# SUBCELLULAR LOCALIZATION OF B APOPROTEIN OF

# PLASMA LIPOPROTEINS IN RAT LIVER

CHERYL A. ALEXANDER, ROBERT L. HAMILTON, and
RICHARD J. HAVEL

From the Cardiovascular Research Institute and the Departments of Anatomy and Medicine,
University of California, San Francisco, San Francisco, California 94143. Dr. Alexander's present
address is the Department of Educational Services, the University of Tennessee, Center for the Health
Sciences, Memphis, Tennessee 38163.

ABSTRACT

Multispecific antigen-binding fragments (Fab) from rabbit antisera against rat very
low density lipoproteins (VLDL) and Fab against rat low density lipoproteins that
were monospecific for the B apoprotein were conjugated to horseradish peroxidase.
Conjugates were incubated with 6-$\mu$m frozen sections from fresh and perfusion-
fixed livers and with tissue chopper sections (40 $\mu$m thick) from perfusion-fixed
livers. In the light microscope, specific reaction product was present in all
hepatocytes of experimental sections as intense brown to black spots whose
locations corresponded to the distribution of the Golgi apparatus: along the bile
canaliculi, near the nuclei, and between the nuclei and bile canaliculi. Perfusion
fixation with formaldehyde produced satisfactory ultrastructural preservation with
retention of lipoprotein antigenic determinants. In the electron microscope,
patches of cisternae and ribosomes of the rough endoplasmic reticulum (ER) and
particularly its smooth-surfaced ends, vesicles located between the rough ER and
the Golgi apparatus, the Golgi apparatus and its secretory vesicles and VLDL
particles in the space of Disse all bore reaction product. The tubules and vesicles of
typical hepatocyte smooth ER did not contain reaction product, nor did the
osmiophilic particles contained therein. The localization obtained in this study
together with other evidence suggests a sequence for the biosynthesis of VLDL that
differs in some respects from that proposed by others: (a) the triglyceride-rich
particle originates in the smooth ER where triglycerides are synthesized; (b) at the
junction of the smooth and rough ER the particle receives apoproteins synthesized
in the rough ER; (c) specialized tubules transport the particle, now a nascent
lipoprotein, to the Golgi apparatus where concentration occurs in secretory
vesicles; (d) secretory vesicles move to the sinusoidal surface where the particles are
secreted into the space of Disse by fusion of the vesicular membrane with the
plasma membrane of the hepatocyte.

Very low density lipoproteins (VLDL)[1] of verte-
brate blood plasma are triglyceride-rich particles

250–800 Å in diameter. Primarily, they transport
triglycerides from liver to those tissues that store
their constituent fatty acids or utilize them as fuel

[1] Abbreviations used in this paper: DAB, 3,3'-
diaminobenzidine tetrahydrochloride; EDTA, disodium
(ethylenedinitrilo)-tetraacetic acid; ER, endoplasmic re-
ticulum; Fab, fragment antigen binding; Fc, fragment

crystallizable; LDL, low density lipoproteins; HDL, high
density lipoproteins; VLDL, very low density lipo-
proteins.

(24). The oily core of these particles is covered by a coating of several apoproteins, phospholipids, and cholesterol. This shielding layer permits the hydrophobic triglycerides to be transported to recipient tissues (10). Since phospholipids alone can stabilize microemulsions of fat, it is generally assumed that the apoproteins of VLDL serve additional functions. One of these apoproteins activates the enzyme lipoprotein lipase which hydrolyzes VLDL triglycerides at the walls of capillaries of recipient tissues (26). It has been suggested that another VLDL apoprotein, the B apoprotein, is required for secretion of triglyceride-rich lipoproteins from liver and intestinal mucosa because plasma of patients with a genetic deficiency of this protein also lacks those lipoprotein species that normally contain the B protein (VLDL, chylomicrons, and LDL) (19).

Electron microscope studies have indicated that particles resembling VLDL that are frequently seen in the smooth endoplasmic reticulum, Golgi apparatus, and space of Disse of liver represent nascent VLDL (23, 27, 48). Nascent VLDL separated from the Golgi apparatus isolated from rat liver (33) contain B apoprotein and other plasma VLDL apoproteins although they are comparatively deficient in the C apoproteins (22). Technical limitations have prevented the use of cell fractionation to characterize the particles contained in the smooth endoplasmic reticulum of normal rat liver. Although the endoplasmic reticulum seems to be specialized for lipid synthesis (20, 47) and the rough endoplasmic reticulum in particular synthesizes VLDL apoproteins (6, 34), the initial site of particle formation and the site where lipid is bound to protein are uncertain (9, 22, 27).

Since the techniques utilized to date have not answered these questions, we have used a new method to study the problem. Antisera were prepared against plasma VLDL and, therefore, contained antibodies against several VLDL apoproteins. Antisera were also prepared against LDL, which contains mainly the B apoprotein. Consequently, this antiserum was directed primarily against the B apoprotein. Fab from both antisera were conjugated to peroxidase and used for immunocytochemical localization by light and electron microscopy. The findings reported here represent the first subcellular localization of plasma lipoprotein apoproteins by this technique (1).

## MATERIALS AND METHODS

### Separation of Lipoproteins

Lipoproteins were separated from the plasma of male Long-Evans rats (300-400 g) by sequential preparative ultracentrifugation (25) for $1.0-1.2 \times 10^8$ g min average at 4°C in a Beckman ultracentrifuge with the 40.3 rotor (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.). The density ranges 1.025-1.045 g/cm$^3$ and 1.075-1.175 g/cm$^3$ were chosen for LDL and HDL, respectively, to maximize electrophoretic and antigenic purity (30). VLDL ($d < 1.006$ g/cm$^3$) and HDL were recentrifuged twice and LDL once to free them of contaminating serum proteins. Lipoproteins were dialyzed for at least 24 h against 0.15 M sodium chloride containing 0.04% disodium EDTA.

### Preparation of Antisera

Antisera were produced in adult male New Zealand white rabbits weighing 3-4 kg. Lipoprotein protein was measured by a modification (45) of the method of Lowry et al. (32). Lipoprotein solutions containing 0.1-1 mg of protein for LDL and 1.5-2.7 mg for VLDL were emulsified with an equal volume or 1 ml maximum of Freund's complete adjuvant (Difco Laboratories, Inc., Detroit, Mich.) and injected subcutaneously at the back of the neck. At least two injections were given and antiserum was obtained from blood collected 7-10 days after the last injection.

### Preparation of Labeled Antibodies

IgG was precipitated from serum at 4°C with a saturated solution of ammonium sulfate pH 6.5 (51) and purified on diethylaminoethyl cellulose (Whatman DE 52, W. and R. Balston Ltd., Springfield Mill, Kent, England) by a batch method (57). After dialysis at 4°C against 0.1 M phosphate buffer, pH 7.0, the purified IgG was digested with papain (40). For every 10 ml of protein solution, 0.25 ml of 0.1 M EDTA was added at room temperature with stirring, followed by 1.16 ml of 0.1 M solution of cysteine in 0.1 M phosphate buffer, pH 7.0. 0.5 mg of a 34 mg/ml suspension of papain (PAP 2LX, Worthington Biochemical Corp., Freehold, N. J.) in 0.05 M sodium acetate, pH 4.5 was added per 100 mg of IgG. The mixture was incubated at 37°C for 4-9 h and then dialyzed for at least 16 h against 0.01 M acetate buffer, pH 5.5. Ion exchange chromatography of the papain digest was carried out on a 2.5 × 25 cm column of carboxymethyl cellulose (Cellex CM, Bio-Rad Laboratories, Richmond, Calif., precycled according to the manufacturer's instructions). Fractions were eluted with a stepwise gradient of acetate buffers of increasing ionic strength (7). The first fraction containing the bulk of the Fab was used.

Horseradish peroxidase (Worthington) was conju-

gated to Fab by the two-step method of Avrameas and Ternynck (2). Conjugates were separated from unreacted peroxidase on a column (110 × 2 cm) of Sephadex G-200 (Pharmacia Fine Chemicals, Piscataway, N. J.). Fractions in the high molecular weight shoulder were pooled and concentrated by diafiltration on a UM 10 membrane (Amicon Corp., Lexington, Mass.). All conjugates were frozen in a dry ice-acetone bath and stored at −20°C. They usually contained 1–3 mg of protein per ml.

## Immunological Techniques

Immunodiffusion and immunoelectrophoresis were carried out by the methods of Ouchterlony (38). A 1% gel medium was prepared by dissolving agarose powder (Bio-Rad Laboratories) in 0.05 M barbital buffer, pH 8.6 (37). The design for immunodiffusion was cut into the solidified agarose with a die producing a large central well of 50 μl capacity surrounded by six smaller wells of 20 μl capacity. For immunoelectrophoresis, the pattern consisted of five troughs of 250 μl capacity and six wells of 20 μl capacity. Electrophoresis was maintained for 1 h at 40 mA total current. Both types of plates were developed for 48 h at room temperature before they were photographed.

## Light Microscopy

To test both the antigen-binding and enzymatic activities of the conjugates, frozen sections 6 μm thick were cut from livers of male Long-Evans rats (250–325 g) and mounted on glass slides subbed with chrome alum-gelatin (39). They were fixed for 10 min in cold (4°C) 100% acetone, rinsed in 0.135 M phosphate buffer, pH 7.4, and incubated under a 20-μl drop of conjugate in a moist chamber for 60 min at room temperature. The sections were then rinsed in buffer for 15 min and incubated in a solution of peroxidase substrate for 30 min at room temperature. This solution consisted of 0.5 mg of 3,3′-diaminobenzidine tetrahydrochloride (Sigma Chemical Co., St. Louis, Mo.) per ml of 0.05 M Tris-HCl buffer (Sigma), pH 7.5–7.6, plus 0.01 ml of 1% hydrogen peroxide (J. T. Baker Chemical Co., Phillipsburg, N. J.) per ml of solution. The sections were then rinsed in 0.135 M phosphate buffer, pH 7.4, and fixed for 10–15 min with a few drops of 3% osmium tetroxide (Goldsmith Division, NL Industries, Inc., Chicago, Ill.) in the same buffer containing 0.25 M sucrose. They were dehydrated in a graded series of acetone solutions, cleared with xylene, and mounted with Permount (Fisher Scientific Co., New York). A Zeiss photo microscope was used for photography.

## Electron Microscopy

Long-Evans rats (200–300 g) were fasted overnight and anesthetized with sodium methohexital (Eli Lilly and Co., Indianapolis, Ind.) by intraperitoneal injection of 0.3 ml/100 g body weight. The portal vein was cannulated with a 16-gauge teflon catheter with a removable needle (Angiocath Intravenous Placement Unit, Deseret Pharmaceutical Co., Sandy, Utah). The catheter was tied in place and connected to a Holter peristaltic pump (Series 1100, Extracorporeal Medical Specialities, Inc., King of Prussia, Pa.) operating at 12–15 ml/min. Simultaneously, the abdominal vena cava was cut below the liver. Blood was flushed from the liver with 10–15 ml of buffer before perfusion for 5–10 min with 1% or 4% formaldehyde freshly prepared from paraformaldehyde (Matheson, Coleman and Bell, Manufacturing Chemists, Los Angeles, Calif.) in 0.135 M phosphate buffer, pH 7.4). The liver was then flushed for 3–10 min with the same buffer to remove excess fixative. Slices were removed from the right lateral lobe for further processing. Cubes of tissue were frozen in freon or liquid nitrogen and 6-μm frozen sections were cut and processed as described above for light microscopy. These served to evaluate retention of antigenic determinants in the tissue after a given fixation procedure. Some 0.5-mm cubes of tissue were postfixed for 1–2 h in 2% glutaraldehyde (Ladd Research Industries Inc., Burlington, Vt.), fixed again overnight in 3% osmium tetroxide, dehydrated, and embedded for electron microscopy. These unincubated controls served to define the best preservation of ultra-structure obtainable for a given liver with the described methods of fixation.

For electron microscope localization of apolipo-proteins, sections 40 μm thick were cut from the liver slices on a Smith-Farquhar tissue chopper (TC-2, Ivan Sorvall Inc., Norwalk, Conn.). The sections were rinsed in 0.135 M phosphate buffer and incubated for 18–24 h at 8°C with gentle shaking in Fab conjugates made from either anti-LDL or anti-VLDL. Tissue chopper sections were also incubated in identical fashion either in a conjugate made from the Fab of an unimmunized rabbit (normal conjugate controls) or in a 5 mg/ml solution of peroxidase in 0.135 M phosphate buffer (peroxidase controls). After incubation, the sections were rinsed in cold 0.135 M phosphate buffer for 1 h with six changes. They were then postfixed for 1 h in 2% glutaraldehyde in the same buffer and rinsed for 1 h with six changes of this buffer. This was followed by preincubation for 15 min in a 0.5 mg/ml solution of DAB in 0.05 M Tris buffer, pH 7.5–7.6, containing 0.25 M sucrose (55) but without hydrogen peroxide (35). Finally, the sections were incubated for 15 min in fresh DAB solution of identical composition but containing 0.01% hydrogen peroxide. To minimize diffusion of reaction product (31) all buffers used beyond this step contained 0.25 M sucrose. The sections were rinsed in 0.05 M Tris buffer and in 0.135 M phosphate buffer, then fixed overnight in 3% osmium tetroxide in 0.135 M phosphate buffer. They were dehydrated in acetone and infiltrated with Epon 812 (Miller and Stephenson Chemical Co., Danbury, Conn.).
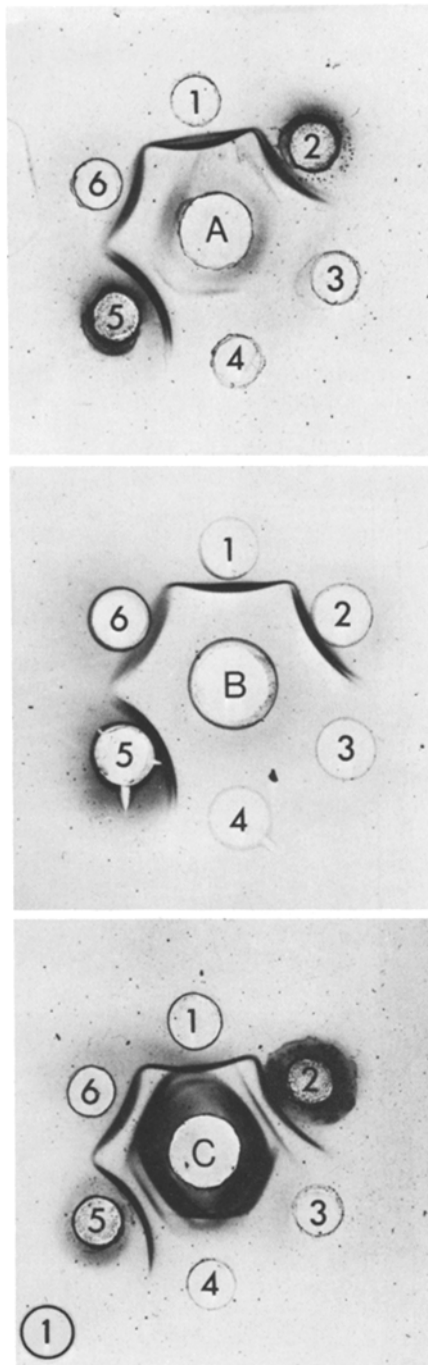
Each section was then laid flat inside the lid of an inverted BEEM capsule (Better Equipment for Electron Microscopy, Bronx, N. Y.) from which the tip had been removed with a razor blade. The capsules were filled with Epon and polymerized at 60°C for 2 days.

Sections of 600-800 Å thickness were cut with diamond knives on a Porter-Blum MT2-B microtome (Ivan Sorvall, Inc., Norwalk, Conn.). Except for unincubated controls which were handled in the same manner as tissue for routine microscopy, sections were always cut as close to the surface of the slice as possible. As sectioning proceeded from the surface to the interior, preservation of ultrastructure improved but the incidence of reaction product decreased. Each ribbon was viewed in the electron microscope (Siemens Elmiskop 101) and photographs were taken in a region having both acceptable preservation of ultrastructure and specific reaction product. Sections were always photographed before staining with lead citrate so that very small deposits of reaction product could be detected (31). This also prevented the spurious identification of lead stain precipitate as reaction product. After this initial photography, the sections were stained for 5 min in lead citrate (43) to improve contrast, and photographed again.

## RESULTS

### Specificity of Antisera

More than 85% of the apoprotein of LDL used in this research ($d = 1.025-1.045$) was insoluble in tetramethylurea (28) and only trace amounts entered the running gel upon electrophoresis in 7.5% polyacrylamide containing 8 M urea (4, 28). On double immunodiffusion, antisera against rat LDL formed one major arc with VLDL, LDL, and whole rat serum (Fig. 1). In each case, the major arcs formed with these three antigens show a reaction of identity. The identity of this as the B protein was established by its characteristic position on both immunodiffusion and immunoelectrophoresis (11). Antiserum against rat VLDL produced the B arc as well as three other arcs with VLDL and two additional arcs with HDL. Two of these arcs represent VLDL proteins which are also present in HDL. One of the latter is also found in the fraction of density greater than 1.21 g/cm³.

### Light Microscope Localization

In the light microscope, brown to black reaction product was present as discrete spots in all

FIGURE 1 Top: immunodiffusion pattern obtained with antiserum A (center well) against LDL (well 1). The major precipitin arc formed by LDL and its antiserum, representing the B apoprotein, is also present in VLDL (wells 2, 5) but is absent from HDL (well 3) and lipoprotein-free serum (well 4). Whole rat serum (well 6) was tested against each antiserum. Middle: immunodif- fusion pattern obtained with antiserum B against LDL. Trace contaminants, present in antiserum A, are absent. Bottom: immunodiffusion pattern obtained with anti- serum C against VLDL. In addition to the one represent- ing the B apoprotein, several other ones are present.
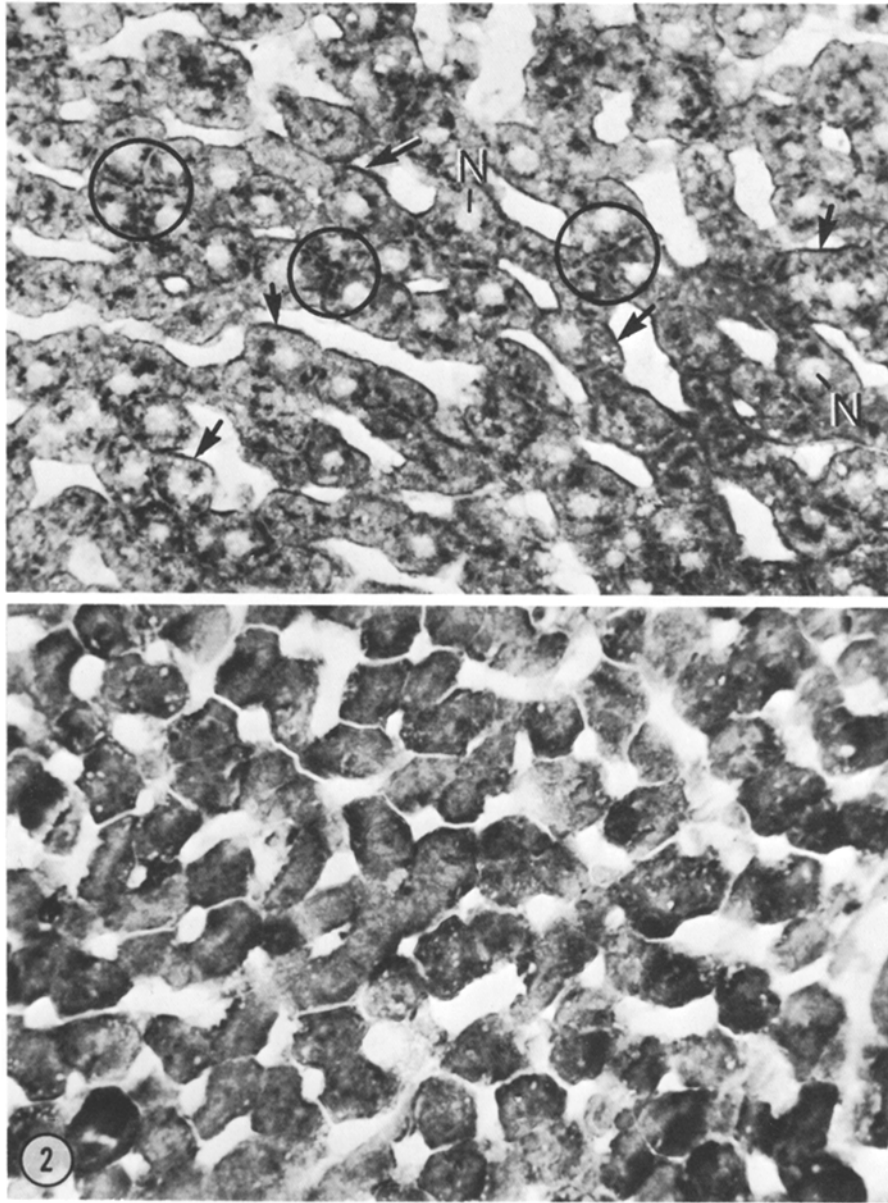
*Abbreviations:*

| | | | | | |
|---|---|---|---|---|---|
| *BC* | bile canaliculus | *LD* | lipid droplet | *RER* | rough endoplasmic reticulum |
| *DB* | dense body | *M* | mitochondrion | *SD* | space of Disse |
| *G* | Golgi apparatus | *N* | nucleus | *SER* | smooth endoplasmic reticulum |
| *K* | Kupffer cell | *P* | peroxisome | *SV* | secretory vesicle |
| *L* | lysosome | | | | |

FIGURE 2 Top: this 6-$\mu$m frozen section is from a liver perfused for 5 min with 4% formaldehyde, and incubated in a conjugate made from the Fab of a rabbit immunized against rat LDL. Specific reaction product is deposited along the bile canaliculi (circles) and close to the nuclei (*N*). Reaction product is also present along the sinusoidal borders of the cells (arrows). $\times$ 105. Bottom: this 6-$\mu$m frozen section from the same liver shown above was incubated in a conjugate made from the Fab of an unimmunized rabbit. Note the absence of specific reaction product. $\times$ 130.
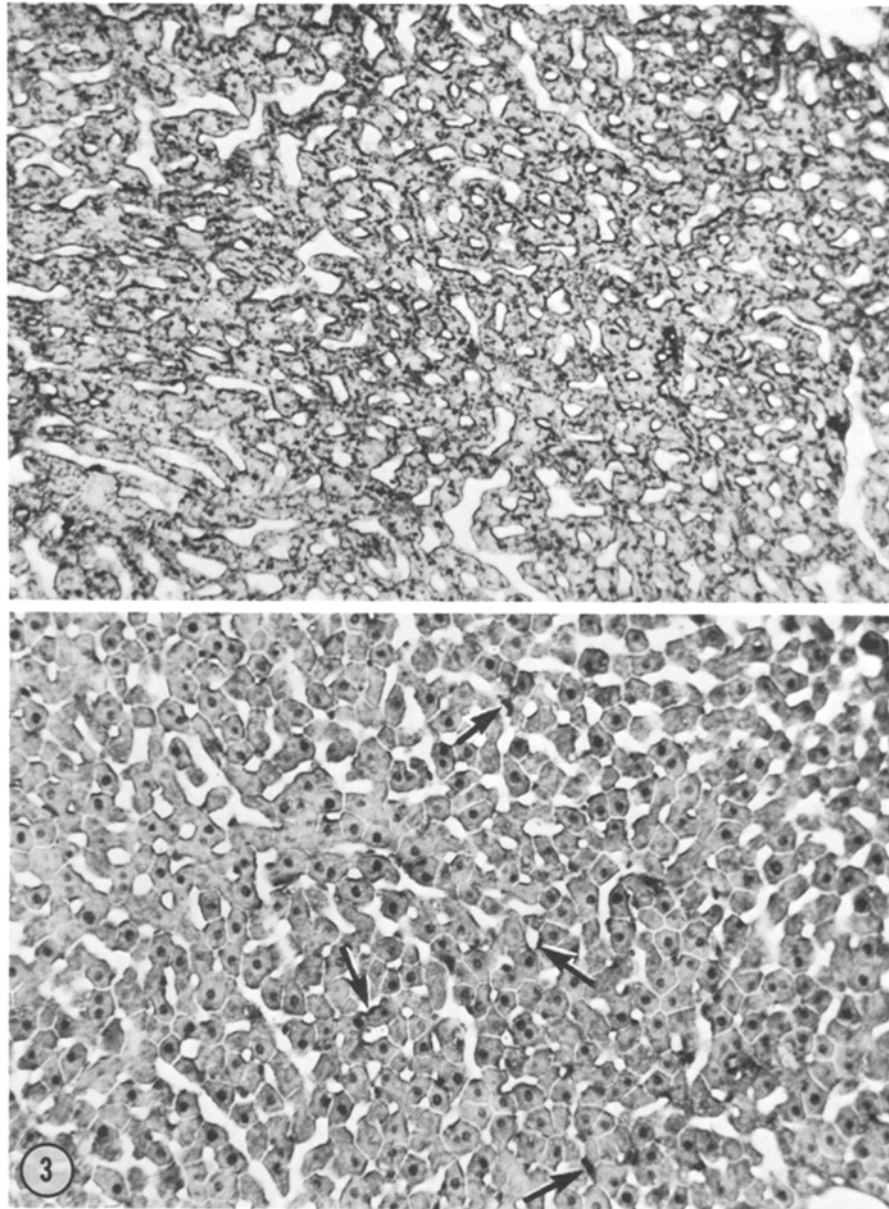
FIGURE 3 Top: this 6-μm frozen section was incubated in a conjugate made from an antiserum against rat VLDL. Specific reaction product occurs in the same locations as in Fig. 2 and is present in all hepatocytes. × 55. Bottom: this 6-μm frozen section was incubated in a solution of Worthington peroxidase 5 mg/ml in 0.135 M phosphate buffer pH 7.4. There is no specific reaction product within the hepatocytes. Kupffer cells in the sinusoids (arrows) contain reaction product due to endogenous peroxidase. × 55.

parenchymal cells of experimental sections. These deposits had characteristic and unvarying locations which correspond to the distribution of the Golgi apparatus in the hepatic parenchymal cell— along the bile canaliculi, adjacent to the nuclei, and between the nuclei and bile canaliculi (Figs. 2 and 3). Reaction product was also present along the sinusoidal borders of most of the cells. In Fig. 2 (top), specific reaction loci along the bile canaliculi are especially prominent. The perinuclear and

perisinusoidal deposits of reaction product are also shown to advantage. Fig. 3 (top) is a low magnification view which emphasizes the widespread occurrence of the specific reaction. The distribution of reaction product was the same whether the Fab used was made from anti-VLDL or anti-LDL. The same pattern and intensity of localization were obtained when the indirect method was used (1). Thus, the use of the direct method did not cause a detectable loss in sensitivity. A generalized brown staining of the cytoplasm was present in both experimental and control sections and was, therefore, interpreted to be nonspecific. Kupffer cells lining the sinusoids of both experimental and control sections contained reaction product in their cytoplasm (Fig. 3, bottom), probably owing to the activity of their endogenous peroxidase.

## Electron Microscope Localization

Successful ultrastructural localization of apoproteins was obtained with livers from four rats. Two of the livers were incubated with conjugates made from anti-LDL Fab, and two were incubated with both anti-LDL and anti-VLDL conjugates. Since reaction product was deposited in the same locations with both these conjugates, the electron micrographs to be presented below were selected from those sections having the best ultrastructural preservation regardless of the type of conjugate used.

The most conspicuous location of specific reaction product was the Golgi apparatus. At low magnifications (Fig. 4), intense deposits were evident in some of its flattened cisternae and in all secretory vesicles. Sometimes the contents of the Golgi apparatus were completely obscured by reaction product, even at higher magnifications so that the organelle could be identified only by its characteristic shape and location (Fig. 6). More frequently, higher magnifications showed that the reaction product was present on the nascent VLDL (Fig. 8). Reaction product was often most intense at the periphery of some of the VLDL particles (Figs. 8 and 13). The Golgi apparatus in control sections contained nascent VLDL whose electron density was due only to osmium tetroxide and lead citrate (Figs. 5, 7, and 9). Fig. 8 (top) shows individually reacted nascent VLDL in some Golgi cisternae whereas a few cisternae of the same Golgi apparatus contain unreacted particles. Such partially reacted Golgi apparatus were seen only occasionally in livers fixed with 4% formaldehyde;

in livers fixed with 1% formaldehyde, all parts of all Golgi apparatus contained reaction product. With both concentrations of fixative, the membranes of some Golgi cisternae and secretory vesicles appeared to have greatly increased electron density (Fig. 8, bottom). In both experimental and control sections, including unincubated controls, autolysosomes containing highly electron-dense round bodies of unknown origin were consistently found adjacent to the Golgi region (Figs. 8, top, and 9).

Some areas of rough ER contained reaction product (Figs. 4, 10, and 11). This was usually present only along a portion of the cisternal length, quite frequently at one of the blind ends (Figs. 10, bottom, and 11). When there was a large amount of reaction product, it filled and extended beyond the cisternae, completely obscuring the ribosomes (Figs. 10, bottom, and 11). However, when the reaction was less intense, the cisternae appeared empty and only the cisternal membrane and attached ribosomes seemed to contain reaction product as evidenced by increased electron density (Figs. 8, top, 10, bottom, 11, and 12). This is especially striking in Figs. 8, top, and 11, bottom, where the selectivity of the phenomenon and the absence of lead staining reduce the possibility that this image is artifactual.

Numerous images showed secretory vesicles packed with nascent VLDL just beneath the sinusoidal surface of the hepatocytes (Fig. 13). VLDL were also present among the microvilli of the space of Disse. In all these locations, the VLDL in experimental sections were heavily invested with reaction product. The electron density of the walls of the secretory vesicles was often greater than that of other cytoplasmic membranes and corresponding structures in control sections (Figs. 8, bottom, and 13). This was also true of the plasma membrane of the sinusoidal surface and the microvilli at regions where particles were present in the space of Disse (Fig. 13).

The smooth ER in all control sections was typical of rat liver after an overnight fast; it appeared as a fine lacework of tubular and vesicular profiles, many of which contained osmiophilic particles similar to the nascent VLDL in the Golgi apparatus (Figs. 7 and 9). In experimental sections the smooth ER was often difficult to identify. When preservation of ultrastructure and background density permitted, similar particles could be identified within cisternae of the smooth ER in
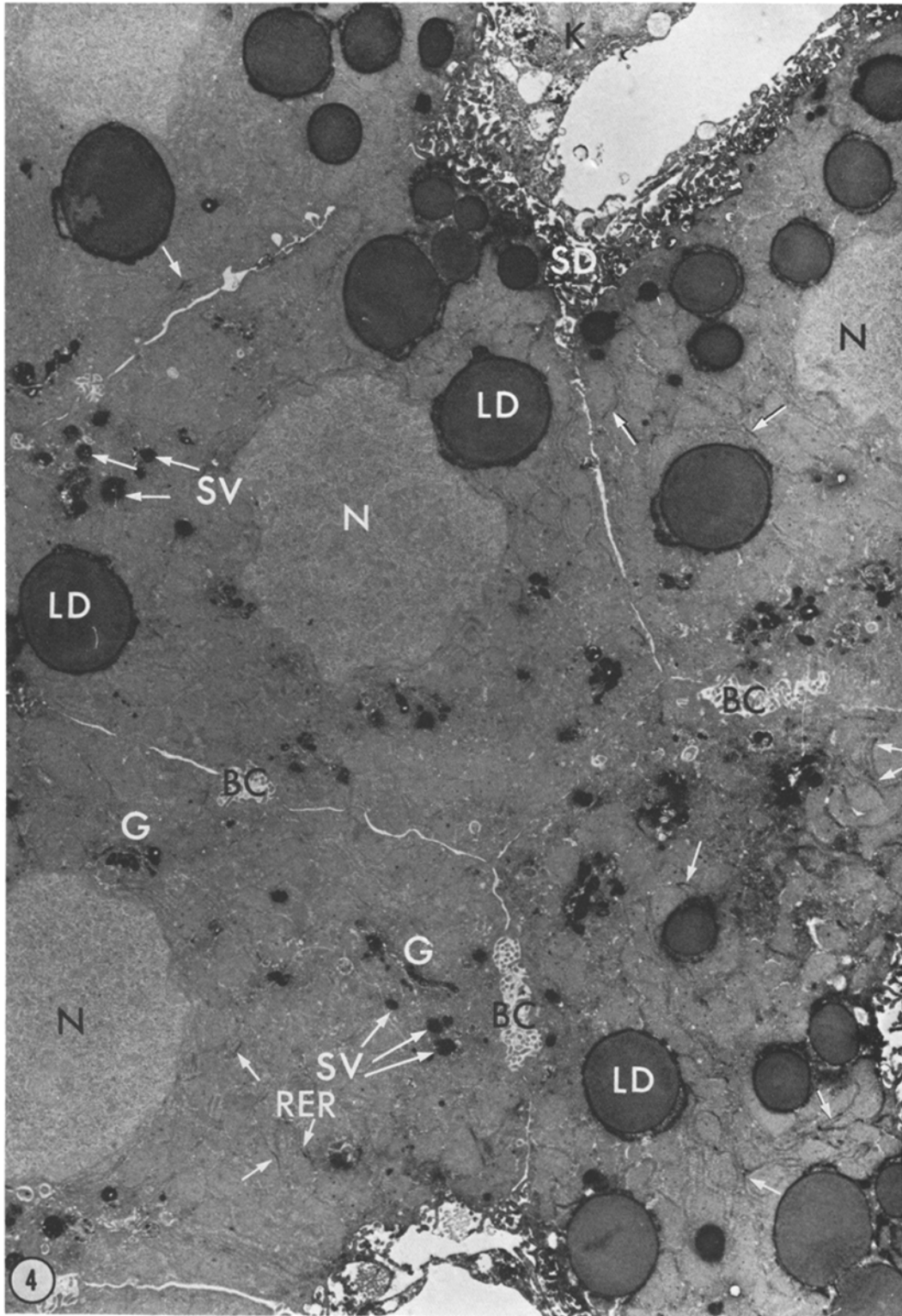
FIGURE 4 This experimental section is from a liver perfused for 10 min with 4% formaldehyde. Reaction product fills secretory vesicles of the Golgi apparatus obscuring nascent VLDL and also is present in some of its flattened cisternae. A more subtle staining reaction appears in the cisternae of the rough ER (arrows). Reaction product also occurs in the space of Disse among the microvilli. The electron density of the cytoplasmic lipid droplets is nonspecific since it is similar to that of droplets in control sections (Fig. 5). The increased electron density at the surfaces of the lipid droplets is an artefact of lead staining since it is absent before staining the sections with lead citrate. Stained with lead citrate. × 7,200.
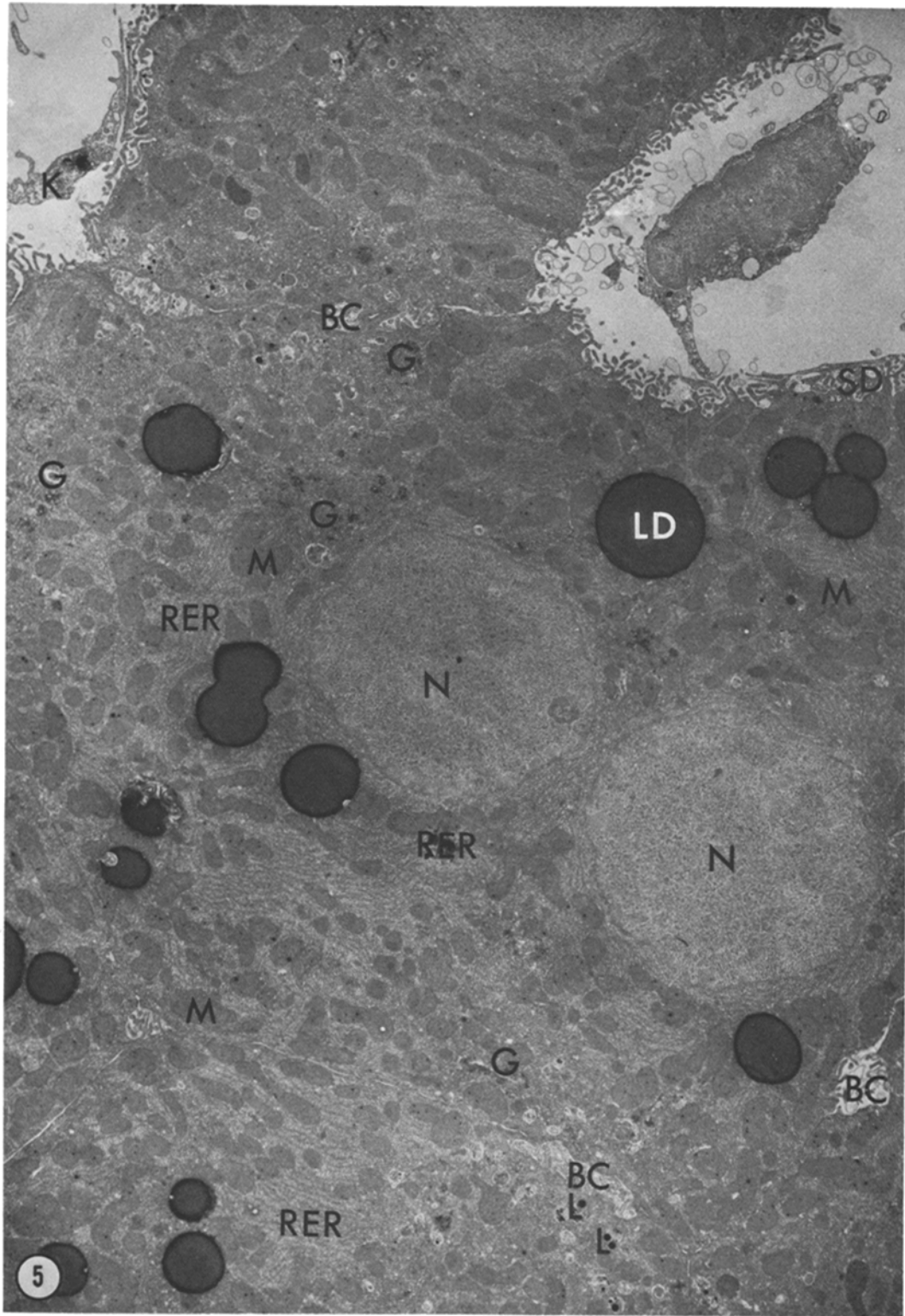
FIGURE 5  This section is from the peroxidase control of the same liver shown in Fig. 4. Note the excellent preservation of ultrastructure, especially the flattened cisternae of the rough ER and the Golgi apparatus which contain unreacted nascent VLDL particles. Stained with lead citrate. × 6,000.
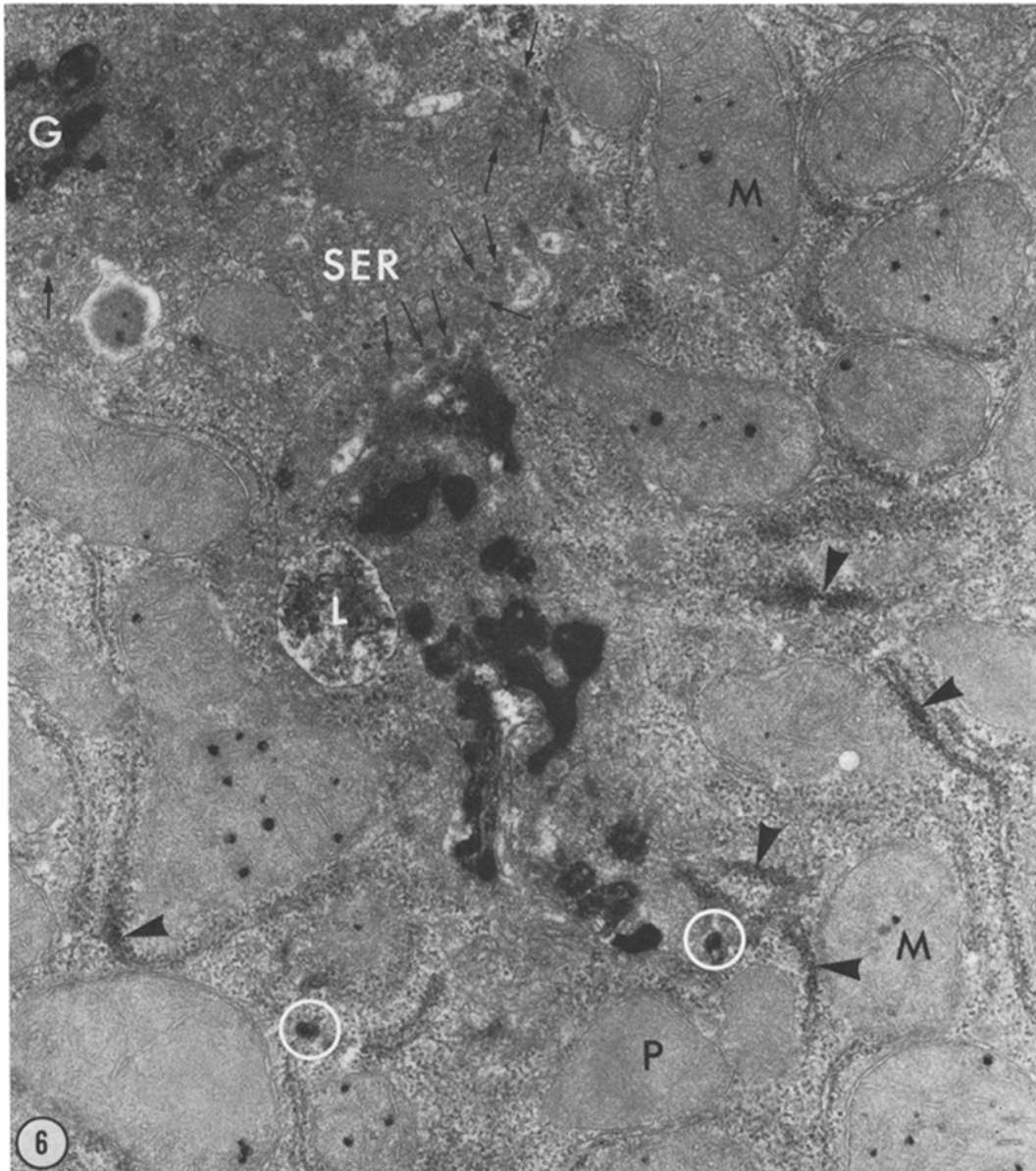
FIGURE 6   Intense deposits of reaction product completely fill many compartments of this experimental Golgi apparatus. Reaction product also stains some rough ER cisternae (arrowheads) and particles contained within the vesicles near the rough ER (circles). Note the absence of reaction product from the smooth ER and particles contained therein (arrows). Stained with lead citrate. × 30,000.

experimental sections. However, these particles did not bear reaction product (Fig. 6). The only portions of the smooth ER which bore reaction product were the smooth-surfaced ends of the rough ER and nearby vesicles (Figs. 6, 8, bottom, 11, and 12). Reaction product at these sites was sometimes clearly associated with a particle (Figs. 6, 8, 11, and 12). Often, however, the intensity of

the reaction product or plane of section made it impossible to establish the presence or absence of a particle (Fig. 11).

## DISCUSSION

In this study, the preservation of ultrastructure was felt to be superior to that reported in previous studies in which enzyme-labeled antibodies were
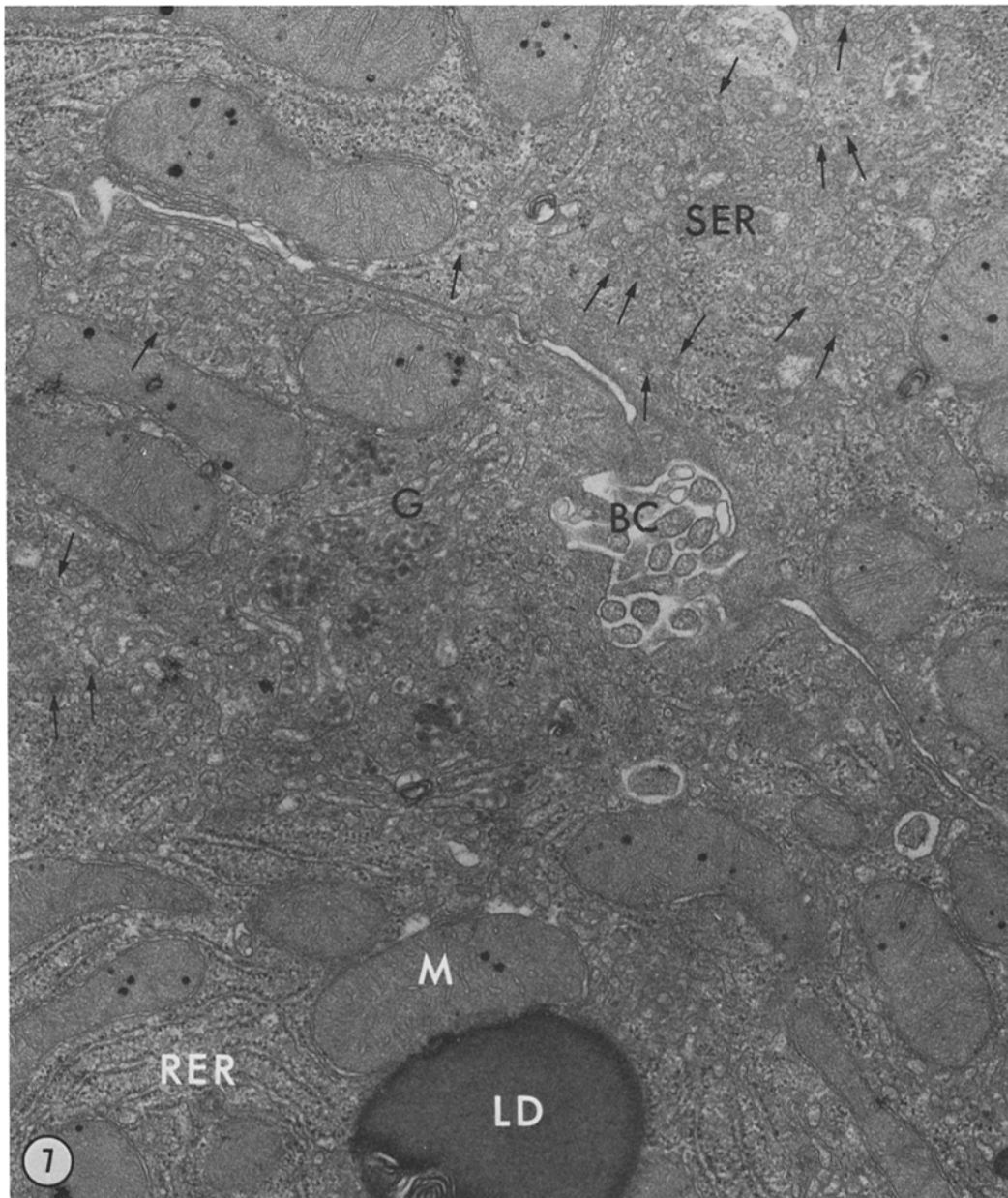
FIGURE 7  Golgi apparatus and smooth ER from a normal conjugate control section of a liver perfused with 4% formaldehyde. Nascent VLDL in the Golgi apparatus are devoid of reaction product. The network of tubular and vesicular profiles of smooth ER contain single osmiophilic particles (arrows). In size and electron density, these particles are similar to those in the Golgi apparatus and similar to the unreacted particles in the smooth ER shown in Fig. 6. Stained with lead citrate. × 30,000.

used on bulk tissues which still retained antigenic determinants. Moreover, the electron microscope localization of specific reaction product on individual VLDL particles suggests an increased sensitivity for localizing small amounts of intracellular protein since nascent VLDL are only about 400 Å in diameter and contain only 5–7% protein by weight (22). These improvements probably result from the method of fixation by perfusion through the hepatic vascular bed. Perfusion of only 0.25%
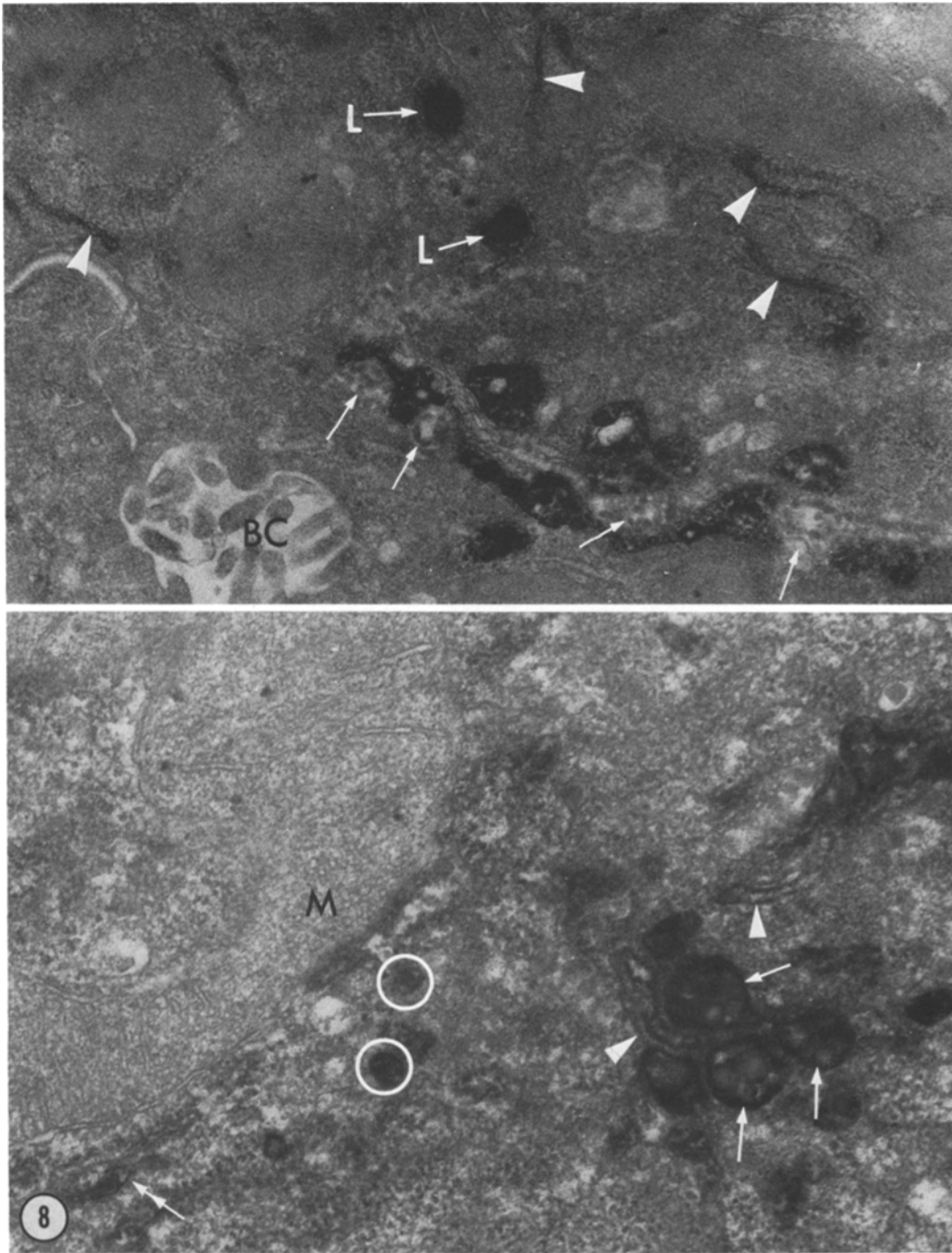
FIGURE 8 Top: in this experimental section (4% formaldehyde), most particles within the flattened cisternae and secretory vesicles of the Golgi apparatus bear reaction product. A few of the cisternae and secretory vesicles contain unreacted particles (arrows). Reaction product is also present on some of the membranes and ribosomes of nearby rough ER cisternae (arrowheads). No lead stain. × 30,000. Bottom: reaction product appears on the walls (arrows) of the secretory vesicles of this experimental Golgi apparatus from a liver fixed in 1% formaldehyde for 10 min. Note the increased electron density of the membranes of its flattened cisternae (arrowheads). The circled structures are cytoplasmic vesicles, each containing a single highly reacted particle. The double arrow denotes a structure which appears to be a smooth-surfaced end of a rough ER cisternae containing a reacted particle. Note the increased electron density of the membranes of these structures. No lead stain. × 48,000.
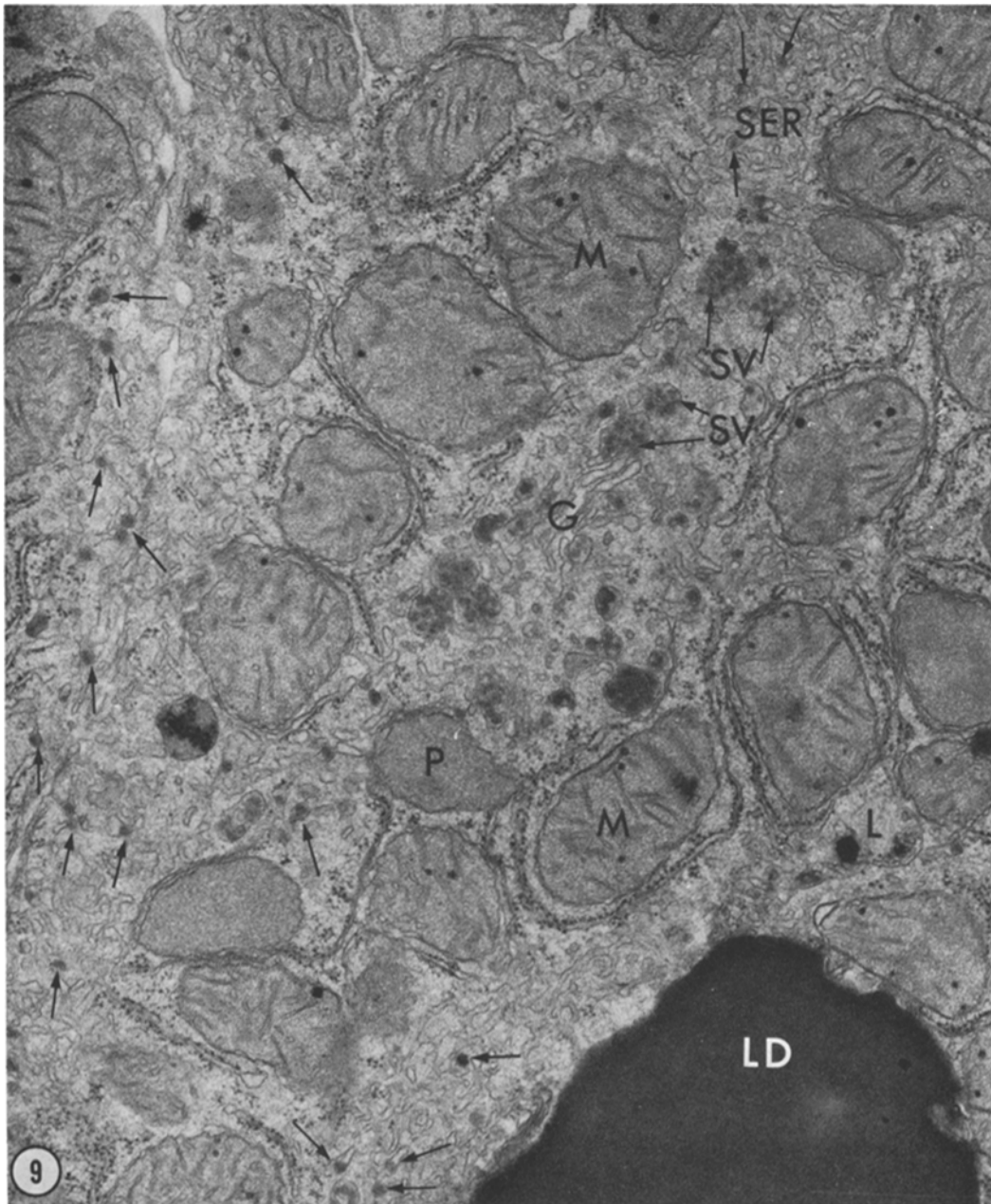
252

FIGURE 9   This is an unincubated control section from a liver fixed in 4% formaldehyde. The electron density of the particles within the Golgi apparatus and its secretory vesicles is due solely to their interaction with osmium tetroxide and lead citrate. Similar particles are present within the tubular and vesicular profiles of the smooth ER (arrows). Stained with lead citrate. × 30,000.

glutaraldehyde for 5 min or 1% paraformaldehyde for 30 min completely destroyed the antigenic determinants as assessed by light microscopy of frozen sections. Perfusion fixation with either 1% or 4% paraformaldehyde for 5–10 min followed immediately by perfusion of buffer for 5–10 min was the only procedure that gave adequate preservation of both cellular details and antigenic deter-
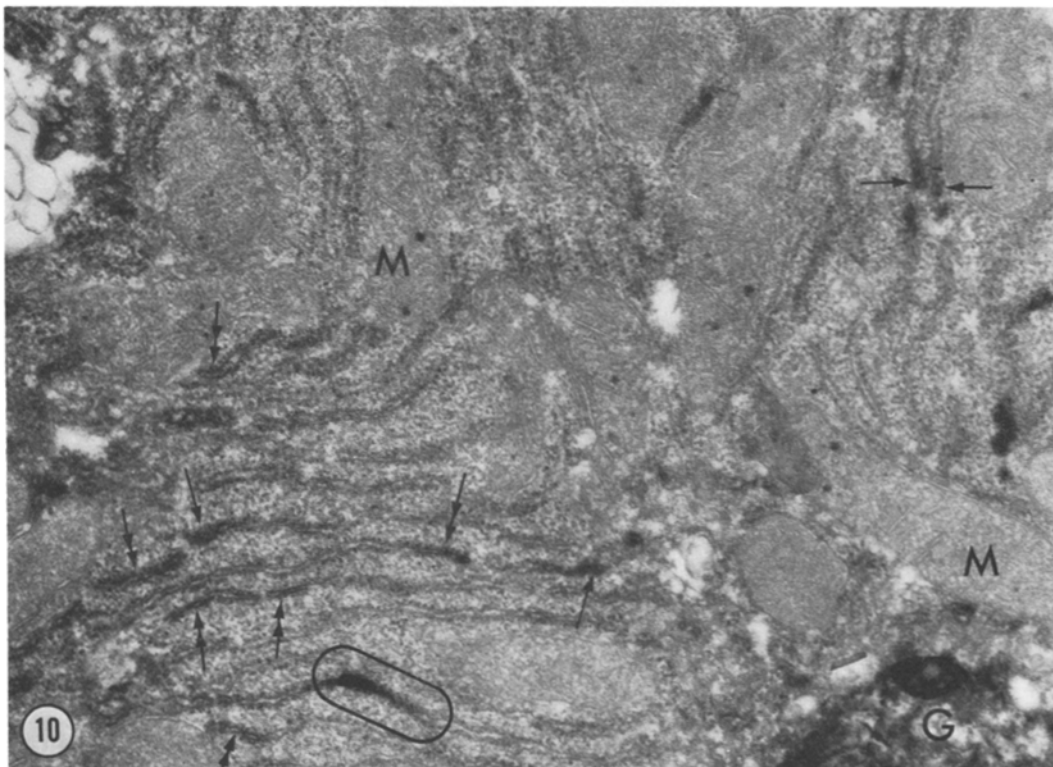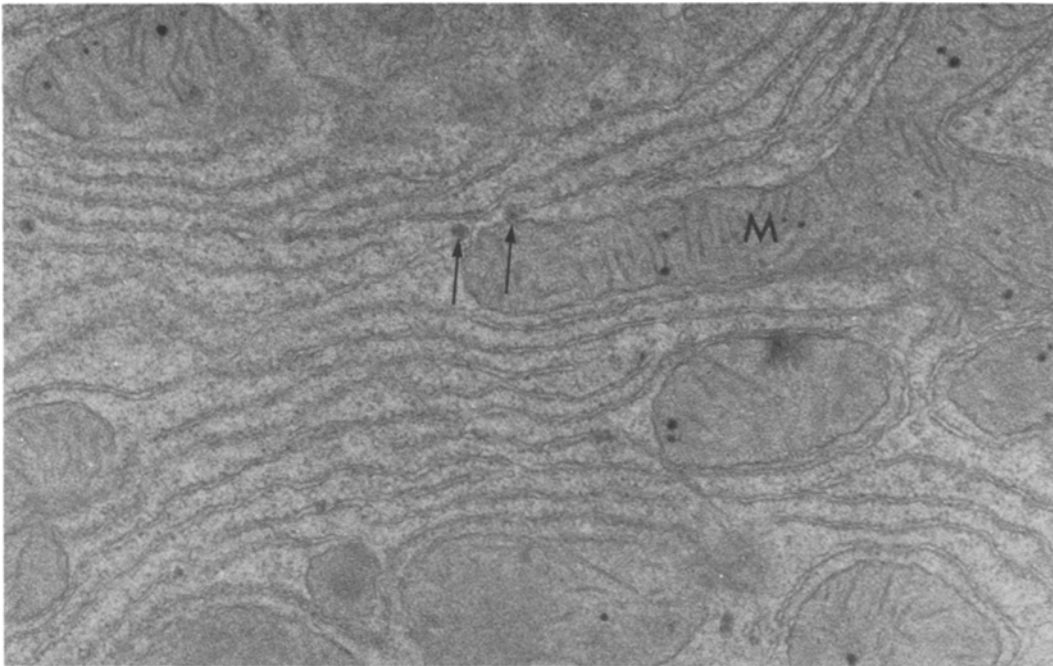
FIGURE 10  Top: rough ER from an unincubated control section from a liver perfused with 4%
formaldehyde. Note that some of the cisternae have smooth-surfaced ends which contain a single
osmiophilic particle (arrows). No lead stain. × 30,000. Bottom: rough ER of an experimental section from
a liver perfused with 1% formaldehyde. Some cisternae have reaction product only at their ends (arrows).
Portions of some cisternae are completely filled with reaction product which obscures the ribosomes
(circle), whereas other cisternae seem to have reaction product on their membranes and ribosomes (double
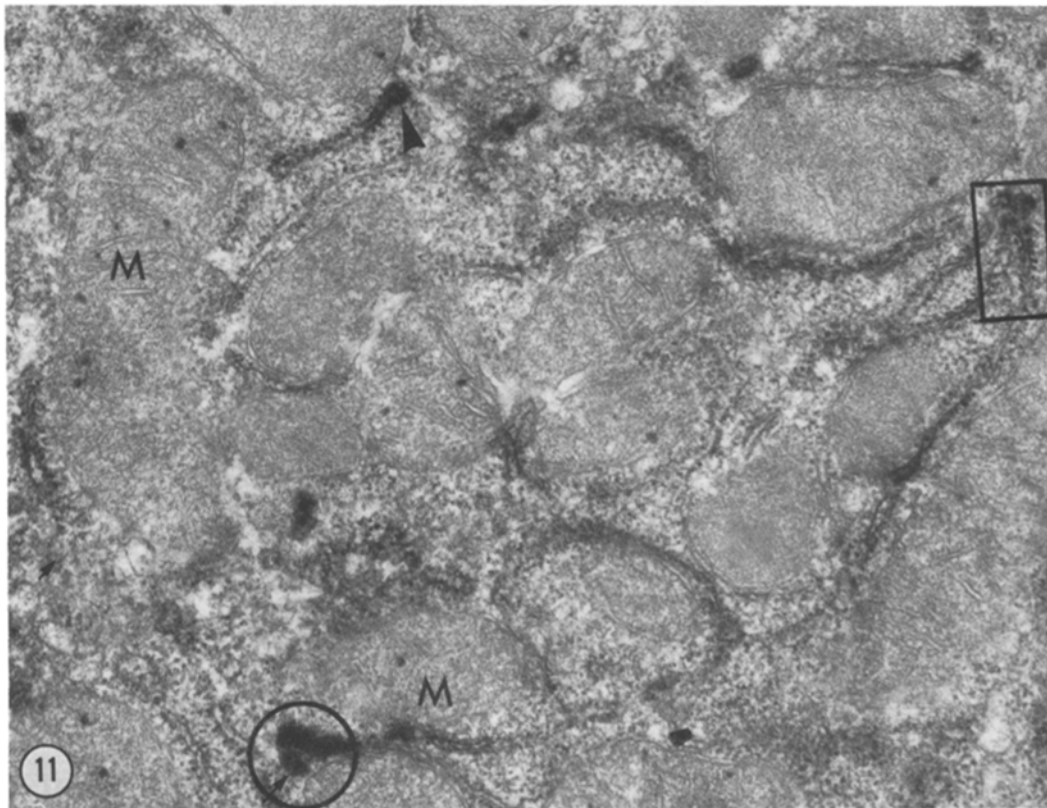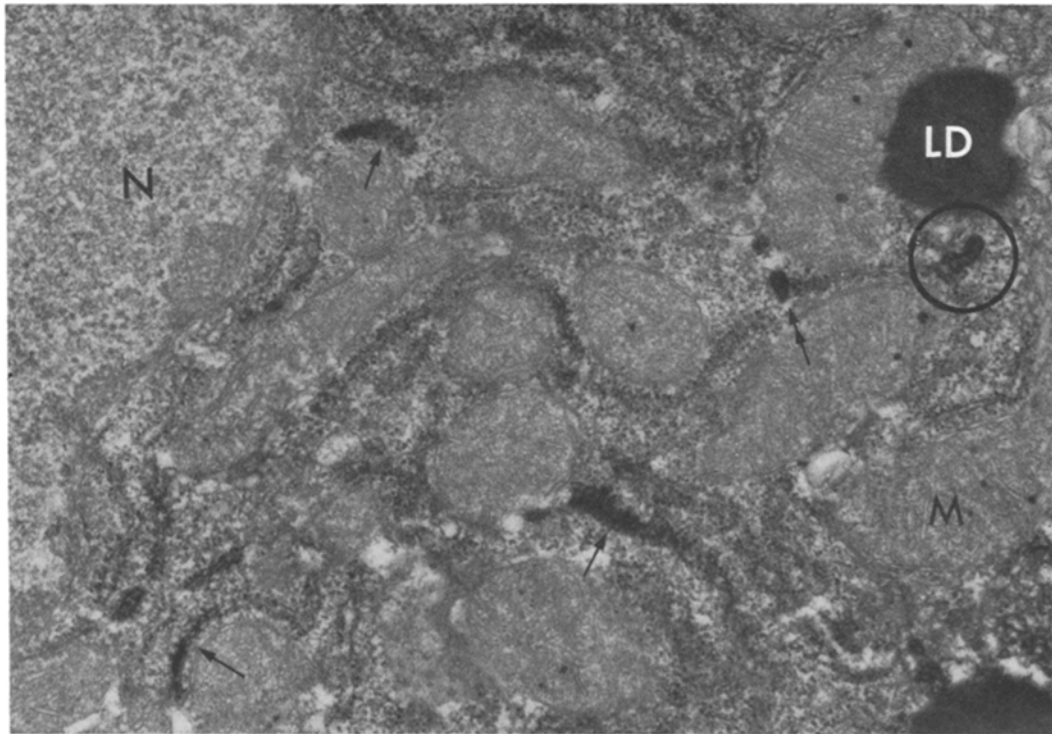arrows). Stained with lead citrate. × 30,000.

254

FIGURE 11 Top: reaction product is present in the rough ER cisternae of this experimental section from a liver fixed with 1% formaldehyde (arrows). The circled structure seems to be a highly reacted particle within the smooth-surfaced end of a rough ER cisterna. The double arrow points to a vesicle which is so full of reaction product that it is impossible to determine whether it contains a particle. No lead stain. × 30,000. Bottom: another area from the same section. Reaction product completely fills the blind end of one cisterna (circle) while the vesicle beneath it seems to contain a highly reacted particle (arrow). The arrowhead indicates what appears to be a reacted particle within the smooth end of a rough ER cisternae. The four cisternae converging at the upper right bear reaction product on their ribosomes and membranes. The ribosomal reaction is particularly striking in the region within the rectangle. No lead stain. × 38,400.
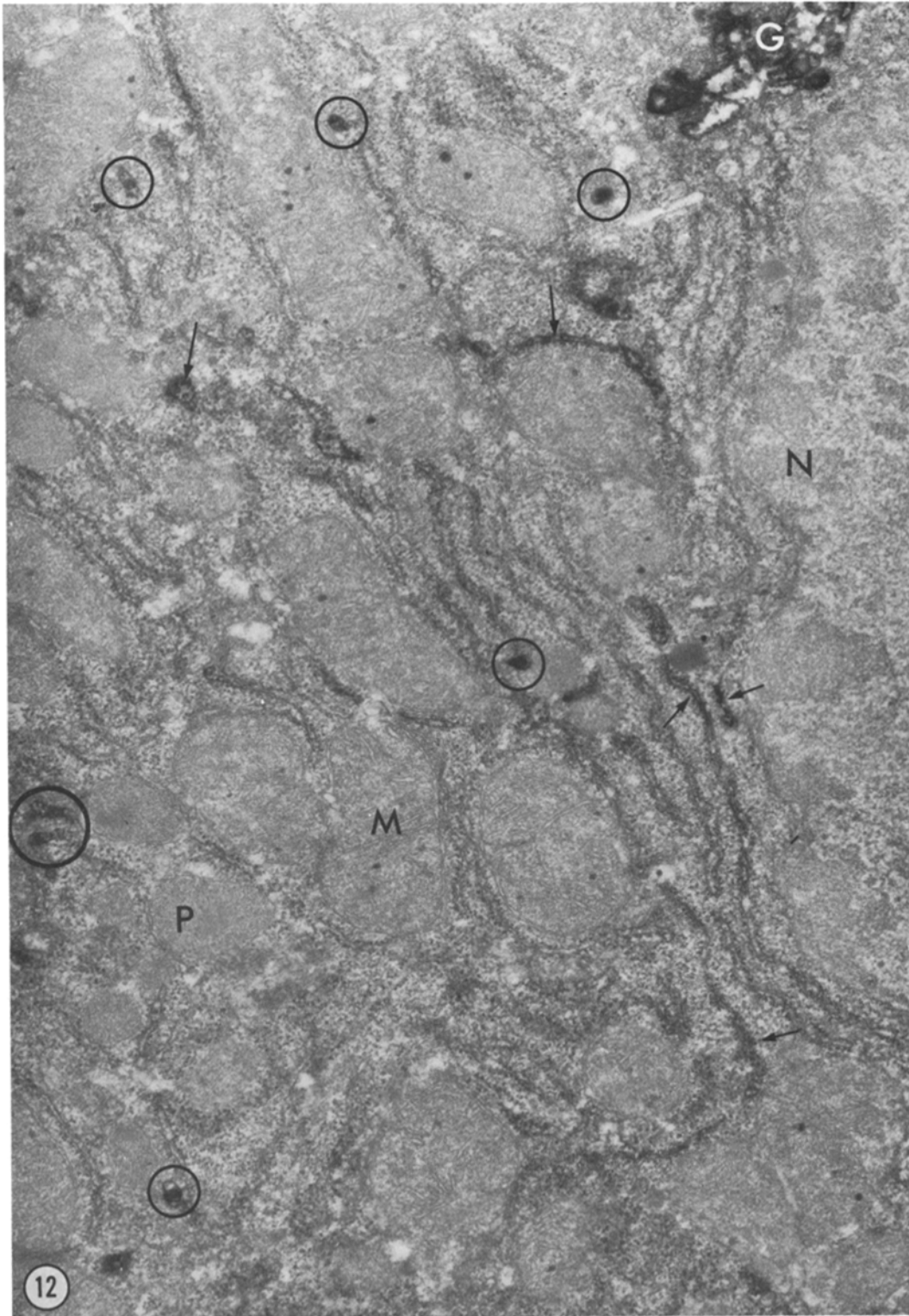
FIGURE 12  Another region from the section shown in Fig. 11. Reaction product is present on the ribosomes and within membranes of the rough ER (arrows). The circled structures are vesicles, most of them smooth surfaced, which contain particles bearing reaction product. Part of a Golgi apparatus is seen at the upper right. Stained with lead citrate. × 30,000.
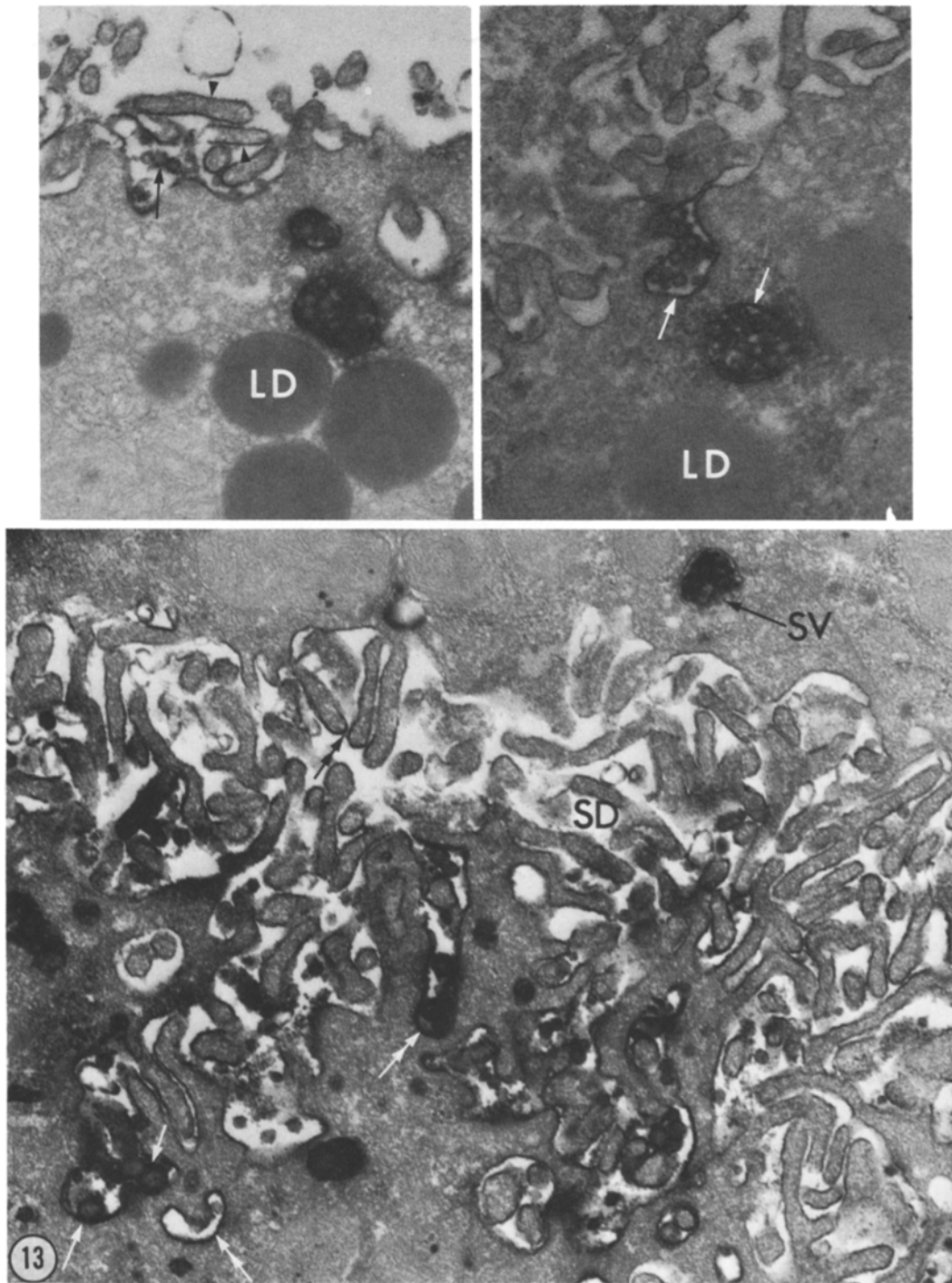
FIGURE 13  Top, left: secretory vesicles just beneath the sinusoidal border in an experimental section contain highly reacted VLDL particles (perfused with 1% formaldehyde). Similar particles are seen among the microvilli at the space of Disse (arrow). Note the increased electron density of the plasma membrane of the microvilli (arrowhead). Stained with lead citrate. × 30,000. Top, right: the secretory vesicle at the top of the field has fused with the plasma membrane, releasing reacted VLDL particles into the space of Disse. Note the increased electron density of its membrane as well as the membrane of the vesicle just below it (arrows). Liver perfused with 1% formaldehyde. No lead stain. × 30,000. Bottom: highly reacted VLDL particles among the microvilli of the triangular space of Disse between hepatocytes. The arrows indicate particles on which the reaction product is much denser at the periphery, indicating that the reaction takes place at the surface of the particle. Note the increased electron density of the microvillous plasma membrane (double arrows). Liver perfused with 4% formaldehyde and stained with lead citrate. × 32,000.

minants (1). Apparently, there is a very critical degree of fixation which permits retention of both structural details and antigenic sites, and this depends on the chemical nature and concentration of the fixative, fixation time, and nature of the antigen (29). Our results suggest that perfusion fixation followed by perfusion of buffer to remove excess fixative and thus arrest further denaturation provides the best mechanism of controlling the fixation process within bulk tissue (1). Immersion fixation of even tiny cubes or thin slices of tissue did not give uniform results (1). The frozen sections of perfusion-fixed livers provided the best test of both the quality of a given conjugate and the retention of antigenic determinants after a given fixation procedure. Unfortunately, although freezing facilitated penetration of the conjugate throughout all parenchymal cells (Figs. 2 and 3, top), it destroyed the ultrastructure (1).

The B apoprotein, which is virtually the sole apoprotein of LDL, is identical to a protein that comprises 25% of rat VLDL (4) and 40% of human VLDL (12, 28). This apoprotein forms a characteristic arc on Ouchterlony immunodiffusion and immunoelectrophoresis (11), is insoluble in tetramethylurea (28), and does not enter 7.5% polyacrylamide gels during electrophoresis (4, 28). The protein of the lipoprotein isolated within the density range 1.025–1.045 g/cm$^3$ in this study had all of these properties. Antiserum raised against this LDL fraction produced immunodiffusion arcs of similar appearance and position with both LDL and VLDL which fused in a reaction of identity (38). Antiserum against VLDL formed this same arc in immunodiffusion against LDL and VLDL. Thus, both antisera contained antibodies to the B apoprotein. Fab conjugates made from either anti-LDL or anti-VLDL gave identical intracellular distribution of reaction product. Therefore, if apoproteins other than the B apoprotein contributed to the reaction product when antiserum to VLDL was used, they evidently did not occupy additional sites.

Reaction product in the rough ER suggests that the B apoprotein is synthesized there. This is in agreement with published evidence that secreted proteins are synthesized on membrane-bound rather than free ribosomes (41, 42, 46) and that rat liver ribosomes and rough microsomes can synthesize plasma lipoprotein apoproteins (6, 34). The rough ER can also store secretory proteins (31), and accumulations of reaction product within the

rough ER cisternae may indicate storage of B apoprotein. Storage of VLDL apoprotein has been proposed to explain the continued secretion of VLDL by perfused rat liver 50–60 min after inhibition of protein synthesis by cycloheximide (3). Images showing empty cisternae but small amounts of reaction product on the membrane-bound ribosomes may indicate the presence of VLDL apoproteins at the ribosomal level (Figs. 8, top, 10, bottom, 11, and 12). Reaction product was also found on ribosomes by Feldmann et al. (17) in a study of albumin synthesis in human liver with peroxidase-labeled antibodies and by Leduc et al. (31) in a study on immunoglobulin synthesis in rabbit spleen with the same method. More recently, ribosomes and membranes of the rough ER of hepatocytes were shown to stain with Fab-peroxidase conjugates specific for fibrinogen (18). Reaction product reportedly was not present in the cisternae of rough ER unless the tissue was prepared from rats injected with colchicine, suggesting that the staining of ribosomes and rough ER membranes by specific conjugates of peroxidase-Fab molecules occurs at this site. We cannot explain how nascent polypeptide chains attached to ribosomes become accessible to conjugates; possibly it results from structural changes of ribosomes produced by fixation. The greatly increased electron density of some of the membranes of the rough endoplasmic reticulum indicates that the apoprotein may exist within the membrane. Redman and Cherian (42) suggested that glycoproteins (to which class some VLDL apoproteins including the B apoprotein belong) are delayed within the membrane of the rough endoplasmic reticulum where mannose and N-acetylglucosamine are added before they pass into the intracisternal space. Alternatively, one must consider the possibility that the apparent staining of these sites represents a fortuitous artifact after translocation of the specific protein during the prolonged incubation procedures or perhaps diffusion of the reaction product in incompletely "fixed" microenvironments.

A number of factors could account for our failure to observe reaction product in the smooth ER. First, the particles in this compartment may contain immunologically reactive VLDL apoproteins that fail to react because these membranes are impermeable to conjugates. Second, the apoprotein may be in a different conformation that is not recognized by antibody. Third, fixation may

not retain the antigenic determinant equally well in different cell compartments. These possibilities seem unlikely because the membranes of the smooth ER are evidently quite labile and vulnerable to structural damage which would be expected to increase their permeability to conjugate molecules. Also, the antigenic site of the apoproteins is retained and is recognized after fixation in several different locations and, probably, in different chemical associations. However, immunocytochemical techniques are difficult to standardize and negative results must always be interpreted with caution.

Specialization of the smooth ER for synthesis of neutral lipid for export is suggested by numerous studies on fatty liver, the common early morphologic feature of which is the accumulation of triglyceride-rich particles within vesicles of smooth ER (9, 16, 23, 27, 52, 53). In normal liver, particles the size of VLDL do not occur in the flattened cisternae of the rough ER except at the smooth-surfaced ends, some of which are sites of continuity with smooth ER (9, 27). Therefore, protein synthesis and lipid synthesis may occur in separate compartments. If so, either protein or lipid must travel to a specific site in the cell, apart from its site of production, to form a lipoprotein particle. Two observations were made in this study that suggest the site of this union. The first was the common localization of intense deposits of reaction product at smooth-surfaced terminal ends of rough ER cisternae in both the presence and absence of a particle. The second was the absence of reaction product from virtually all particles in typical smooth ER tubules and vesicles of experimental sections. Some vesicles in close proximity to rough ER or interposed between rough ER cisternae and the Golgi apparatus contained reaction product associated with a particle but these were not identifiable as typical smooth or rough ER. These structures could represent special smooth-surfaced tubular elements that transport nascent VLDL to secretory vesicles of the Golgi apparatus (9, 22). The absence of reaction product in typical smooth ER is particularly striking because numerous particles were present in the smooth ER of all control sections from the same livers (Figs. 7 and 9). These findings suggest that triglyceride particles in the smooth ER (presumably stabilized by a coat of polar lipid) do not contain VLDL apoproteins, but rather obtain their complement of apoproteins subsequently from the

rough ER. These particles in the smooth ER could, however, be associated with proteins other than those present in serum VLDL. Images of single osmiophilic particles 300-800 Å in diameter in the smooth-surfaced ends of rough ER cisternae are evident in several published reports (5, 8, 9, 27). They are, however, much less common than the similar particles seen in tubular and vesicular profiles of typical smooth ER. Our findings confirm this observation. In the present study, a few images of particles were seen in the smooth ends of rough ER in control sections (Fig. 10, top) although the more common image was that of a particle in a vesicle quite close or immediately adjacent to the ends of rough ER cisternae (Figs. 7 and 9). The images seen in this and other studies cannot distinguish between a continuous system of smooth ER, rough ER, and Golgi apparatus and three discontinuous systems that are intermittently connected through brief membrane fusions. We favor the former possibility based on the tubular structures demonstrated in serial sections of fixed liver described by Claude (9), but the precise mode of transport among these organelles remains uncertain.

Our findings appear to be inconsistent with those of Glaumann et al. (21) who concluded that "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." Their conclusions were based on experiments in which [$^3$H]glycerol was injected into the portal circulation of rats followed by isolation of smooth and rough microsomes at different times from livers. Measurements were then made of the radioactivity of triglycerides separated from the microsomes after washing the membranes twice, sonicating the washed pellet, and subsequently floating the released material at a density of 1.03. Those authors found that triglyceride from smooth and rough microsomes was labeled about equally at the earliest time that livers were sampled after injection of isotope (3-5 min). Reference was made to previous studies by Stein and Stein (49) and van Golde et al. (54) who concluded that both rough and smooth ER take part in triglyceride synthesis. Following these studies, it has generally been assumed that synthesis of VLDL-triglycerides occurs generally in the rough ER compartment as well as in the smooth ER of hepatocytes. Because of our observations we have re-examined the

evidence from which this concept evolved. Stein and Stein localized radioautographic grains over subcellular structures after the intravascular injection of [³H]glycerol and palmitic acid. Although the interpretation was made initially that grains were localized over the rough endoplasmic reticulum by 2 and 5 min, the published images show grains only in the vicinity of the ends of parallel arrays of RER or of single RER cisternae, and no grains were clearly associated with flattened cisternae containing ribosomes (49). Although the grain size was too large for precise localization, these investigators have recently stated that grains were seen often in the regions of transition between the rough and smooth ER (50). Thus, contrary to previous interpretations, the radioautographic studies suggest to us that the ribosome-studded, flattened cisternae of the rough endoplasmic reticulum do not synthesize triglycerides. The presence of label clearly localized in regions of transition between smooth and rough endoplasmic reticulum, particularly at the earliest times of sampling of liver (2-5 min after injection), suggests that triglyceride synthesis occurs either in this transitional compartment or in nearby smooth ER cisternae (and is then transported to the transitional zone) or in both sites. Glaumann et al. found no clear precursor-product relationship between the rates of labeling of triglyceride obtained from rough- and smooth-surfaced microsome fractions at the earliest times of sampling, 3-5 min (21). Triglyceride synthetase activity is distributed about equally between rough and smooth microsome fractions isolated from rat livers (54). Although these observations have been interpreted to indicate that triglyceride is synthesized in both compartments in intact cells, it is possible that mechanical rupture of membranes of the intact endoplasmic reticulum does not successfully separate the critical subcompartments. Smooth-surfaced ends of rough ER may form vesicles either with or without ribosomes attached so that the enzymes, apoproteins and lipids contained therein are distributed between smooth and rough microsomes. In both the radioautographic and cell fractionation studies, there was adequate time for triglyceride synthesis to have occurred in the smooth ER and for triglyceride-rich particles to be transported to the transitional compartment. We conclude that none of the techniques used to date (radioautography, cell fractionation or immunoelectron microscopy)

presently provides adequate resolution to establish the sequence of events in the assembly of VLDL. However, we believe that the pathway outlined in Fig. 14 provides a reasonable explanation for our own observations and that this pathway is not excluded by data obtained with other methods.

Previous studies suggested that the Golgi apparatus of the liver concentrates nascent VLDL into
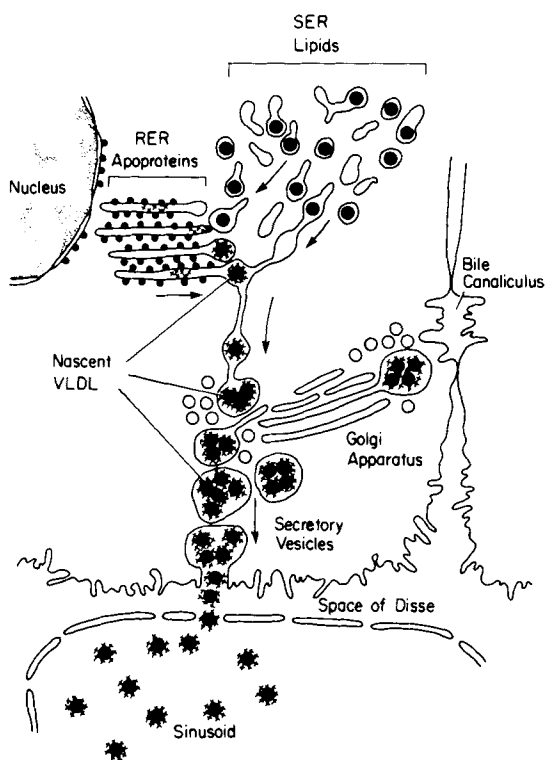


FIGURE 14   This drawing summarizes our present interpretations of VLDL secretion by hepatocytes. It is suggested that the nascent particle originates within cisternae of the smooth ER, after synthesis by enzymes in these membranes (16, 47). The lipid particle subsequently moves to the smooth surfaced ends of rough ER cisternae either through tubules connecting the smooth and rough ER or by brief fusion of the two compartments. At this junction between smooth and rough ER (9, 27), B apoprotein and probably other VLDL apoproteins are bound to the lipid particle to form a nascent lipoprotein. The nascent VLDL are then transported within a tubular membrane compartment to the Golgi apparatus and are collected in secretory vesicles developing from this organelle (9, 22). After concentration and glycosylation (56), these vesicles transport nascent VLDL to the cell surface where release into the space of Disse results from membrane fusion between secretory vesicles and the plasma membrane (22, 23).

secretory vesicles which transport them to the cell surface for release into the space of Disse by exocytosis (8, 9, 22, 23, 47, 49). The Golgi apparatus has been shown to be the site at which the terminal carbohydrate moieties are added to the VLDL apoprotein (56). In the present study, the Golgi apparatus and Golgi-derived secretory vesicles contained the most intense deposits of reaction product, thus confirming the importance of this organelle in lipoprotein secretion. All the Golgi apparatus in the livers fixed with 1% formaldehyde contained reacted particles. Therefore, the lack of reaction product in some Golgi cisternae of livers fixed in 4% formaldehyde may have resulted from their impenetrability to conjugate.

The increased electron density of the membrane of some Golgi cisternae and secretory vesicles may result from adsorption of reaction product from nascent VLDL. Loss of some lipid from nascent VLDL during processing and embedding for electron microscopy may have caused them to shrink away from the walls of the secretory vesicle, leaving some of the reaction product from their surfaces adherent to the wall. Alternatively, B apoprotein, reaction product, or both may have become detached from the particle surface and adhered to the wall of the vesicle.

The increased electron density of the plasma membrane at the sinusoidal border and that of the microvilli and, in some cases, the presence of a layer of reaction product on these surfaces (Fig. 13) are of interest in the light of recent findings by Eisenberg and Rachmilewitz (13, 14), Roheim et al. (44), and Faergeman et al. (15). They found that, in the rat, most of the apolipoprotein B of VLDL is cleared from the circulation with a half-life of only a few minutes and that most of an injected dose of radioactive apo-B can be recovered in the liver within one hour. This finding is in contrast to that with most of the other VLDL apoproteins, which have half-lives of 8–11 h. The increased density of the plasma membrane and the layer of reaction product on its surface could reflect this B protein in the process of its uptake into parenchymal cells. Particulate remnants of VLDL, low in triglycerides and enriched in cholesteryl esters and B apoprotein, are formed by the action of lipoprotein lipase (36) and are rapidly taken up by the liver (15). Since the diameter of VLDL decreases with the cube root of volume as triglyceride is removed from their apolar core, these particles are only slightly smaller than the

parent VLDL. Some of the reacted particles in the space of Disse could therefore be VLDL remnants rich in the B apoprotein.

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