SITES OF LIPOPROTEIN PARTICLES IN NORMAL RAT HEPATOCYTES

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ABSTRACT

Very low density lipoprotein (VLDL) particles are packaged by the Golgi apparatus into vacuoles which move to the plasma membrane and empty the particles into the space of Disse, via exocytosis. Traditionally, all lipoprotein-containing cisternae and vacuoles are thought to be parts of this pathway. Observations reported here demonstrate that there is a second population of lipoprotein-containing cisternae and vacuoles. This population is part of GERL, an organelle we consider to be a specialized hydrolase-rich region of the endoplasmic reticulum (ER). To our knowledge, this is the first systematic study of GERL in normal rat hepatocytes.

KEY WORDS lipoproteins · GERL · Golgi apparatus · hepatocytes · acid phosphatase · thiamine pyrophosphatase

In a variety of cell types it has been possible to discriminate between the innermost (trans [10]) element of the Golgi apparatus and adjacent GERL, considered by us to be a specialized area of endoplasmic reticulum (ER); that distinction is based on different cytochemically demonstrable phosphatase (Pase) activities. The trans Golgi element exhibits thiamine pyrophosphatase (TPPase) activity, but not acid phosphatase (AcPase) activity. The reverse is true of GERL; it shows AcPase and no TPPase activity.

Recently, we reported the cytochemical identification of GERL in hepatocytes of rats in experiments in which the organelle is much enlarged. By feeding rats orotic acid (OA), markedly fatty livers develop, and subsequent addition of chlorophenoxyisobutyrate (clofibrate or CPIB) to the OA diet is followed by clearing of the lipid from the hepatocytes. The morphological signs of Golgi apparatus involvement in very low density lipoprotein (VLDL) secretion disappear in OA-fed rats (38) and return to normal when the rats are fed the reversal (CPIB + OA) diet (36); also, apoprotein B disappears from the serum during OA feeding and reappears with CPIB + OA feeding. With the CPIB + OA diet, GERL elements are much enlarged, distended with lipoprotein (LP) particles. Morphological identification of GERL was recently confirmed by cytochemistry (35, 39).

These studies led us to extend fragmentary observations made in various laboratories, including our own, on hepatocytes of normal rats. To our knowledge, the present report is the first systematic study of GERL in untreated rats. In normal hepatocytes, the organelle is less extensive than in rats on the CPIB + OA diet, but the pattern of cytochemically demonstrable phosphatase activities is the same. This study demonstrates that hepatocytes, as in other cell types (34, 29,

33, 31, 17, 18), show TPPase activity in the trans element[1] of the Golgi apparatus and AcPase activity in GERL.[2] The study shows AcPase reaction product to accumulate in the same structures in which LP particles are present. The observations suggest that LP may undergo transformations inside GERL and inside the residual bodies which appear to be derived from GERL. For a review of earlier observations dealing with probable transformations of LP and other substances in GERL of various cell types, see reference 29.

MATERIALS AND METHODS

Six male Sprague-Dawley rats (Holtzman Co., Madison, Wis.), 350–400 g when sacrificed, were used. The rats were maintained under standardized lighting conditions, with ready access to water and Purina rat chow (Teklad Mills, Winfield, Iowa). All liver samples were removed from etherized rats between 10 a.m. and 12 noon. Fixations were performed in four ways: (*a*) Perfusion fixation in aldehyde. Under light ether anesthesia, isotonic saline (room temperature) was perfused, via the portal vein, for 2 min at a flow rate of 8 ml/min followed by perfusion of fixative at room temperature for 6 min. The fixative, a modification (24) of that introduced by Karnovsky (21), consists of 2.5% glutaraldehyde (Ladd Research Industries, Inc., Burlington, Vt.)–2% formaldehyde (prepared from paraformaldehyde)–, 0.09 M cacodylate buffer, pH 7.4, 0.025% $CaCl_2$, and 5% sucrose. After this brief perfusion fixation, at room temperature, thin slices were made and fixed by immersion for another 80 min in the same fixative, but at ice temperature. (*b*) Fixation as in (*a*), followed by postfixation for 60 min in cold 1% OsO_4 buffered with 0.1 M cacodylate buffer, pH 7.4. (*c*) Immersion fixation directly in cold 1, 2, or 4% OsO_4 buffered with either 0.1 M phosphate buffer, pH 7.4, or veronal-acetate buffer, pH 7.4, and containing 5% sucrose. The tissue was diced immediately into pieces ca. 1 mm^3. (*d*) Immersion fixation of small pieces directly in cold aldehyde fixative, with 5% sucrose, which were sliced and kept in fixative for 90 min. Fixation procedures (*b*) and (*c*) were among the attempts at achieving better preservation and greater electron opacity of the VLDL particles in the Golgi elements, e.g., use of 60 kV in electron microscopy (10), in unincubated tissue. All procedures yielded negative results in our hands.

The slices fixed by procedures (*a*) and (*d*) were used to prepare nonfrozen sections with a Sorvall TC-2 tissue sectioner (DuPont Instruments, Sorvall Operations, Newton, Conn.), set at 25 μm, by the procedure of Smith and Farquhar (42). These sections were incubated, for either AcPase or TPPase activity, in Stender dishes, shaken in a Dubnoff Metabolic Shaking Incubator, at 37°C. The TPPase medium is that of Novikoff and Goldfischer (30). It consists of 25 mg of TPP, sodium salt (Sigma Chemical Co., St. Louis, Mo.); 7 ml of distilled water; 10 ml of 0.2 M Tris-maleate buffer, pH 7.2; 5 ml of 0.025 M manganese chloride; 3 ml of 1% lead nitrate; and 1.25 g of sucrose. The medium is filtered before use and renewed every 30 min of incubation. The AcPase medium is that of Novikoff (26).[3] It consists of 25 mg of CMP, sodium salt (Sigma Chemical Co.); 12 ml of distilled water; 10 ml of 0.5 M acetate buffer, pH 5.0; 3 ml of 1% lead nitrate; and 1.25 g of sucrose. The medium is filtered after a precipitate forms.

In the study of unincubated tissue, desired areas of the liver lobules were selected by preparing 1- to 2-μm thick Epon sections stained with 1% toluidine blue, in 1% borate buffer, pH 10.0 (Sigma Chemical Co.), briefly heated on a hot plate.

Incubated nonfrozen sections were rinsed in cold 7.5% sucrose and fixed in cold 1% OsO_4 buffered with 0.1 M cacodylate buffer, pH 7.4, and containing 5% sucrose, for 1 h. Subsequent processing for electron microscopy was the same as with unincubated tissue (36) except that the sections were embedded in Epon lying flat in BEEM capsule covers (Better Equipment for Electron Microscopy, Inc., Bronx, N. Y.). For selection of desired areas, 1- to 2-μm Epon sections were heated in ca. 12% ammonium sulfide, on a hot plate, until visualization of lead phosphate sites was complete, generally about $^1/_2$ min.

Hepatocytes in centrolobular, midzonal, and periportal areas were studied, in unincubated tissue, in sections

[1] The present purpose requires that the trans element shows TPPase activity, as it does, so that it is distinguishable from GERL which lies adjacent to it. Whether another TPPase-positive element is present adjacent to the trans element is irrelevant to this purpose. Intensive studies by one of us (P. M. Novikoff) established that in the Golgi apparatus of neurons in rat dorsal root ganglia (37) there is a geometrically complex single TPPase-positive element (see Fig. 40 in reference 37) although in any given thin section this element might appear as two or more elements, as in Figs. 3, 4 and 8–10 in reference 37). In the present manuscript, the micrographs (particularly Figs. 7 and 8) suggest that the same may be true in rat hepatocytes. However, the procedures used in the neuron have not been performed and thus we simply refer to "the TPPase-positive element."

[2] Some of these results have been presented in abstract form (40).

[3] In our laboratory, cytidine-5'-monophosphate (CMP) rather than β-glycerophosphate (βGP) is used routinely as the substrate for the AcPase reaction. There is less diffusion artifact, and crisper localizations are obtained. Since βGP is often used as substrate in biochemical assays of lysosomes, in some experiments βGP as well as CMP was used. The localizations of reaction product were the same with both substrates.

incubated for AcPase activity and in sections incubated for TPPase activity.

RESULTS

Unincubated Tissue

The structures in the Golgi zone are essentially similar in hepatocytes irrespective of their position in the hepatic lobule (Figs. 1–6). These observations, as well as those on incubated tissue described below, were made on a great many hepatocytes of the six animals.

Thin sections used in electron microscopy show random views of portions of the Golgi apparatus, each portion designated as a Golgi stack. Golgi stacks of hepatocytes, when sectioned transversely, usually show three or four smooth-surfaced cisternae, or elements, closely apposed to each other. These elements possess numerous dilatations which contain VLDL particles, best seen in Figs. 1, 2, 5, and 6. GERL is situated trans to the Golgi stack. Some portions are closely juxtaposed to the trans Golgi element (Figs. 3, 5, and 6). Other portions of GERL are separated from the Golgi stack by variable distances, with smooth-surfaced vesicles or tubules located between the two organelles (Figs. 1–6).

In hepatocytes, GERL consists of smooth-surfaced cisternae and tubules, anastomosing in areas (Figs. 2 and 3–6), dilated by LP particles in numerous regions (Figs. 1–6), and compacted in other places to form "rigid lamellae" (a term adopted [29] from Claude [6] who described such compacted ER in the Golgi zone of hepatocytes) (Figs. 5 and 6). In some regions, finely granular material accumulates within GERL (Fig. 5, small arrow). Some areas of GERL may be coated (C in Figs. 2 and 4). The configurations of these coated areas suggest that they are forming coated vesicles by the criteria of Palade and Bruns (41). As will be seen below, all the components of GERL and all derivatives of GERL (coated vesicles and residual bodies) display AcPase activity. An electron-lucent area, or "halo" (7, 28), is often seen beneath the inner leaflet of the delimiting membrane of GERL (Figs. 2 and 4) and residual bodies (Fig. 4).

The LP particles in GERL tend to appear more electron-opaque than the VLDL particles in the Golgi elements (Figs. 1–6). As noted earlier, in rats fed the CPIB + OA diet (38, 36), images of LP particles in GERL and residual bodies apparently forming from GERL suggest that they are undergoing transformation. They are irregular in size and shape (Fig. 4; also Figs. 7 and 8 in reference 36, and Fig. 1 in reference 39). Often the residual bodies show a finely granular material together with the LP particles (Fig. 4).

Figs. 1 and 3 strongly suggest that LP-containing elements of GERL are continuous with smooth ER that, in turn, continues to rough ER, i.e., ribosome-studded ER.

Other types of residual bodies are seen, as in Figs. 3 and 6.

Incubation for TPPase Activity

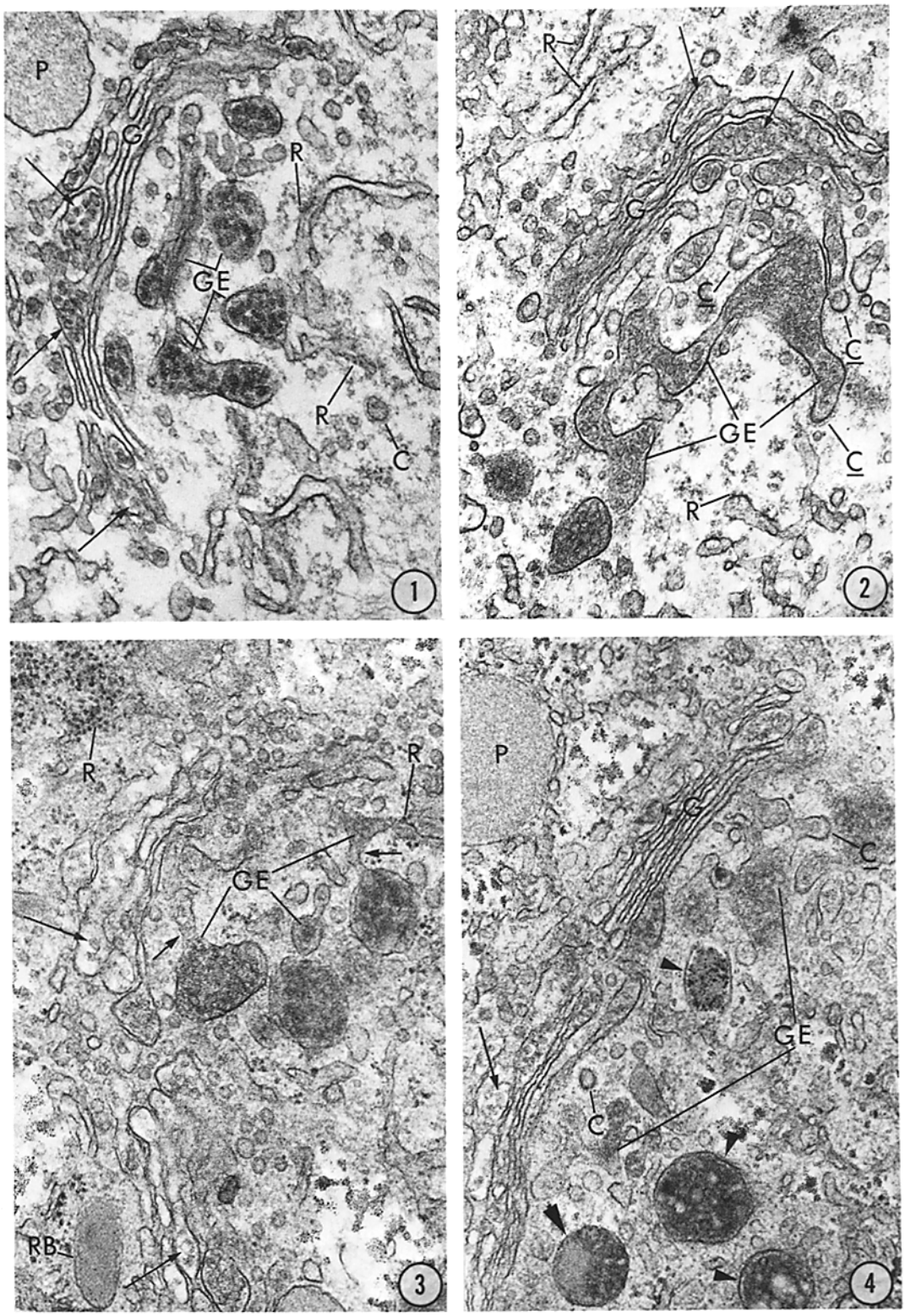
Typical results of incubating sections of normal liver for TPPase activity are seen in Figs. 7–9. Reaction product is present in the ER (Figs. 8 and 9), as previously noted (27, 5, 14). Dense reaction product is present in the trans element of the Golgi stack, with the VLDL particles standing out in negative relief. The arrowhead in Fig. 7 indicates a dilatation interpreted as a forming secretory vacuole; presumably it would have detached and moved to the space of Disse where it would empty its VLDL. The same is probably true of the dilatations indicated by arrowheads in Fig. 8.

As in the unincubated material, LP particles usually show a greater electron opacity in GERL than in the Golgi stack. The particles are heterogeneous in size and shape, as seen in Figs. 7 and 9. Such heterogeneity is clearly evident in Fig. 4 of Cheetham et al. (5).

Incubation for AcPase Activity

The appearances of hepatocytes in sections of liver incubated for AcPase activity are seen in Figs. 10–12. These greatly strengthen the identification of the components and derivatives of GERL in unincubated material (Figs. 1–6): cisternae distended by LP particles (Figs. 10 and 11), LP-containing residual bodies (arrowheads in Figs. 11 and 12), and coated vesicles (Fig. 11). In sections of AcPase-incubated material, the LP particles can be seen only if the tissue has not been overincubated to the point where they are obscured by reaction product, e.g., the components of GERL at the left side of Fig. 10.

What appear as separate vacuolar structures, such as those in Figs. 11 and 12, might prove by serial sectioning (not performed on this material) to be attached to GERL in some cases, as seen in Fig. 10.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Continuities between GERL and Golgi elements were not found despite intensive search, especially between the trans Golgi element and GERL, in both unincubated and incubated tissue.

DISCUSSION

The observations presented above establish the existence of GERL in normal rat hepatocytes. As in other cell types (28, 33), GERL possesses ultrastructural features and demonstrable cytochemical enzyme activities characteristic of residual body-type lysosomes. Ultrastructurally, in hepatocytes it includes anastomosing tubules and cisternae that may be dilated by LP particles or compacted to form "rigid lamellae" (Figs. 5 and 6) (see also Fig. 6 in reference 33) and coated areas. As in pancreatic exocrine cells (31), these coated areas appear to give rise to coated vesicles (Figs. 2 and 4). These vesicles show AcPase activity (Fig. 11) and would be considered as primary lysosomes (28), but their functional roles in hepatocytes are not clarified by the present observations. It might be argued that the coated vesicles, rather than separating from GERL, in fact merge with GERL. If so, the possibility of some kind of crinophagy (13) would need to be entertained. However, the numerous micrographs we have are more readily viewed, using the criteria of Palade and Bruns (41), as vesicle separation rather than merger. The delimiting membranes of both GERL and residual bodies are thicker than that of ER generally, and an electron-lucent region, often referred to as a halo, lies under the inner leaflet of the membrane (Figs. 2 and 4). Both organelles, GERL and residual bodies, show no TPPase activity but do display AcPase activity and, it is assumed, other hydrolases as well. Aryl sulfatase and thiolacetic esterase activities have been demonstrated in GERL of neurons and megakaryocytes (9, 2). In hepatocytes, there would appear to be a developmental continuum between LP-filled dilatations and residual bodies containing LP undergoing transformation in normal rats and in rats in which marked lipoprotein mobilization is induced (39). Other origins of residual bodies are present in hepatocytes (19).

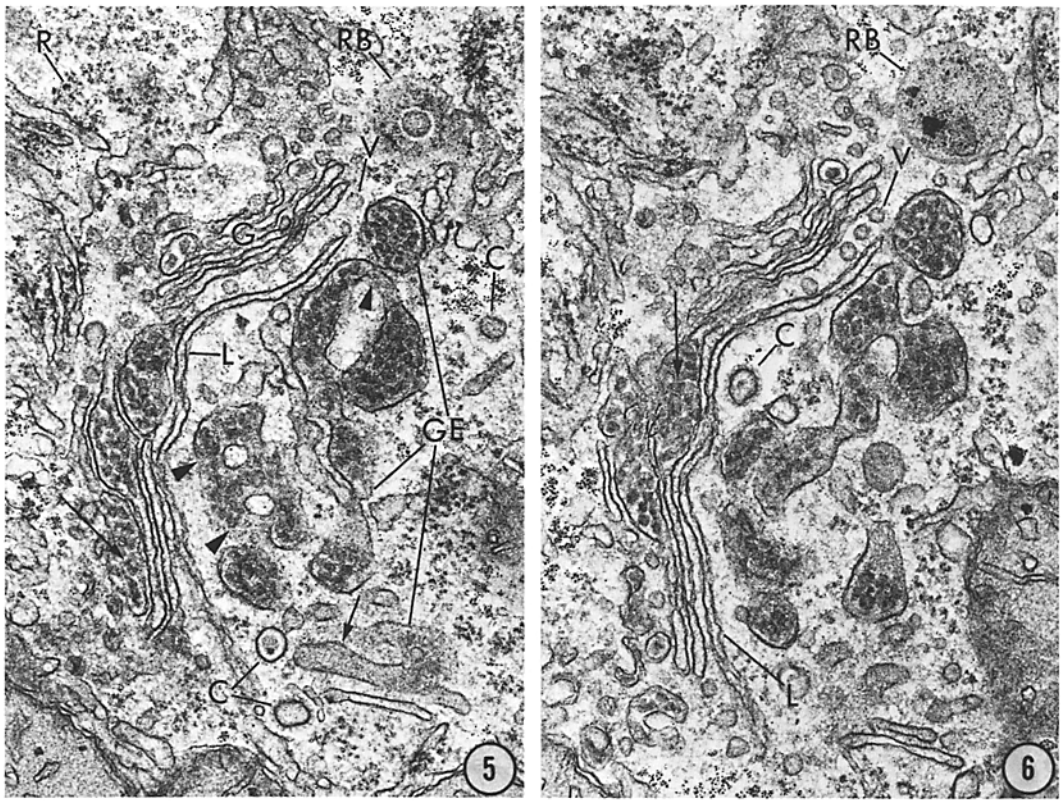
FIGURES 1–4 Unincubated material. × 44,000.

FIGURE 1 Periportal hepatocyte. Fixation: 4% OsO_4, phosphate buffer [Fixation (c)]. VLDL particles are present in dilated elements (arrows) of the Golgi stack (*G*). In GERL (*GE*) the LP particles are more electron-opaque. Ribosomes are marked by *R* in two regions of rough ER that appear to be continuous with GERL; a portion of the ER in the upper region is out of the plane of section. A coated vesicle is seen at *C*. Also in the field is a peroxisome (*P*).

FIGURE 2 Centrolobular hepatocyte. Fixation: 2% OsO_4, veronal-acetate buffer [Fixation (c)]. The arrows indicate dilated elements of the Golgi stack (*G*) containing VLDL particles. Three coated areas of GERL are indicated by C. Ribosomes (*R*) are seen on rough ER.

FIGURE 3 Centrolobular hepatocyte. Fixation: aldehyde perfusion fixation followed by postfixation in 1% OsO_4, cacodylate buffer [Fixation (d)]. The long arrow indicates a dilated portion of a Golgi element containing VLDL particles. The LP particles in GERL (*GE*) are more electron-opaque than the VLDL particles. At upper left, *R* indicates ribosomes in tangentially sectioned rough ER; at upper right, *R* indicates ribosomes on membrane of perpendicularly sectioned rough ER. The two short arrows indicate continuities of GERL cisternae with dilated regions containing LP particles; above the arrow to the right, GERL appears to be continuous with rough ER. A residual body is seen at *RB*. Its delimiting membrane is seen below the leader; note that the membrane is more distinct than in the ER and that a narrow electron-lucent zone is present beneath the membrane.

FIGURE 4 Centrolobular hepatocyte. Fixation: as in Fig. 3. A long arrow indicates a dilated element of the Golgi stack (*G*) containing VLDL particles. The LP particles are seen in GERL (*GE*) and in three apparent vacuoles in the lower portion of the field. The smaller arrowheads indicate where the relatively thick membrane and halo are evident in GERL (near center) and two apparent vacuoles (near bottom). The term "apparent vacuoles" is used because serial sections were not performed to determine whether they would be attached to GERL in other section planes as in Figs. 1 and 3. A coated vesicle is seen at *C*, and at C a coated area of GERL. Note the size heterogeneity of the LP particles in the two apparent vacuoles. The larger arrowhead indicates an apparent vacuole in which the left portion is occupied by a granular material.



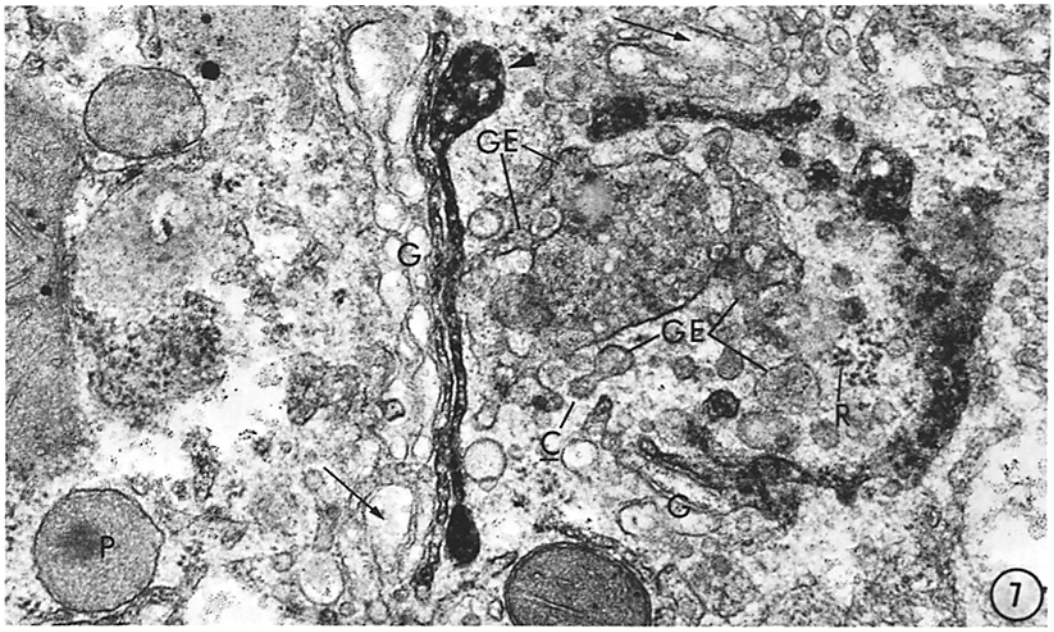
FIGURES 5–6 Unincubated material. Fixation: as in Fig. 2. Serial sections. Long arrows indicate dilatations of Golgi elements containing VLDL particles. The anastomosing nature of GERL (*GE*) is more apparent in Fig. 5 (arrowheads) than in Fig. 6. The LP particles in GERL are electron opaque (cf. Fig. 1). The short arrow in Fig. 5 indicates a cisterna of GERL dilated by finely granular material. Note the length of the compacted cisterna of GERL or "rigid lamella" (*L*), and the small vesicles (*V*) between it and the Golgi stack. Three coated vesicles are seen in Fig. 5 (*C*), and another one in Fig. 6 (*C*). × 43,000.

FIGURES 7–9 TPPase-incubated material. Fixation: aldehyde perfusion and immersion [Fixation (a)]. Incubation: TPPase medium, 90 min, 37°C. × 44,000.

FIGURE 7 The TPPase-positive trans element of the Golgi stack (*G*) is sectioned transversely at the left and tangentially at the right. The arrowhead is directed towards a dilatation of the trans element that would probably have formed a secretory vacuole to be transported to the space of Disse. Note the VLDL particles in negative relief in the vacuole and elsewhere in the trans element. The dilatations of the other Golgi elements containing VLDL particles are indicated by arrows. Note the extensive nature of GERL (*GE*) and the heterogeneity in size of the LP particles within its dilatations. A coated region of GERL is indicated by C. Some ribosomes are indicated by *R* and a peroxisome by *P*.

FIGURE 8 The ER shows some reaction product (*ER*) as well as the trans element of the Golgi stack (*G*). Arrowheads indicate dilatations of the trans element that would probably have formed secretory vacuoles. GERL (*GE*) is devoid of reaction product and contains LP particles.

FIGURE 9 The *ER* shows light deposits of reaction product (*ER*) whereas the reaction product in the trans Golgi element is dense. The long arrows indicate dilatations of other Golgi elements containing VLDL particles. The area circumscribed by the trans element is occupied by GERL, except for small lengths of ER. The short arrows indicate some of the dilatations of GERL filled with LP particles. The arrowhead indicates an apparent vacuole containing LP particles which appear heterogeneous in size.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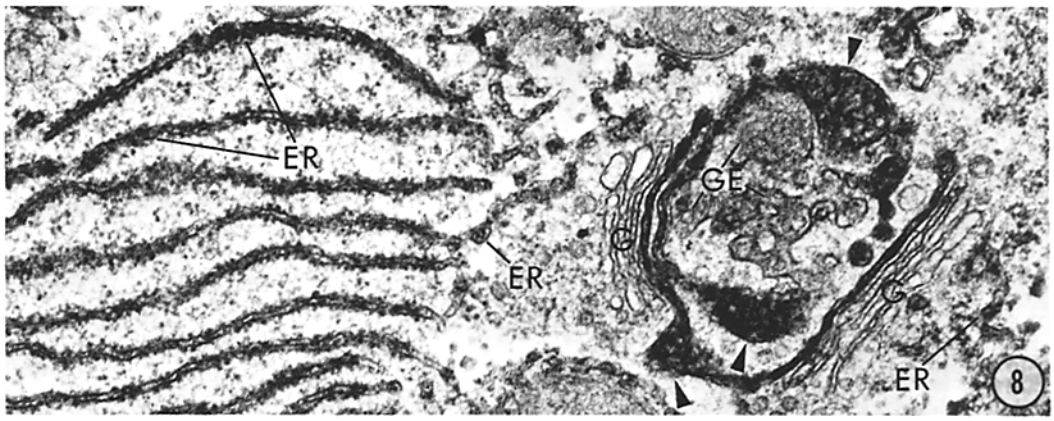
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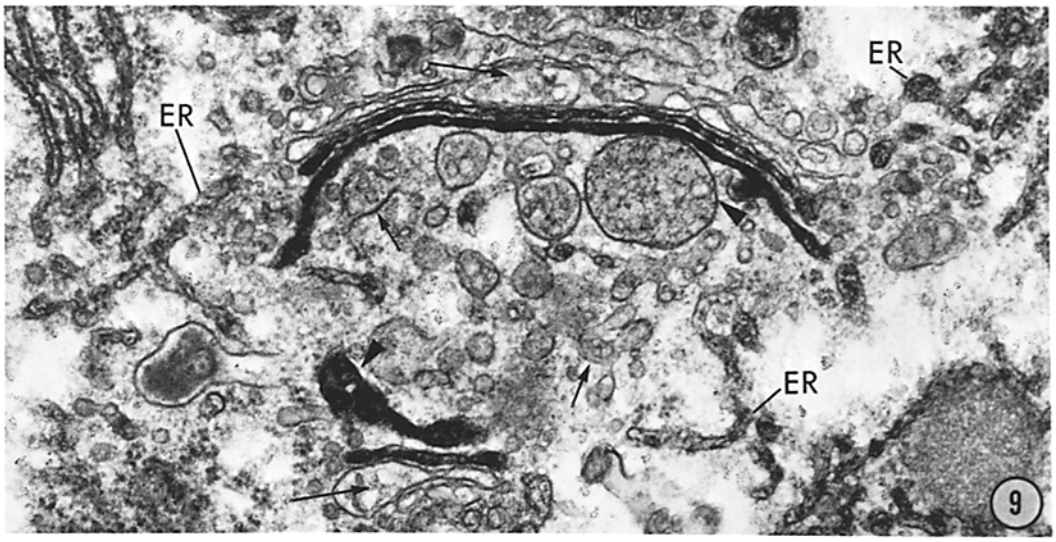
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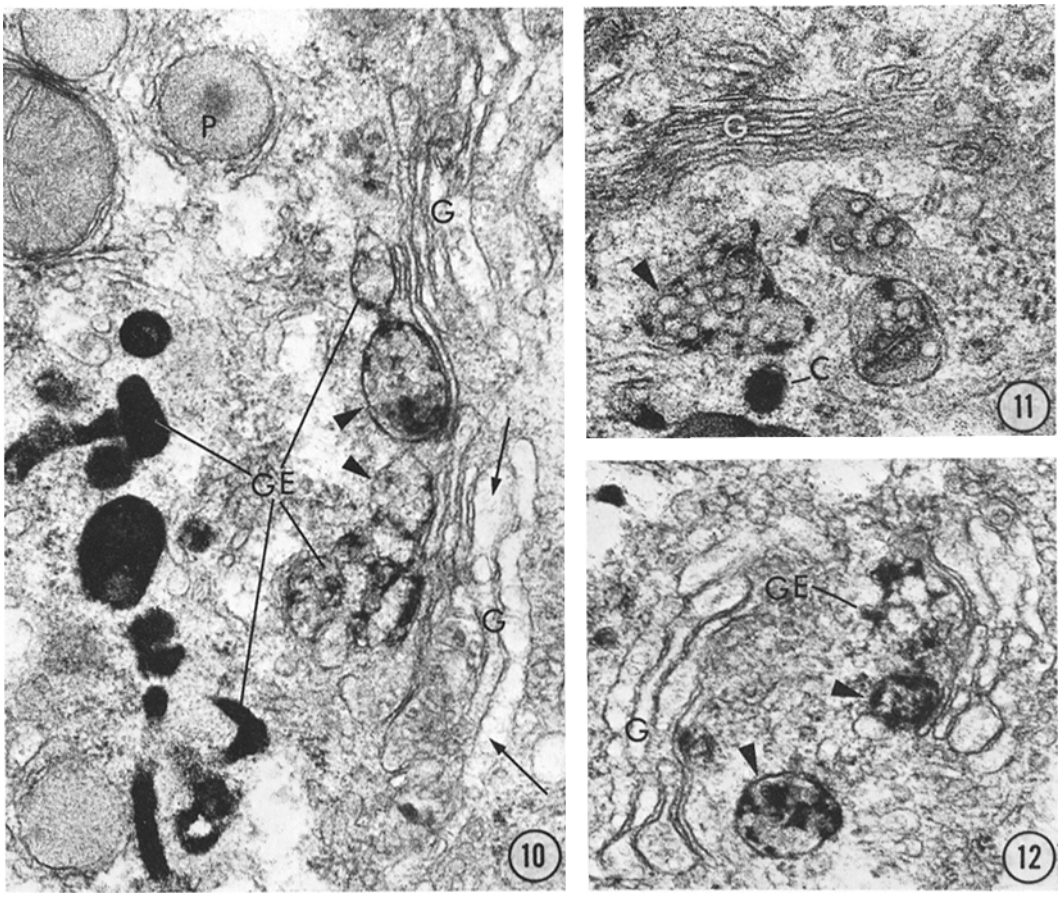

FIGURES 10–12 AcPase-incubated material. Fixation: aldehyde perfusion and immersion [Fixation (a)]. Incubation: AcPase medium, 40 min (Figs. 10 and 12) or 13 min (Fig. 11), 37°C. Fig. 10, × 45,000; Fig. 11, × 57,000; and Fig. 12, × 42,000.

FIGURE 10 Reaction product obscures the contents of GERL in the left portion, but not in the right portion. This difference in quantity of reaction product probably results from different penetration of reagents into different portions of the nonfrozen section. The extent of GERL (*GE*) is impressive. The two arrowheads indicate dilatations of GERL which show both reaction product and LP particles. Note the absence of reaction product from the Golgi stack (*G*). The arrows indicate dilatations of the Golgi elements, with the VLDL particles barely evident.

FIGURE 11 Reaction product is absent from the Golgi stack (*G*), is abundant in a coated vesicle (*C*), and is present in a dilatation of GERL containing LP particles (arrowhead).

FIGURE 12 Reaction product is absent from the Golgi stack (*G*) but is present in GERL (*GE*). Arrowheads indicate two apparent vacuoles containing both reaction product and LP particles.

Those residual bodies in Fig. 3 (*RB*) and Fig. 4 (small arrowhead in center of field) may have originated by accumulation of electron-opaque grains within GERL. The residual body in Fig. 6 may well have arisen from an autophagic vacuole.

Cytochemistry has helped identify the diversity of morphological forms and possible functional activities of GERL among the cell types in which this organelle is present (29, 33). The conversion of the TPPase-positive trans elements of the Golgi stacks to GERL, the possibility of which cannot be excluded, seems most unlikely for reasons discussed by Novikoff et al. (31).

Farquhar et al. (12) have reported cytochemical

studies of sections and subcellular fractions of liver from ethanol-treated rats. Though scantier, reaction product localizations were consistent with those reported here. These authors noted that reaction product was not found in "the stacked cisternae" of the Golgi apparatus. They wrote that product was present in "some of the VLDL-filled secretory droplets" . . . "along the secretory Golgi face," and in some VLDL-filled vacuoles present in the Golgi-enriched fractions. Ma and Biempica (23) have described similar TPPase and AcPase localizations in a human liver biopsy specimen.

An important unsettled issue is the manner by which LP particles gain access to GERL. We interpret the areas marked *R* in Fig. 1 and the short arrow in Fig. 3 at the right as showing loss of ribosomes (degranulation) where rough ER transforms into GERL; for similar interpretations in pancreatic exocrine cells and other cell types, see references 34, 33, and 31. If this interpretation is correct, then lipoproteins could gain access to GERL directly from the ER. The presence of apoprotein B in GERL of rat hepatocytes is suggested to us by Fig. 6 in Alexander et al. (1) where peroxidase-labeled antibody was used to reveal intracellular sites of the apoprotein.

The LP transformations which appear to occur in GERL and in residual bodies that may arise from it involve their size and shape. The LP particles become irregular and larger, and it is common to find residual bodies (at large arrowhead, Fig. 4) in which the particles are replaced by a homogeneously electron-opaque material in portions of the residual bodies. Such transformation of LP particles could involve their protein components (25) and other moieties as well as their lipids. That lysosomal activity can yield products with biological activity is now well known. An early example, and still one of the most interesting, is the release of thyroid hormone by partial hydrolysis of thyroglobulin within the residual bodies of thyroid epithelial cells (43, 32). It remains to be learned whether products of LP transformation (catabolism, etc.) in hepatocytes are secreted into the circulation and, if so, what the mechanisms are by which such secretion occurs.

It has long been known that lipases are present in liver, including one or more localized in lysosomes (see references 22–27 in Debeer et al. [8]). That interconversions of VLDL and low density lipoprotein (LDL) may occur in the liver is suggested by the data of Chapman et al. (3, 4). These authors showed the presence of LDL as well as VLDL in a Golgi-enriched fraction isolated from homogenates of guinea pig liver. In the rat, it is not known whether LDL is synthesized de novo or formed from VLDL in the liver (11, 19).

The findings recorded here, which demonstrate the existence of new sites of LP particles, need to be validated by isolating GERL, and the residual bodies apparently derived from it, from rat liver homogenates. Even where the LP particles appear to be in dilatations of GERL (Fig. 10) rather than in separated residual bodies (Fig. 12), homogenization might separate the dilated areas with their contents preserved. This would involve rupture of the delimiting membranes with immediate resealing, in the manner that ER, during homogenization, gives rise to microsomes. Knowing that some VLDL-containing vacuoles show TPPase activity and that other LP-containing vacuoles show AcPase activity might help to separate the two types of vacuoles. Their abilities to liberate phosphate ions under specified conditions can be used, as Leskes et al. (22) did for glucose-6-phosphatase-rich microsomes; the phosphate is trapped by the lead in the medium and the lead increases the density of the vacuoles. However, separations by isopycnic centrifugation on sucrose gradients might suffice.

Were such isolation successful, the characterization of the LP particles might enable direct search for secretion products of their transformation, through the use of isolated perfused livers as was established definitively for VLDL by Hamilton et al. (16) and Jones et al. (20).

It is to be noted that the hepatocyte differs from the other secretory cell types in which GERL, rather than the Golgi apparatus, has been shown to be the site of packaging secretory material (34, 29, 33, 31, 17, 18). VLDL is packaged by the Golgi apparatus and, as noted above, whether there is secretion of any materials from GERL is unknown. On the other hand, hepatocytes are like the other cell types studied by Novikoff (29), Novikoff et al. (34, 38, 31), and Hand and Oliver (17, 18), in that morphologic continuities between Golgi stack and GERL have not been found. This, however, does not exclude functional interrelationships between the two organelles.

There is a minor discrepancy between the reports of the Novikoff group, who find that the trans Golgi element shows no AcPase activity, and Hand and Oliver who report that "occasion-

ally" the TPPase-positive trans element also shows AcPase activity (18). However, we would consider that the structure in the micrograph illustrating such AcPase activity (Fig. 15 in reference 18) is part of GERL rather than the "innermost Golgi saccule" (18). If so, it would be consistent with our findings in hepatocytes.

As investigations of GERL and its derivatives are pursued in different cell types, our horizons concerning possible metabolic events within these structures will expand. A striking example is the finding of Gonatas et al. (15) that lectin-bound plasma membrane is internalized to GERL in cultured neurons and neuroblastoma cells.

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