 5 mM $MgCl_2$) per 10 g of liver, and homogenized for 30 sec with a Polytron homogenizer at setting No. 1 (model PT 10; Brinkmann Instruments, Inc., Westbury, N.Y.). The homogenate was filtered through Miracloth (Chicopee Mills, New York) and centrifuged in a Spinco centrifuge, model L2-65B, in the SW-25.1 or SW-27 rotor at 10°C in the following manner: 3000 rpm for 5 min, 7000 rpm for 10 min, and 25,000 rpm for 20 min in the same tube. The upper one-third of the stratified pellet was resuspended in 3 ml of supernatant solution and placed on top of a discontinuous sucrose gradient consisting of 5 ml of 1.6 M, 10 ml of 1.5 M, and 12 ml of 1.25 M Tris–malate-buffered sucrose. The resuspended pellet, representing about 20 g of homogenized liver, could be placed on one gradient. After centrifugation for 2 hr at 25,000 rpm, the Golgi apparatus cell fraction appeared as a white band and was removed from the upper 1.25 M sucrose interface with a Pasteur pipette. This fraction was diluted approximately 1:1 with 0.15 M sodium chloride and centrifuged at 25,000 rpm for 40 min in either rotor. The resulting pellet contained Golgi apparatus and associated Golgi particles. Little or no mitochondria or rough endoplasmic reticulum was present.

An alternative barbital-buffered homogenizing medium at pH 8.4 (37.5 mM sodium diethyl barbiturate; 7.3 mM diethyl barbituric acid; 0.5 M sucrose; 1% dextran; and 5 mM $MgCl_2$) was used to isolate the Golgi apparatus for immunodiffusion and electrophoresis. An unbuffered sucrose gradient was used for these same studies.

Electron Microscopic Techniques

To monitor the composition of the Golgi apparatus cell fraction, we fixed small portions from various regions of the pellet with 2% osmium tetroxide in Millonig's phosphate buffer (14) overnight at 4°C and processed them for study as described before (1). Thin sections were examined with a Hitachi HU 11B electron microscope.

Isolation of Golgi Particles

The pellets containing Golgi apparatus were resuspended in 2 ml of 0.15 M sodium chloride or barbital buffer (37.5 mM sodium diethyl barbiturate; 7.3 mM diethyl barbituric acid; pH 8.4) and were frozen, thawed, and sonicated to disrupt membranes. The fractions were frozen slowly (20–40 min) 4–6 times at −25°C in a Revco deep-freezer (Revco, Inc., Deerfield, Mich.) and thawed at room temperature. During alternate cycles of freezing and thawing, the thawed fractions were sonicated by continuously varying the intensity, during 30 sec, of a Bendix ultrasonic oscillator (Bendix Corp., Davenport, Iowa) at room temperature. The particles were separated from the Golgi membranes by centrifugation at d = 1.006 at 100,000 *g* for 16 hr in a 40.3 rotor. We monitored the purity of the Golgi particle fraction by negative staining as previously described (1).

Isolation of Plasma VLDL

The plasma VLDL from rats fasted for 3–4 hr were isolated by ultracentrifugation at 100,000 *g* for 16 hr. 4.5 ml of plasma was overlaid with 1 ml of 0.15 M sodium chloride, 1 mM with respect to disodium EDTA. After centrifugation, the floated VLDL were carefully removed with a Pasteur pipette in a volume of about 0.5 ml. The remainder, referred to as VLDL-freed plasma, was used in the studies to be discussed later. In certain studies of immunodiffusion and electrophoresis, plasma VLDL resuspended in the barbital-buffered homogenizing medium at pH 8.4 were subjected to homogenization, freezing, thawing, and sonication as used in the isolation of the Golgi apparatus and Golgi particles. In other studies, plasma VLDL were resuspended in Tris–malate-buffered homogenizing media at pH 6.4.

Analytical Methods

Total lipids were extracted from the lyophilized Golgi particles and plasma VLDL according to the method of Folch, Lees, and Sloane Stanley (15), dried under nitrogen, and redissolved in chloroform. The neutral lipids and free fatty acids were separated from the phospholipids on small silicic acid columns as described by Lombardi and Ugazio (16). The neutral lipid eluate was further separated by thin-layer chromatography on Silica Gel G (20 × 20 cm plates; Brinkmann Instruments, Inc., Westbury, N.Y.) into triglycerides, free fatty acids, free cholesterol, and cholesteryl esters with petroleum ether–ethyl ether–acetic acid 80:20:1. The lipids and standards were identified by exposure of the thin-layer plates to iodine vapor. Individual lipid classes were scraped from the plate into Kontes glass recovery tubes and eluted from the gel with chloroform–methanol 2:1 followed by ethanol–acetone 1:1. The eluates were dried under nitrogen and the residues were dissolved in appropriate solvents for chemical determinations. Standards were carried through the same procedures as the unknowns.

Free cholesterol and cholesteryl esters were determined colorimetrically by the method of Searcy and Bergquist (17). Triglycerides were determined by hydrolysis and titrimetry of the fatty acids by the method of Albrink (18), free fatty acids by the method of Dole and Meinertz (19), and lipid phosphorus by wet digestion in sulfuric acid (20) followed by the colorimetric procedure of Fiske

and SubbaRow (21) with a factor of 25 to convert lipid phosphorus to phospholipid. Protein was determined by the method of Lowry, Rosebrough, Farr, and Randall with bovine serum albumin as standard (22).

Immunochemical Procedures

Antiserum was prepared by intravenous injection of rat VLDL into New Zealand white rabbits. Shear's polysaccharide (10 μg/kg of body wt) was injected intravenously as an adjuvant (23) immediately after the injection of the lipoprotein fraction (about 2 mg of protein/injection). Three injections of rat VLDL were given about 3 wk apart, and about 30 ml of blood was collected 10 days after the last injection. The antiserum was stored at 5°C in sealed tubes.

Gel immunodiffusion was performed at room temperature on Ouchterlony plates (24) of 1% agarose prepared by Mann Research Laboratories, Inc. (pattern A), New York.

Electrophoretic Procedure

Paper electrophoresis of the plasma lipoproteins and Golgi particles was performed by the method of Lees and Hatch (25). The lipoproteins were made visible by staining the strips with purified Fat Red 7B (26) for 3 hr followed by decolorizing the background in an aqueous solution of 0.5% sodium hypochlorite and 2% acetic acid. In one electrophoretic study, Golgi particles were incubated with VLDL-freed plasma at 37°C for 3, 6, or 18 hr in a shaking water bath. Golgi particles were reisolated by centrifugation as described and subjected to electrophoresis.

RESULTS

Electron Microscopy of the Isolated Golgi Apparatus

Fig. 1 shows a section of the pellet of Golgi apparatus cell fraction obtained from rat liver. Numerous Golgi apparatus were recognized by the parallel cisternae and associated small vesicular profiles. Larger secretory vesicles containing particles tentatively identified as VLDL are prominent in this cell fraction. This was the typical appearance of the cell fraction isolated in the Tris–malate homogenizing medium at pH 6.4. When barbital was substituted for Tris–malate in the homogenizing medium, we obtained a cell fraction, at the same location on the gradient, which had a similar appearance. However, morphologically the parallel cisternae were not easily identified and appeared dilated, but vesicles containing particles were abundant. A particle fraction similar in appearance was obtained in both cases from the isolated Golgi fraction, and it was assumed that the fractions isolated in exactly the same manner except with different buffers were comparable.

Chemical Composition

Table 1 presents data on the chemical composition of the Golgi particles and plasma VLDL from rats fasted for 3.5 hr. The protein component of both the Golgi particles and plasma VLDL was approximately 10% of dry wt. Triglycerides comprised 59.8% of the total lipid of the Golgi particles and 56.2% for the plasma VLDL. The phospholipid content was about 25% in both cases. However, the percentages of cholesteryl esters and free cholesterol of the Golgi particles were significantly lower than in the plasma VLDL. The lipid composition of serum VLDL obtained from rats fasted for 16 hr and VLDL floated at d = 1.019 (16) is shown for comparison.

Immunochemistry

Precipitin lines between the Golgi particles and anti-rat plasma VLDL merged with those between plasma VLDL and the same antiserum (Fig. 2). Only particles obtained from the cell fraction that had been isolated in barbital buffer at pH 8.4 diffused adequately in the agarose media and established lines of identity. The presence of Tris-buffered homogenizing medium at pH 6.4 did not interfere with the diffusion or immunochemical reactivity of plasma VLDL but does appear to affect the reactivity of the Golgi particles.

To determine if the immunochemically reactive particles were within the tubules and vesicles of the isolated Golgi apparatus, we resuspended the Golgi apparatus-rich pellets (whose membranes had not yet been disrupted) from four rat livers in 2 ml of 0.15 M saline. One-half of this resuspended fraction was frozen, thawed, and sonicated to fragment the membrane as described under Methods. The other half was not subjected to this treatment. An equal portion of each was placed on an Ouchterlony immunodiffusion plate. A precipitin line was formed with the anti-rat plasma VLDL and the frozen, thawed, and sonicated Golgi fraction but not with the untreated fraction; this suggested that the particles are inaccessible to antisera in the intact Golgi apparatus and must first be released from the membrane compartment. Also this indicates that the immunochemical results are not due to plasma lipoproteins remaining in the livers from which the Golgi apparatus had been isolated.

Electrophoretic Mobility

Golgi particles did not migrate to the pre-β position in paper electrophoresis as expected. Only particles obtained from the cell fraction isolated in barbital buffer at pH 8.4 migrated at all on paper; many of these remained near the origin or trailed behind the pre-β position (Fig. 3). Golgi particles isolated in Tris buffer at pH 6.4 showed only a sharp lipid-staining band at the point of application. Incubation of Golgi particles in plasma (previously freed of VLDL) at 37°C for 3, 6, or 18 hr did

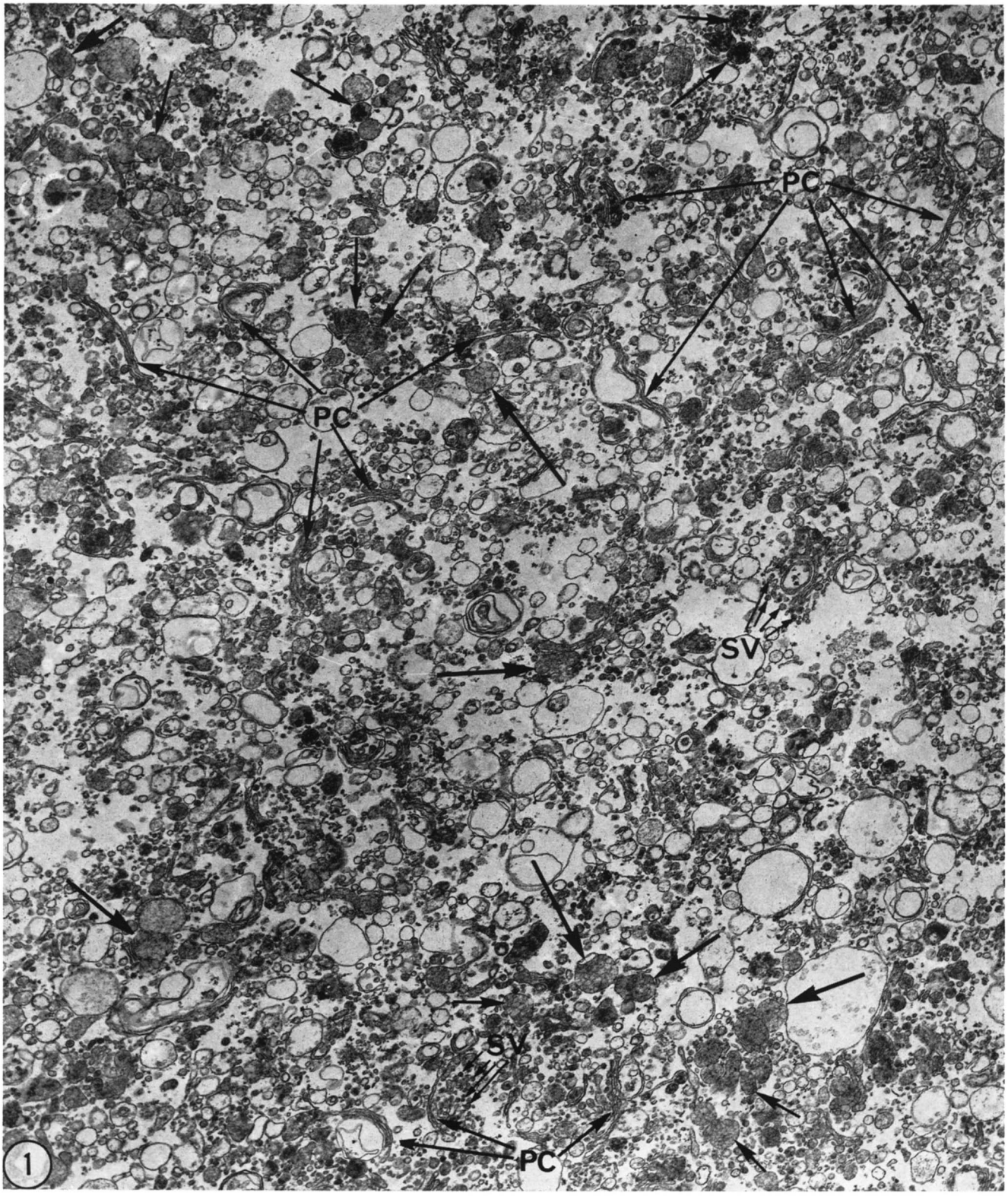

FIG. 1. Electron micrograph of the pellet of the Golgi apparatus cell fraction. Numerous Golgi apparatus can be recognized by the parallel cisternae (*PC*) and associated small vesicles (*SV*). Larger vesicles containing particles tentatively identified as VLDL are indicated by arrows. It can be seen that the predominant organelle of this cell fraction is the Golgi apparatus. ×20,000.

TABLE 1 CHEMICAL COMPOSITION OF GOLGI PARTICLES AND VERY LOW DENSITY LIPOPROTEINS FROM THE RAT

	Triglyceride	Phospholipid	Cholesteryl Ester	Free Cholesterol	Free Fatty Acids	Protein
			mg/100 mg lipid			*mg/100 mg dry wt*
Golgi particles* (d < 1.006)						
Mean	59.8	24.9	8.3	3.7	3.4	10.8 (rat 1)
±SEM	±2.0	±0.9	±0.7	±0.6	±0.5	8.9 (rat 2)
						9.3 (rat 3)
Plasma VLDL† (3.5-hr fasted; d < 1.006)						
Mean	56.2	24.9	12.7	6.2	0.7	10.8 (rat 1)
±SEM	±2.3	±2.4	±2.9	±0.5	±0.1	9.4 (rat 2)
						11.2 (rat 3)
Serum VLDL‡ (16-hr fasted; d < 1.019)	44.6	23.1	24.7	6.4	1.1	

* Results obtained from six experiments.
† Results obtained from four of the six groups of rats from which the Golgi particles were obtained for the lipid analysis reported here.
‡ Calculated from Lombardi and Ugazio (16).

not alter their mobility. Concentration of Golgi particles obtained from the cell fraction isolated in barbital buffer by dry dialysis with Sephadex G-75 further reduced their migration. By negative-staining electron microscopy, these concentrated particles appeared to have aggregated. In contrast, most VLDL obtained from plasma did migrate to the pre-β position although they exhibited some trailing and diffusion behind the origin (Fig. 3). After plasma VLDL had been mixed with the barbital-buffered homogenizing medium, homogenized, frozen, thawed, and sonicated, they continued to migrate to the pre-β position. Golgi particles appeared, therefore, to be more unstable than plasma VLDL.

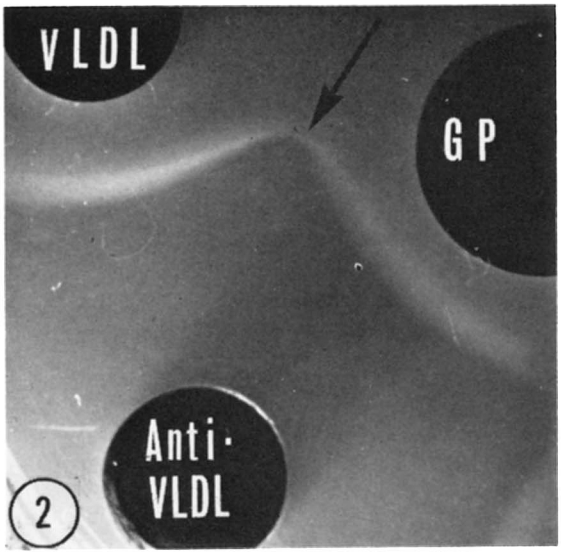

FIG. 2. Precipitin lines were formed when particles associated with the Golgi apparatus (*GP*) and VLDL were tested with antiserum to rat plasma VLDL (*Anti-VLDL*) by immunodiffusion in agarose. An arrow indicates the point of deviation and fusion of the precipitin lines indicating identity of the Golgi particles with the *VLDL*.

DISCUSSION

Electron microscopic studies of livers of rats perfused with free fatty acids (FFA) led to the hypothesis that particles of 300–1000 A diameter in the Golgi apparatus of liver cells are precursors of plasma VLDL (1, 27). Similar in vivo studies of cortisone-treated rabbits (2), norepinephrine-infused dogs (3), and ethanol-treated rats (28) gave further support to this conclusion. Previously, we have shown that the tubules and large vesicles of the isolated Golgi apparatus contain particles morphologically identical with plasma VLDL (11, 12). Stein and Stein showed by radioautography of rat liver that similarly located particles contained lipid and became radiolabeled 10 min after the injection of radioactive precursors of VLDL lipids (8). Of interest in this regard was the work of Schlunk and Lombardi (29), who showed that "liposomes," isolated from the endoplasmic reticulum of rat liver after ethionine poisoning, were triglyceride-rich lipoprotein particles. The relationship between ethionine-produced "liposomes" and Golgi particles, however, has not been shown.

The present report describes a technique for isolating lipoproteins from the Golgi apparatus of normal rat livers in amounts sufficient for physical and chemical examination. This method is a modification of the procedure for isolating Golgi apparatus from liver by Morré (to be published).[1] Larger rotors (Beckman SW-25.1 and SW-27) were used to accommodate homogenates from up to 12 rat livers (125–150 g wet wt) and the centrifugal force used to sediment the Golgi apparatus fraction was increased.

Golgi particles were released from the Golgi apparatus cell fraction after fragmentation of the membranes by freezing, thawing, and sonication. It was found that the Golgi particles freed from the membranes floated at

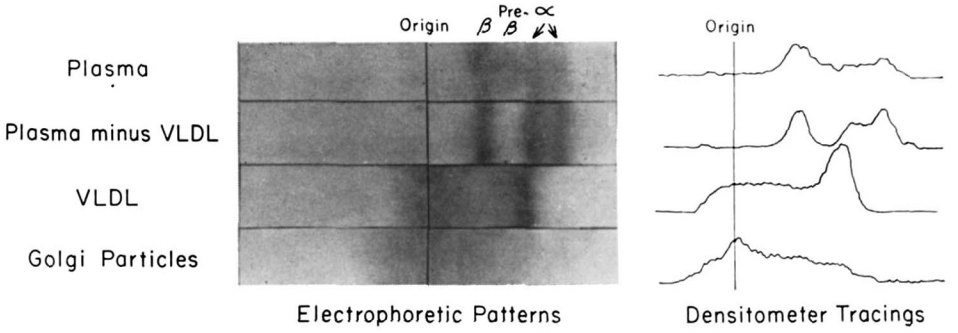

FIG. 3. Electrophoretic patterns and corresponding densitometric tracings showing the various lipoprotein classes in rat plasma (β, pre-β, and two α-bands) and the "pre-β" migration of VLDL isolated from plasma. Some particles isolated from the Golgi apparatus cell fraction migrated from the origin to the pre-β position but the bulk trailed and diffused behind the origin. Note that plasma VLDL also exhibited some trailing and diffusion but that most form a pre-β band.

d = 1.006 in the preparative ultracentrifuge and were 300–1000 A in diameter in negatively stained preparations (12, 30). Other investigators have been unable to detect plasma lipoproteins in rat liver homogenates (31). To isolate plasma lipoproteins or their precursors from cells, it is probably necessary to first isolate the organelle containing the lipoproteins from the other cell organelles and cytoplasm in order to protect them from the lytic enzymes of the cell, and then to release them from their cytomembrane compartments. Possibly the homogenizing media, the homogenization procedure, and the choice of pH and buffer system could all be critical in obtaining reactive particles. Our results show that the particles are not accessible for immunoprecipitin reactions until the membranes of the Golgi apparatus have been fragmented.

We considered the possibility that some of the reactive particles were lipoproteins from residual plasma within the livers from which the Golgi apparatus had been isolated. In the first place, the procedure for exsanguinating the rats and draining the livers of blood was very effective. Secondly, during the procedure for the isolation of the Golgi apparatus, the organelle was sedimented at 100,000 *g*. At this centrifugal force, VLDL would float and could be sedimented only if adsorbed or occluded by the pellet. Also we have shown that the Golgi apparatus-rich cell fraction did not react with anti-VLDL and hence did not contain plasma VLDL; only after the fragmentation of the Golgi membranes could immunochemically reactive particles be demonstrated.

The chemical composition of the floated Golgi particles was strikingly similar to that of the plasma VLDL obtained from normal rats (Table 1). The contents of protein and phospholipid were virtually identical; the content of triglyceride was slightly greater in the Golgi particles than in rat plasma VLDL obtained from the same rats 3.5 hr after fasting; 16 hr after fasting the serum VLDL are, according to Lombardi and Ugazio (16), still lower in triglyceride. This is consistent with the concept that VLDL transport triglyceride from liver to extrahepatic tissues. Our results showed that cholesteryl esters were already present in particles isolated from liver. This agrees with the work of Gidez, Roheim, and Eder, who showed (32) that cholesteryl esters of plasma VLDL are derived largely from newly synthesized cholesterol in liver, and is consistent with the role attributed to the particulate fraction of rat liver in the esterification of free cholesterol (33). Our results, however, differ from those of Heimberg, Van Harken, and Brown, who were unable to detect cholesteryl esters in d < 1.02 lipoproteins released from perfused rat livers (34).

Several factors could account for the small differences in chemical composition between Golgi particles and plasma VLDL. First, the triglyceride content of the particles was somewhat higher than that of the plasma VLDL, and the FFA content considerably higher. This might indicate that the triglyceride concentration of the particles in vivo is actually still higher than that found and that hepatic lipase might have acted on the particles during isolation and given rise to the FFA. Second, the percentages of free and esterified cholesterol were both lower in the particles than in the plasma VLDL. However, the free:esterified cholesterol ratios are almost identical. The cholesterol content of the particles could be altered by transfer and exchange reactions (35–39) once the particles reach the plasma. A third possibility is that the particles isolated from the tubules and vesicles of the Golgi apparatus fraction are heterogeneous in the sense that they may be in various stages of assembly.

The immunochemical data give strong support to the hypothesis that the Golgi particles are indeed precursors of plasma VLDL. The Golgi particles and VLDL from plasma formed lines of identity when tested against VLDL antiserum. The peptides of rat plasma VLDL have not been identified, but Camejo reported that the bulk of rat VLDL proteins were indistinguishable from the protein of rat high density lipoprotein (40).

VLDL from both rat (31) and human (24, 41) plasma migrate to the pre-β position on paper electrophoresis. In the present study, VLDL from rat plasma migrated as pre-β lipoproteins. Some of the particles isolated from the Golgi apparatus migrated to the pre-β position, but the majority trailed from the point of application. It appeared from other evidence that the Golgi particles were unstable and more susceptible to coalescence or denaturation than plasma VLDL, but the reasons for this instability are not known.

The data presented in this study show that particles isolated from tubules and secretory vesicles of rat hepatocyte Golgi apparatus are VLDL and are, in all likelihood, the precursors of plasma VLDL. It has now been shown that the Golgi particles share similar morphologic, flotational, chemical, and immunochemical properties with plasma VLDL. The difference observed could be attributed to subsequent alterations in the physical and chemical properties of the Golgi particles after secretion from the liver and circulation in the plasma compartment.

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