GROWTH AND DIFFERENTIATION OF CYTOPLASMIC MEMBRANES IN THE COURSE OF LIPOPROTEIN GRANULE SYNTHESIS IN THE HEPATIC CELL

I. Elaboration of Elements of the Golgi Complex

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ABSTRACT

The synthesis of "very low" density lipoprotein in liver cells is characterized by the fact that the synthesized products, mostly triglycerides, are processed in the form of discrete, size-limited granules or globules, about 400 A in diameter. The present investigation has been made possible in part by the use of a fixative (OsO_4 in bidistilled H_2O at pH 6.0, in the absence of electrolytes) particularly effective in preserving cytoplasmic membranes and lipids, and giving them high stainability and differential contrast. Under these technical conditions, the lipoprotein granules retain their morphology and high density to electrons practically unaltered, and may serve as tracers in determining their route of transport from the sites of synthesis, starting at the rough-smooth ER junctions, to the lumen of Golgi concentrating vesicles. From the observations, it may be deduced that, along with lipoprotein granule synthesis and transport, there are also production and transfer of new membranes in the form of tubular extensions of smooth ER network which, by progressive fusion and coalescence, participate in the elaboration of fenestrated plates and solid Golgi sacs. In contradistinction to the entire process of liver lipoprotein granule synthesis, transport, and segregation, as reported in the present paper, appears to constitute a developmental sequence which includes the following communicating compartments, in consecutive order: cisternae of rough ER where proteins and possibly phospholipids are synthesized, smooth ER network where triglycerides are synthesized and transported in the form of dense granules, fusion of smooth ER tubular extensions into Golgi fenestrated plates, and further coalescence into solid Golgi sacs, ending in the segregation of the granules in appended concentrating vesicles, or detached "secretory vesicles." It seems that it is this progressive evolution in growth and configuration of membranes which is reflected in the so called polarity, from forming to mature faces, of the Golgi apparatus.

INTRODUCTION

The Golgi apparatus, as revealed by early studies of tissue sections by electron microscopy, appeared as a distinct although complex entity, consisting of three main components: (*a*) the most characteristic, represented by stacks of elements variable in size and number, in the form of flattened sacs or lamellae; (*b*) large "vacuoles"; and (*c*) groups of small vesicles. This general description, presented as typical for the composition of the Golgi apparatus in a variety of animal and plant cells, by

Dalton and Felix in 1956 (11, 12), has been retained as an elementary concept in subsequent works concerned with the fine morphology, origin, and functions of the Golgi structures. A definite, intrinsic polarization of the elements of the Golgi complex, as regards both form and function, has been recognized, the large "vacuoles," interpreted as condensing or secretory vesicles, being generally found next to or at the mature face, the small vesicles being more numerous at the forming face of the Golgi stack. In recent years, considerable progress has been made towards the understanding of particular functions of the Golgi apparatus, namely its specific role in protein-carbohydrate metabolism (36, 35, 39, 40), and the packaging of synthesized products, for storage (3, 4), or for export.

Mollenhauer and Morré (33), for dictyosomes of plant cells, and, more recently Flickinger (14), in the case of the epididymis of rodents, have proposed models, quite similar in their diagrammatic representations, meant to account for certain morphological findings, especially the demonstration of fenestrated cisternae (14) and an anastomosed tubate system (33) occurring in close association with the solid membrane classical Golgi sacs, and observed in both microscopical sections and negatively stained preparations (10). To date, unequivocal information concerning the origin, growth, and differentiation of the membrane structures which constitute the Golgi apparatus is still lacking (5, 14, 33).

In the present work, advantage has been taken of the fact that, in the course of lipoprotein granule synthesis in the hepatic cell, the synthesized product may be used as a marker during its transport, in a particulate form directly detectable by electron microscopy, from its site of origin through the successive compartments of the smooth endoplasmic reticulum, Golgi cisternae, and concentrating vesicles. During this process, definite correlations have been observed between tubular elements of the smooth ER network, the elaboration of fenestrated Golgi plates, and solid membrane Golgi sacs. Some of these observations and conclusions have appeared in a condensed and abstract form (9, 8).

As observed in the present study, the metabolism of lipids involved in the production and disposal of lipoprotein granules in the hepatic cell includes, morphologically and biochemically, a number of separate steps: (*a*) the uptake of precursors at the cell border, and their transport to the endoplasmic reticulum; (*b*) the synthesis of the lipidic and protein moieties and their assembly into lipoprotein elements of definite size, and their transport within channels of the endoplasmic reticulum; (*c*) temporary storage of the granules in vesicular extensions of the smooth ER; (*d*) elaboration of elements of the Golgi complex with participation of the smooth ER system; (*e*) direct disposal of the granules by membrane fusions and extrusion into the space of Disse; (*f*) degradation and relocation of the products, part of these in the form of large liposomes. The present paper will be restricted to the morphological aspects of the (*b*), (*c*), and (*d*) stages of the sequence with special attention to the elaboration of elements of the Golgi complex. Observations on the disposal of lipoprotein granules (steps *e*, and *f*) will be the object of a subsequent report.

MATERIALS AND METHODS

Partial Hepatectomy

20 female albino rats, 72 days old and from 200 to 220 g in weight, fed commercial[1] laboratory chow with water *ad libitum*, were used. Partial hepatectomy was performed under light ether anesthesia, 55% or more of the liver (frontal and left lobes) being excised. The recovery of the treated animals was uneventful. A decrease in body weight was recorded 24 hr after hepatectomy, 7.4 g per rat in average, in addition to the weight of liver removed, a loss to be ascribed in part to the reduction of food intake during the same period. 48 hr after hepatectomy, 45% of the animals had regained weight. 7 days after hepatectomy, healing of the abdominal wound was complete and the body weights, from 206 to 224 g, were about 7 g per rat over the original figures.

Fasting and Feeding prior to Electron Microscopy

Rats from the group just described, i.e. subjected to partial hepatectomy 7 days previously, now 79 days old, and about 210 g each, were selected for the present study.[2] The animals were fasted by complete

[1] Wayne Lab Blox, U.S.A., and "Protector," Brussels, pellets.

[2] Partially hepatectomized rats were prepared because of reports that hepatectomy might increase the incidence of lipoprotein granules in liver cells. Since no significant differences were found, only fully recovered animals were used for the present work.

privation of food, but with water at will, for about 14 hr. They were then fed a meal composed of ground pellets of the laboratory chow, to which 20% of the weight of the pellets in fresh butter had been added as a source of neutral fats. The animals were sacrificed 2–3 hr later, and their livers were removed for electron microscopic study.

Electron Microscopy

FIXATIVES: Specimens from the same liver were systematically fixed by means of three different fixatives which are, to some extent, complementary: (*a*) 1% OsO_4 in 0.1 M phosphate buffer, pH 7.4, according to Millonig (31); (*b*) 2.5% glutaraldehyde in 0.09 M phosphate buffer, pH 7.4, followed by postosmication with 1% OsO_4 in 0.1 M phosphate buffer, pH 7.4 (43); and (*c*) 4% OsO_4 in bidistilled water, in the absence of electrolytes, pH 6.0 (7).

Since all the electron micrographs illustrating the present report correspond to material fixed with the latter technique, only the procedure using a solution of OsO_4 in twice-distilled water as fixative will be described in some detail. Osmium tetroxide is a neutral compound which, when in aqueous solutions, does not affect the pH of the medium. Thus, in the absence of electrolytes or contaminants, the pH of a solution of OsO_4 in distilled water reflects the amount of carbon dioxide which is dissolved in the liquid, under atmospheric pressure. In practice, water freshly distilled and protected from air should have a pH close to 7.0. On standing, the reaction becomes progressively more acid, reaching an equilibrium in CO_2 concentration, at room temperature, corresponding to a pH of about 5.9–6.1. It might be surmised that, in the absence of buffers, this reaction could not be maintained in the OsO_4-H_2O fixative upon the immersion of tissue specimens such as liver blocks. However, measurements during or after fixation show that this is not the case, the reaction of the fixing fluid remaining about pH 6.0 throughout the period of fixation, a fact suggesting that immediate fixation of the surface may seal the tissue blocks, thus preventing the leakage of substances with buffer capacities, including tissue electrolytes. This problem of fixation by OsO_4 in water has been discussed elsewhere in greater detail (7); the use of this fixative has been of definite advantage in the present study.

In addition to being an excellent fixative for cell structures as a whole, the fixative employed, i.e. OsO_4 in distilled water at pH 6.0, is particularly effective in preserving cytoplasmic membranes and lipids, and in giving them relatively high stainability and differential contrast. The apparent contrast is also enhanced in part by the fact that, due to the conditions of fixation, certain cell components such as glycogen, which may be abundant and cumbersome in liver cells, stain poorly or not at all with lead salts, permitting a better definition of the remaining cell structures. An example is provided in Fig. 1 where the lower part of the picture is occupied by glycogen. That glycogen is actually present may be demonstrated by restaining the section, first with uranyl acetate, a treatment which seems to make the glycogen responsive again to the lead stain.

FIXATION: The rats were killed by decapitation and free bleeding from the wound. The liver was exposed, and a lobe was transferred to a slab of dental wax. The lobe was cut in half and a transverse slice was made, about 1 mm thick and close to the center, but avoiding the large vessels. The trimmed slice was covered with the cold OsO_4-H_2O fixative, and cut into small blocks, 1 mm^3 or less in size. About 20–30 of these blocks, selected from different regions of the slice, were transferred into a vial containing 6 ml of the fixative, kept in melting ice. Routinely, the fluid was replaced by the same volume of fresh OsO_4-H_2O fixative within 5–10 min. but this step may not prove necessary. The procedure, including decapitation and deposition of the fixative on the liver slice, took 3½ min. and the cutting and selection of the blocks, 4–5 min. From then on, the vials were placed in a refrigerator while being kept at all times in melting ice, i.e. close to 0°C, for a total fixing time of 2½–3 hr. Direct dehydration in cold 70, 94, and 100% ethanol was also carried out at about 0°C. At the 100% ethanol stage, 12 tissue blocks, among those evenly cut and less than 1 mm^3 in size, were selected for embedding. The same operation was carried out in parallel, in the case of the other two fixatives listed, making in all a total of 36 tissue blocks, from the same liver lobe, and available for examination by electron microscopy. Impregnation in epoxy resin according to Luft (28) was made through successive baths of: (*a*) equal parts 100% ethanol-propylene oxide; (*b*) 100% propylene oxide alone; (*c*) equal parts propylene oxide-complete Epon mixture (Shell Chemical Co., New York), carried out at room temperature, the last mixture being kept overnight in a desiccator, in the presence of silica gel as absorbent for propylene oxide and water. Polymerization in gelatin capsules was carried out at 60°C for 3 days. Sections were cut with diamond knives on a LKB microtome (LKB Produkter, Stockholm, Sweden),

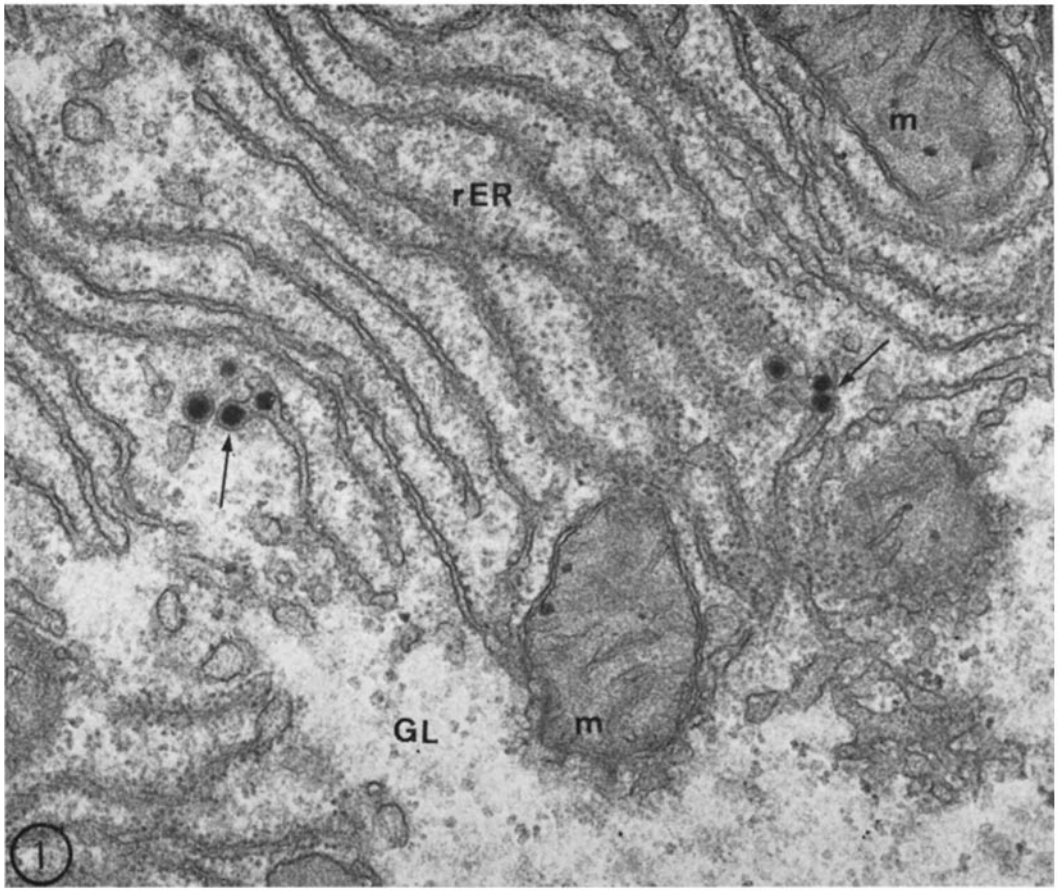


FIGURE 1 Stacks of rough ER lamellae (*rER*), some of them participating in lipoprotein granule synthesis. The granules have never been found in rough ER cisternae, but appear first at the smooth side (arrows) of smooth and rough ER junctions. The large white areas are occupied by particulate glycogen (*GL*) which, under the fixation procedure employed, is not stained by osmium or lead salts. *m*, mitochondria. × 49,500.

and stained with lead citrate, according to Reynolds (41). Electron micrographs were made at 60 kv, with a Philips EM 200 electron microscope.

OBSERVATIONS

Under normal physiological conditions, channels of the endoplasmic reticulum of hepatocytes contain inclusions in the shape of small dense granules, about 400 A in avaerage size. Chemical analysis by Schlunk and Lombardi (45) of such elements isolated by differential centrifugation has shown that they contain neutral fats as the main constituent, together with phospholipids and cholesterol in lesser proportions, and a protein moiety. Owing to their discrete form, definite sizes, and high reactivity with electron stains, these lipoprotein complexes[3] may be readily followed from their site of synthesis either to Golgi regions, or to the cell border.

Synthesis of Lipoprotein Complexes, together with Associated Smooth Membranes

Fig. 1 illustrates lipoprotein granules (arrows) enclosed in smooth ER protrusions, budding at

[3] Depending on technical procedures and the degree of tissue preservation, the size of the lipoprotein granules may vary and get larger. Under the experimental conditions used, their mean diameter has remained relatively constant, within the limits indicated.

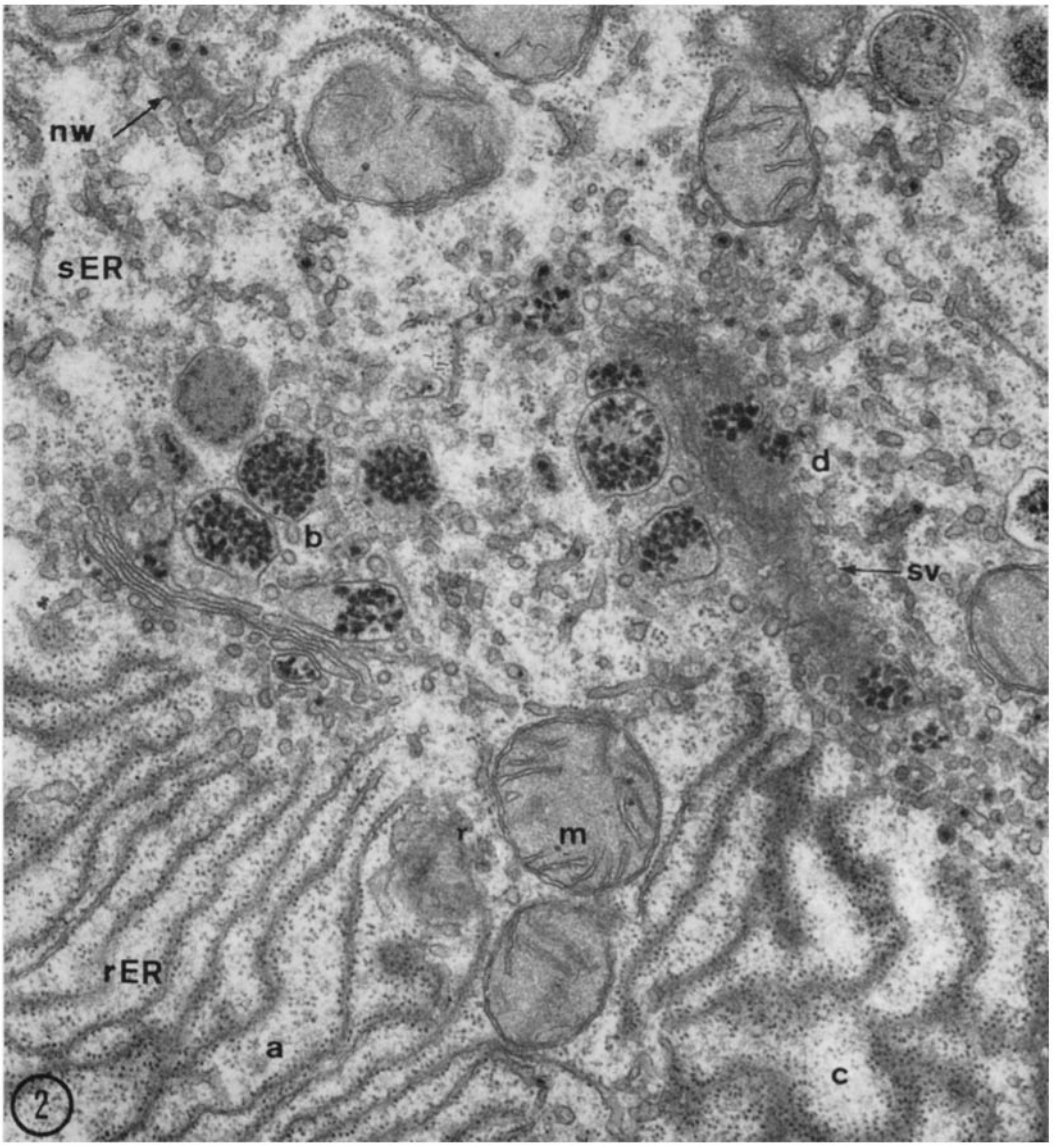


FIGURE 2 The micrograph illustrates two series of rough ER-smooth ER-Golgi complexes, disposed in functional order. At the left, the rough ER lamellae (*a*) and the Golgi elongated cisternae (*b*) have been sectioned crosswise and present sharp profiles, whereas the other group of rough ER (*c*) and Golgi apparatus (*d*) are seen in side view, as a result of oblique sectioning, suggesting the existence of a spatial relationship in the orientation of the two types of structures. Connections between small vesicles (*Sv*) and Golgi cisternae may be detected in oblique sectioning (*d*, and arrow), a relation rarely observed in cross-sections. A group of mitochondria (*m*) is responsible for diverting the rough ER lamellae into two separate groups, an effect leading to the production of two Golgi complexes, instead of one. In the upper part of the picture, a smooth ER network (*sER* and *nw*) from another rough ER (*rER*) stack participates also in the elaboration of the two Golgi complexes from the opposite direction. × 26,500.

the edge of rough ER lamellae. Out of several thousands of liver cell electron micrographs available for inspection in this respect, lipoprotein granules have never been observed within cisternae of the rough ER, their nearest position to the latter being on the smooth side of smooth-rough ER junctions, as shown in Fig. 1.

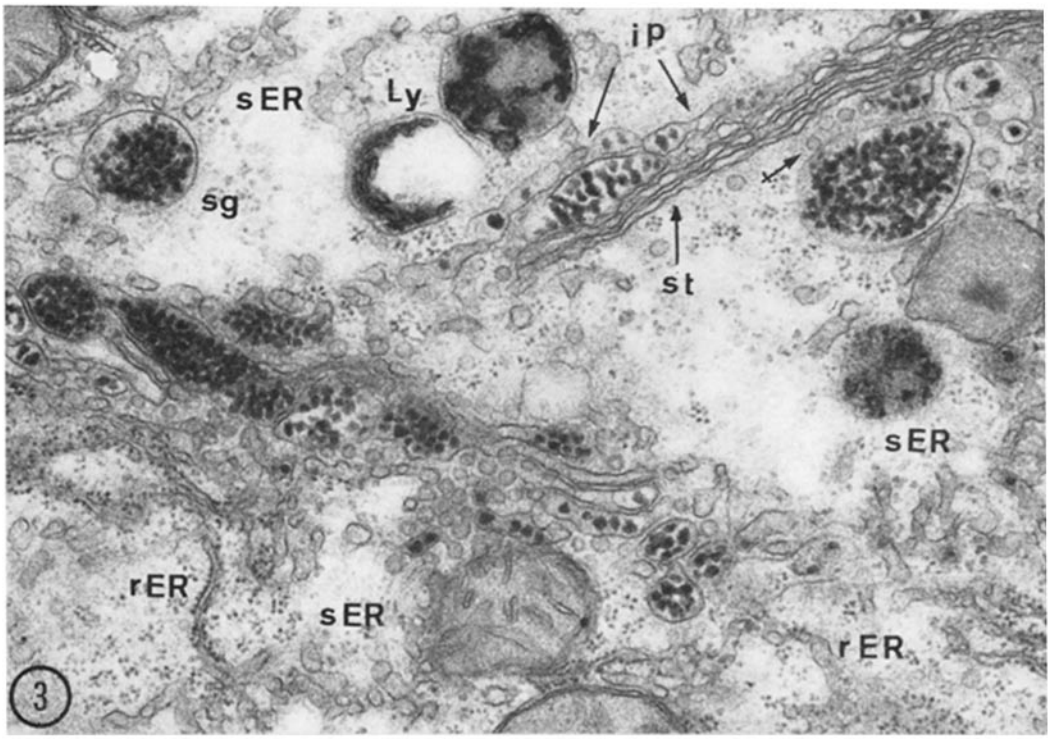
On the other hand, lipoprotein granules are observed at various levels of the smooth endoplasmic reticulum, precisely within a tubular network which is always found interposed between stacks of rough ER lamellae and corresponding Golgi apparatuses in which the granules are being segregated, as illustrated in Figs. 2–5 (also in reference 9, Figs. 5, 7, and 8). Two points of interest are: (*a*) the fact that lipoprotein granules, when in elements of the smooth ER, are never seen in clusters but singly or in rows, and, (*b*) that, as a rule, the granules do not come into direct contact with the profile of the smooth ER tubule, being held back in the center of the lumen by a relatively uniform distance of about 80 A; this particularity is illustrated in Figs. 1–13. These characteristic features may help in distinguishing, especially when dealing with cross-sections, between smooth ER and mature Golgi membrane structures.

Areas occupied by smooth ER networks, or found at the periphery of Golgi complexes, are frequently represented by what appear to be closed vesicles, small or large, whereas they actually represent cross-sections of tubular elements of the smooth ER, or of expanded portions of elongated tubules. The demonstration of continuity and intercommunications in such systems is made difficult by the fact that small tubules constantly weave in and out of the thin microscopical sections. The same difficulties are encountered in the study of the configuration and interrelations of variably oriented Golgi lamellae and Golgi "plates." In both cases, the probability is small of obtaining longitudinal or tangential sections for appreciable distances, or passing unequivocally through the narrow points of contact if they exist. The use of serial and oriented sections, as illustrated in the pairs of electron micrographs shown in Figs. 3 and 4, and Figs. 9 and 10, respectively, seems to be the method of choice in obtaining valuable and ultimately decisive information on the actual spatial configuration of the structures, and the nature of their possible interrelationships.

In cross-sections, the characteristic elements of the Golgi apparatus, i.e. the flattened sacs, appear as relatively straight and more or less rigid profiles, in contrast to the tortuous profiles of smooth ER tubules (Figs. 2–4). As a rule, these profiles are terminated by bulbous extensions filled with lipoprotein granules, whereas the median part often appears empty (Figs. 3–10). Occasionally, a profile applied alongside a Golgi stack assumes an intermediate appearance, as in the serial sections pictured in Figs. 3 and 4 (*ip*, and arrows), in that it contains a row of lipoprotein granules and is continuous (left end of the *ip* profiles in both pictures) with an extension of the smooth ER tubular network.

The foregoing observations indicate that, in liver cells, three different compartments are involved in the synthesis and transport of lipoprotein granules, namely: rough ER, smooth ER, and the Golgi apparatus. The interrelations between the membrane components of these various structures, and the particular role played

FIGURES 3 and 4 Two successive sections of a series, showing ER and Golgi structures at levels about 500 A apart. Occupying most of the pictures are portions of two separate formations consisting of elongated profiles of Golgi cisternae (*st*), large concentrating vesicles filled with dense lipoprotein granules, and communicating with the cisternae, and clusters of small vesicles, especially abundant next to the Golgi apparatus at the left. Comparative study of the membrane configurations in the two sections demonstrates that rows of vesicles in one section may prove to be communicating tubules or cisternae in the other (*st*, arrows and crossed arrows); the same applies also to the smooth ER (*sER*) network: rings of tubules in the center of Fig. 4 (*nw*) are represented in Fig. 3 by a few "isolated" vesicles. Of interest is an intermediate type of Golgi profile (*ip*, and arrows), containing rows of lipoprotein granules and being in continuity (left end in both pictures) with smooth ER type extension. A detached, spherical concentrating vesicle (*sg*), at some distance from the Golgi structures, may correspond, in the classical terminology, to a secretory granule. Two lysosome-like bodies (*Ly*), with slightly different morphology, are present in both pictures. *rER*, rough ER. Figs. 3 and 4: × 34,000.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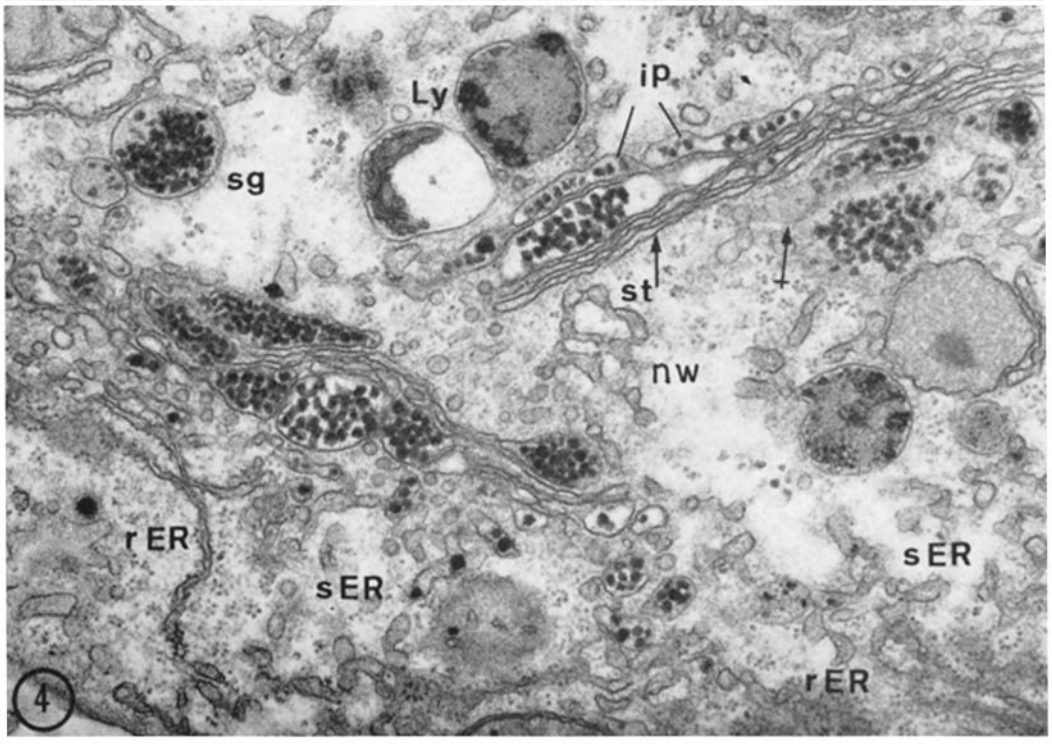
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by the Golgi apparatus in the hepatic cells, will be considered in the following paragraphs.

Elaboration and Distribution of Golgi Complexes in Hepatic Cells

The process of synthesis, transport, and disposal of lipoprotein granules in the hepatic cells includes the participation of groups of structures identified morphologically as Golgi complexes. The present survey indicates that, in the course of this process, there is no constant, preferential orientation of the Golgi complexes with respect to such landmarks as the periphery of the nucleus, the bile ducts, or the space of Disse, as may be the case in typical secretory cells, for example in the case of the exocrine cells of the pancreas (6, 18, 19), or in the mucus cells of the intestinal epithelium (36, 35), where the Golgi complex occupies a definite position between the nucleus and the excretory regions, a disposition in relation to the functional polarization of these cells. It is clear, on the other hand, that in liver cells individual Golgi complexes possess an intrinsic polarity which is originally determined by the particular orientation in the cytoplasm of corresponding stacks of rough ER lamellae, with which they are functionally related. For reasons to be discussed later, the plane of a given Golgi complex is always found oriented at right angles to that of the lamellae of a corresponding rough ER stack: this characteristic disposition is illustrated in Fig. 2, and even more clearly in Claude 1968, reference 9 (Figs. 5, 7, and 8). In each case, a more or less abundant smooth ER, in the form of a tubular network, is always found interposed between the rough ER stacks and the Golgi structures.

In terms of tridimensional arrangement, the situation in liver cells may appear even more complex. Frequently, several separate rough ER stacks engaged in the same lipoprotein synthetic process, and their corresponding Golgi formations, converge on the same cytoplasmic region (Fig. 2), giving rise to a multiple Golgi center, opposite Golgi apparatuses producing the appearance of symmetrical Golgi complexes (Fig. 8, in reference 9). Smooth, tubular ER elements distributed in the upper part of Fig. 2, and coming from a diametrically located rough ER stack (not shown in the picture), are seen to approach the Golgi structures from an opposite direction. A similar complex situation, with the participation of oppositely located rough ER stacks, is illustrated in Fig. 5. Conversely, as in Fig. 2, the same stack of rough ER lamellae is seen to contribute to the elaboration of two distinct Golgi complexes, as a result of the fact that they were incidentally separated, at this level, by the presence of a group of mitochondria. In hepatocytes, the rough ER lamellae engaged in lipoprotein production may be considered as discs, the synthesized products being transported radially in the cisternae, from the center to the periphery, where they find their way into smooth ER extensions bulging from the rim. Under these conditions, a corresponding Golgi apparatus may be expected to be found at any location at the periphery and, in cross-sections, at the opposite sides of a rough ER stack.

Morphology of the Components of the Golgi Complex

The most conspicuous components in the Golgi complexes are vesicular elements of relatively large sizes, filled with dense granules similar in appearance and in size to the lipoprotein granules seen singly or in rows within the channels of the surrounding smooth ER network, as illustrated in Figs. 2–5. A point of interest is the mechanism by which the lipoprotein granules may pass to, and be concentrated in, these elements which constitute a characteristic part of the Golgi membrane system. The fact that the granules retain the essentials of their morphology in the process suggests that they must be transported directly, or, as such, across the Golgi boundary. Fortunately the lipoprotein granules, owing to their discrete shape and particular density to electrons, act as their own markers, so that, under favorable conditions, they may help to identify, by their presence, the pathways they follow. In addition, the use of a favorable fixation technique (7) and of serial and oriented sections has been applied to the study of both the rough and smooth endoplasmic reticulum, and Golgi membrane systems. Different aspects of the results are illustrated in Figs. 2–13.

Two types of membranes have been distinguished in connection with the synthesis, transport, and temporary storage of lipoprotein granules: (*a*) the channels of the smooth ER network containing the newly synthesized granules, and (*b*) elongated, smooth-surfaced profiles, part of the Golgi system, either "empty" or containing rows of lipoprotein granules, and occurring typically in close bundles or stacks. As shown in

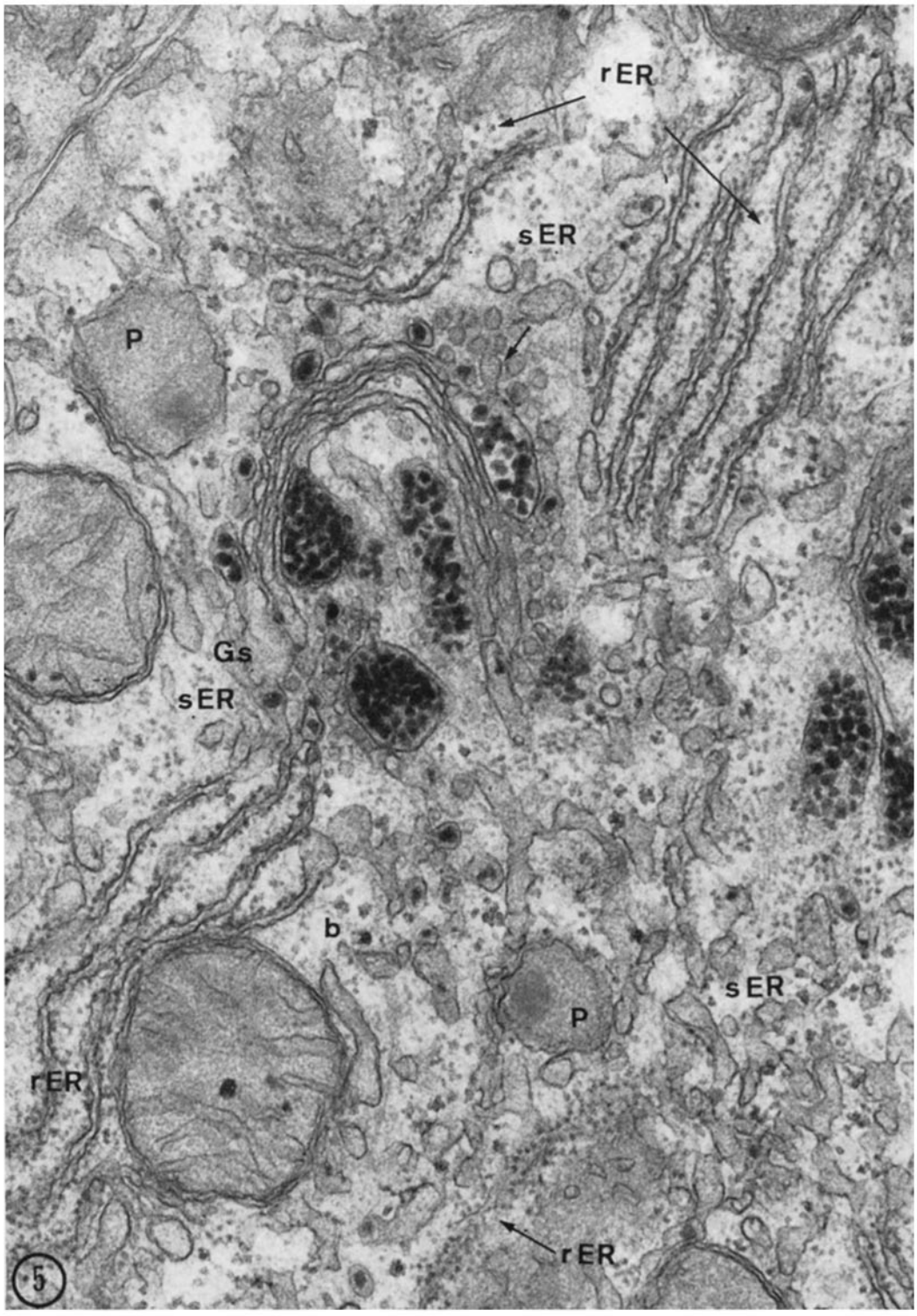
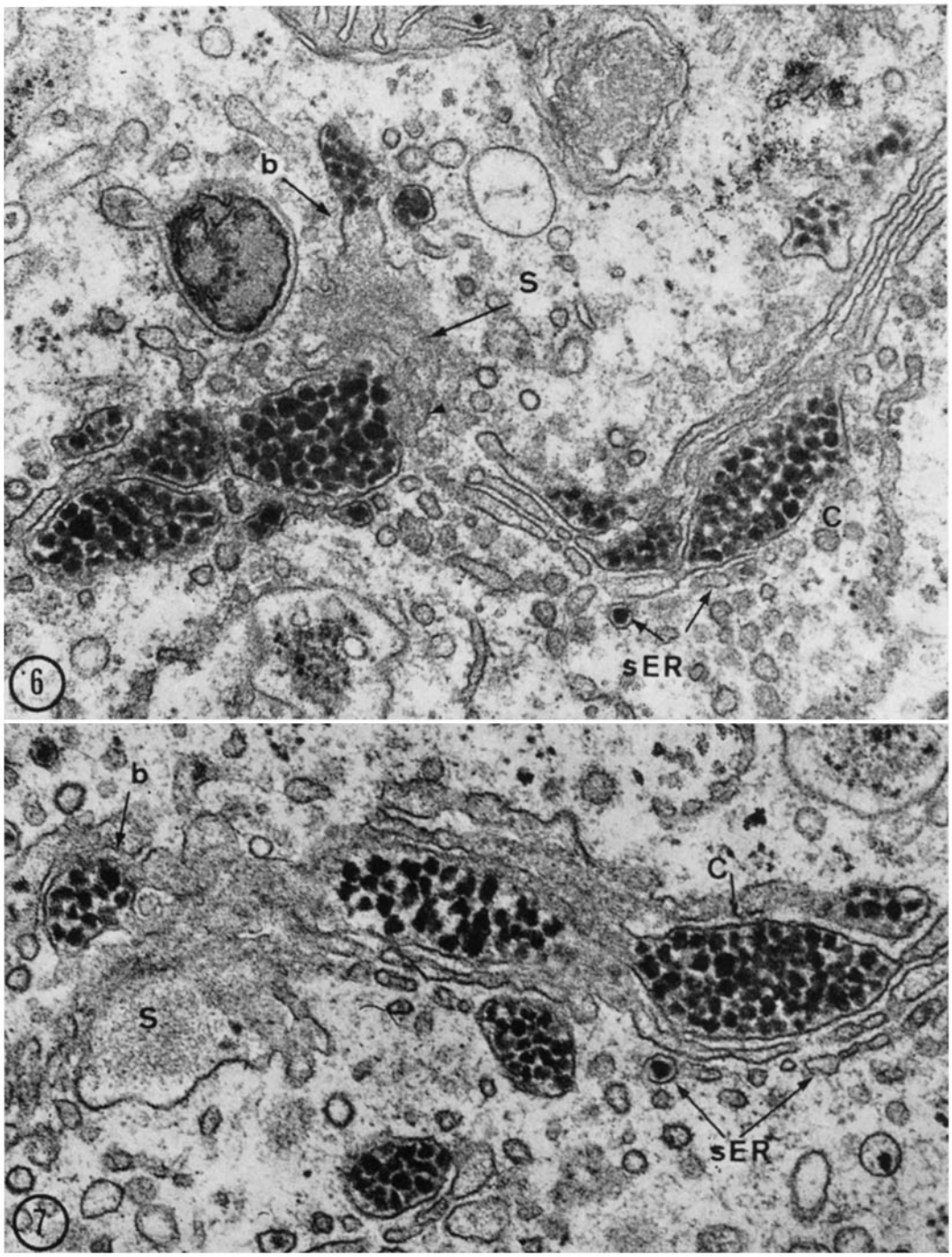


FIGURE 5 The illustration shows two opposite stacks of *rER* lamellae participating in the constitution of a Golgi complex. In this case, the "forming" face is obviously located at the convex side of the apparatus, with extensions from the smooth ER network (*sER*) piling up from below and above, along the curved structure. A cluster of small vesicles, on top of the Golgi structure and next to a concentrating vesicle, is interpreted as representing cross-sections of tubular, smooth ER extensions, with one of them (arrow) connecting with the concentrating vesicle. In the region at the left, between rough ER lamellae and the Golgi apparatus, a smooth ER extension with lipoprotein granules seems to be connected with a widened Golgi saccule (*Gs*). In (*P*), two peroxisomes. × 56,500.



FIGURES 6 AND 7 Both electron micrographs illustrate what appears to be either cross-sections or side views of 3 or 4 elongated Golgi saccules tightly applied along their length, and presenting interposed or "terminal" Golgi concentrating vesicles (C). The gray area at the left in Fig. 6 (*S*, and arrow) may correspond to superimposed side views of three Golgi saccules, one of them widely connected with a concentrating vesicle appended at its rim (*b*, and arrow). The same situation is found, with different configurations, at the left in Fig. 7 (*s*, *b*, and arrow). In both pictures, arrays of small vesicles (*sER* and arrows), terminated at left by a vesicle containing a lipoprotein granule, are interpreted as cross sections of tubular elements of the smooth ER network, or part of a "fenestrated" plate, in parallel position with the Golgi sacs. Fig. 6: × 55,500; Fig. 7: × 61,000.

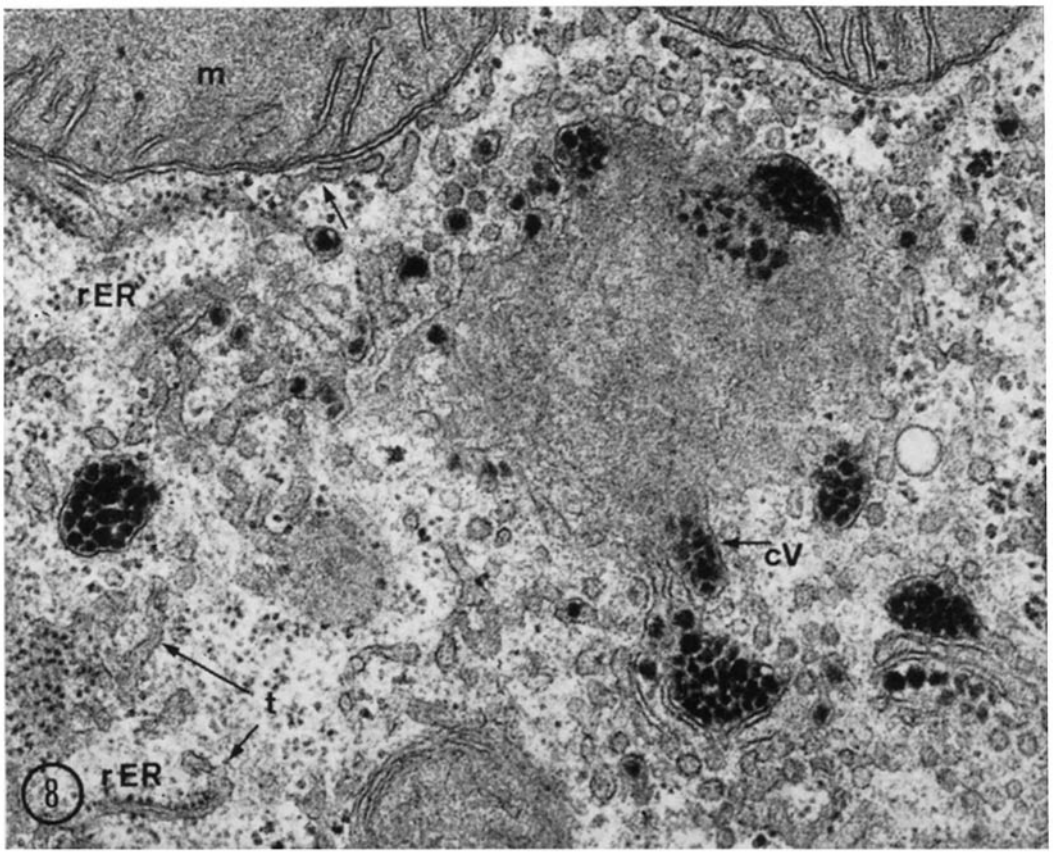


FIGURE 8 The electron micrograph represents a front view of a Golgi sac of somewhat diffuse outline, probably due to the obliqueness, with respect to the boundary, of the grazing section. This Golgi "plate," about 1.2 μ in diameter, is characterized by the presence at its periphery of a number of concentrating vesicles, one of them (*cV*) shown in direct communication with it. On the other hand, numerous smooth ER tubules, with enclosed lipoprotein granules, are in the vicinity of the Golgi element, especially at the left side, and some of them seem to be in communication with it, as in the case illustrated in Fig. 10. At the far left of the picture are found the usual rough ER lamellae (*rER*), in continuity with tubular extensions of the smooth ER network (*t*, and arrows). *m*, mitochondria. × 46,800.

Figs. 3–7, these are interrupted or terminated by expansions which, as a rule, appear in cross-sections in the form of large vesicles filled with lipoprotein granules, referred to in this paper as "concentrating vesicles," or as similar, but rounded vesicles (detached concentrating vesicles), the latter corresponding to secretory granules in other cell types. Figs. 3–7 present evidence that both the elongated profiles and the vesicles are part of the same communicating system; it is this system, consisting of sharply defined narrow cisternae on the one hand, and large, connected concentrating vesicles on the other hand, which constitutes what are considered to be the main components of the Golgi apparatus, i.e. the Golgi sacs and the large Golgi vacuoles, respectively.

The elaboration of these Golgi units and their spatial configuration are best revealed by means of serial sections (Figs. 3 and 4), and by the study of tangential or grazing sections, as illustrated in Figs. 6–10. The serial sections of Figs. 3 and 4 show cross-sections of the narrow profiles of elongated Golgi cisternae, tightly arranged in parallel, some of them containing long rows of lipoprotein granules, others apparently empty but terminated by expanded vesicles filled with the same type of granules.

Figs. 6 and 7 illustrate formations which are

very similar to those shown in Figs. 3 and 4, but which are cut somewhat obliquely with respect to the main plane of the structures, the section, in some parts, grazing the surface of the limiting membranes. As in cross-sections, the Golgi elements appear closely and regularly associated in stacks, with an intervening space of about 75–150 A in width. These illustrations, and the following to be described, show that the elementary constituents of Golgi stacks most frequently seen in cross-section as narrow, double profiles are actually lamellae or narrow sacs, more or less extended laterally and parallel to each other, except for the presence, in different regions, of concentrating vesicles. The electron micrographs in Figs. 6 and 7 demonstrate even more clearly the continuity which exists, sometimes at several points (Figs. 6 and 7), between the narrow regions of the lamellae and the concentrating vesicles, or between the narrow regions of the lamellae and the concentrating vesicles which appear as bulges along the rim of the Golgi sacs (*b* and arrows, Figs. 6 and 7). Darker areas (*s*, and arrow in Fig. 6) correspond to side views of Golgi cisternae, the curved bands of different densities suggesting that several Golgi sacs, close together, have been cut obliquely, and nearly parallel to parts of their limiting membranes. In both pictures, a regular array (*sER* and arrows) of what appear to be small, clear vesicles, one of them with a dense granule (at the left of the row), corresponds probably to an uninterrupted smooth ER tubule with enclosed lipoprotein granules, winding in and out of the plane of the section, as previously discussed.

Origin and Differentiation of Elements of the Golgi Apparatus

The electron micrograph in Fig. 8 represents in the center a side view of a Golgi sac, sectioned parallel and close to its surface, with appended concentrating vesicles at the rim. The point of interest in the present case is the presence of tubular smooth ER extensions in the vicinity of the Golgi apparatus, and especially the close association which these smooth ER extensions, together with their accompanying lipoprotein

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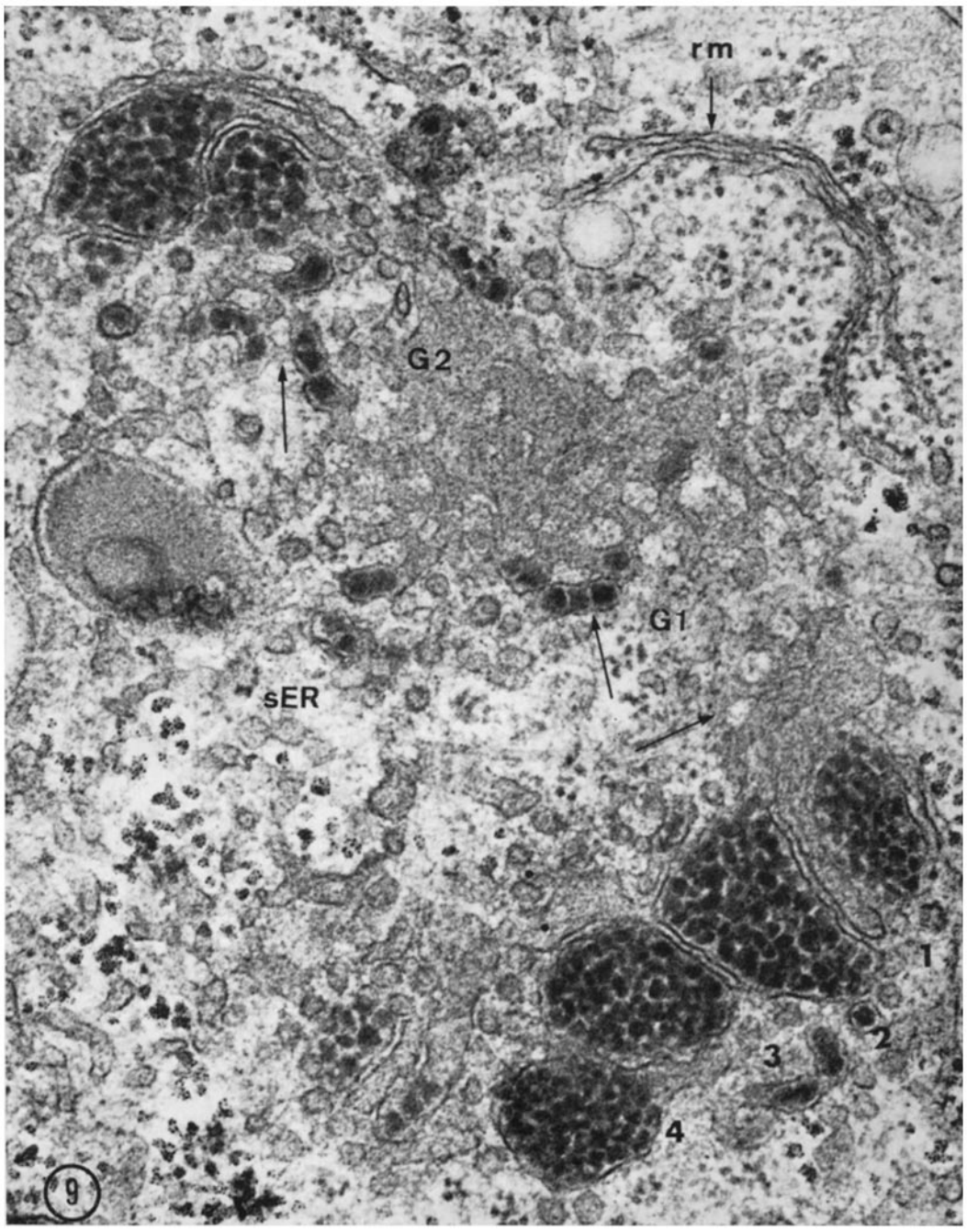
FIGURES 9 and 10 The micrographs represent two sections of a series passing through the same Golgi complex, but about 3–4 section thicknesses apart. The positions of the four concentrating vesicles in the lower right corner of Fig. 9 have been numbered *1 to 4*, to serve as markers in the comparative study of both pictures. The vesicle labeled *4*, and present in Fig. 9, is practically out of the corresponding region in Fig. 10, except for portions of a few lipoprotein granules, indicating that the two figures are separated in space by a distance of about 2,500–3,000 A, including the whole thickness of both sections represented in Figs. 9 and 10.

Perforated plates and Golgi sacs: the central region in Fig. 9 illustrates a fenestrated plate in the process of formation, through the fusion of smooth ER (*sER*) tubules (arrows) carrying rows of dense lipoprotein granules. The dimensions of this perforated plate are about 0.7 × 0.6 μ, and might be as much as 1.1 μ in width, if it is assumed that the plate is continuous, over the *G1* region, with the part of network situated next to the No. 1 concentrating vesicle. Further coalescence of the tubules may lead to the constitution of portions of solid membranes, as is probably the case in the *G2* region. The solid membrane structure in Fig. 10 (*G3*) may be considered to be a face view of a large Golgi sac, 1.2 × 0.5 μ in size, not counting the complement of communicating, concentrating vesicles disposed at the periphery. It is of interest that the two Golgi structures shown in Figs. 9 and 10, roughly 2,000–2,500 A apart, are very similar in extent and general configuration, and are undoubtedly parts of the same Golgi stack. Moreover, it is apparent that the fenestrated plate and the Golgi sac next to it are situated at the forming face and, in this position, must constitute the first or "younger" elements of a growing Golgi apparatus.

Rigid smooth lamellae: The double profiles of curved, smooth surfaced membranes (*rm*) located at the upper right corner of the micrographs present practically identical configurations and sizes in both Fig. 9 and Fig. 10, although extending over the entire thickness of 3–4 successive sections. Under these conditions it is apparent that the profiles correspond to large lamellae, about 0.8 μ in length (the length of the smooth profile) and at least 0.2 μ in width. Both lamellae stand side by side on two sections of rough ER (*rER*) of about equal length and resting close to the same rough ER cisterna (Fig. 10, arrow at right). The possible relationship of this type of smooth lamellae, also shown in Figs. 11–13, with the process of differentiation of the Golgi structures has been discussed. Figs. 9 and 10: × 67,800.

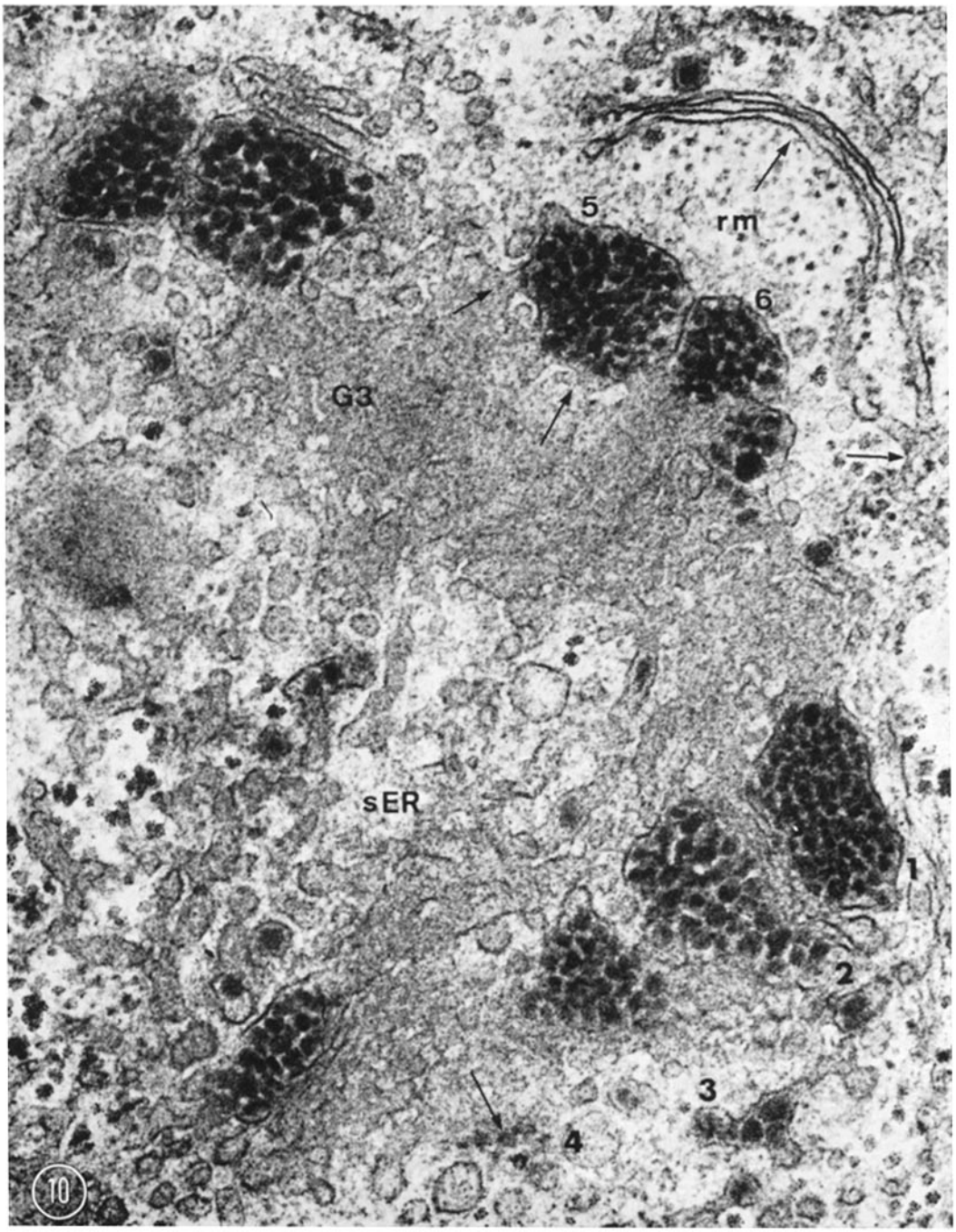

rm
G2
G1
sER
1
2
3
4
9

granules, seem to establish with the periphery of the Golgi disc. At the upper part of the disc, the same limiting membrane of the Golgi sac, seen in cross-section, appears to enclose a number of concentrating vesicles. Elements of rough ER occupy the left of the picture (*rER*), continous with tubular, smooth-surfaced extensions (arrows), close to similar tubules with lipoprotein granules, indicating that lipoprotein synthesis was still in progress in part of the ER at the time of fixation of the specimen.

The electron micrograph shown in Fig. 9 represents a section passing also in the plane of a Golgi structure, but at a somewhat different level. The particularities exhibited in this picture constitute evidence for the fusion of smooth ER tubules (identified, as in Fig. 8, by the presence of lipoprotein granules) with themselves, especially clear in two regions of Fig. 9 (arrows), and in one plane parallel to the corresponding solid membrane shown in Fig. 10. This type of fusion or coalescence of tubules at some points along their lengths tends to leave free spaces between them, giving the resulting structure the appearance of a lacework or fenestrated plate. This process of coalescence of smooth ER tubules, as demonstrated in Figs. 8–10, may be the origin and mode of formation of the laceworks and fenestrated membranes occasionally encountered among Golgi structures, both in sections and in negatively stained preparations (10, 14, 23, 30).

The preceding observations indicate that membrane structures from the smooth endoplasmic reticulum system take an active part, and enter to an appreciable extent, in the constitution of elements of the Golgi apparatus. On the other hand, typical Golgi structures are known to possess morphological and especially biochemical characteristics of their own (13, 37, 38, 40), quite distinct from those exhibited by other cytoplasmic membranes, and which differ, according to function, from one cell type to another. The following observations suggest that the endoplasmic reticulum (rough and smooth) may participate further (Figs. 9–13), at a different or later stage of the lipoprotein synthesis and transport cycle, in the morphological constitution, and possibly the functional differentiation, of the Golgi apparatus.

As already indicated, Figs. 9 and 10 correspond to serial sections a few section thicknesses apart. At the centers and left sides of the pictures are elements of the smooth ER containing lipoprotein granules; some of the tubular elements seem to be continuous with the Golgi plate (Fig. 9), and with the "stem" of concentrating granules in Fig. 10. At the lower right side of the pictures, the positions of four concentrating vesicles have been numbered (*1–4*) in order to serve as markers in the estimation of the level in the specimen, and in the orientation of the same Golgi structures as they present themselves, in Figs. 9 and 10, respectively. The two parallel ER profiles located at the upper right hand corner of Fig. 9 (*rm*) are still found, without much morphological change, in Fig. 10, indicating that they are not tubules, but lamellae extending through 4–5 sections thick and, according to measurements, at least 0.20 μ wide and 0.8–1 μ in length. By comparison, these dimensions are of the order of magnitude of those presented by the Golgi elements and "fenestrated plates," i.e. 0.75–1 μ, as illustrated in Figs. 8–10, respectively. This type of smooth-surfaced lamella, which is also illustrated in Figs. 11–13, has been found to occur in the vicinity of Golgi complexes, and may be described similarly. As a rule, the lamellae are extensions of a segment of rough ER lamellae; two of the pair shown in Fig. 10 seem to have their origin at the same locus on a rough ER cisternae (Fig. 10, arrow), and to differentiate into smooth-surfaced membranes at the same distance from their bases on the rough ER lamellae, possibly extending along a band of rough ER, about 0.25 μ wide. In cross-section, the profiles of these smooth-surfaced lamellae are sharply defined, delimiting narrow cisternae which are empty in appearance, in contrast to the usual tubular, smooth ER network, which appears denser to electrons, presents wider and more tortuous outlines. These differences are demonstrated in Figs. 12 and 13, where the two types of straight (*rm*) and contorted (*sER* in Fig. 12, and arrow in upper part of Fig. 13) structures are found extending side by side. A feature which may be distinctive for this type of lamella is its rigid and somewhat angular appearance in some part of the length (*rm*, in Figs. 12 and 13). The possibility that lamellae of the type just described may become associated with elements of a Golgi complex is demonstrated in Fig. 11 (*rm*). In this case a smooth-surfaced lamella, still attached to its base of rough ER, is applied alongside a stack of Golgi elements identi- by the presence of terminal concentrating vesicles with lipoprotein granules. The view that lamellae of this type may play a specific role in the elabora-



Legend Fig. 10 on page 756.

tion and differentiation of the Golgi complexes should be substantiated by appropriate histochemical tests, which we hope to be able to adapt to this particular problem, in combination with our technique of fixation.

DISCUSSION

Low density lipoprotein synthesis in liver cells is characterized by the fact that the synthesized product, mostly triglycerides, is processed in the form of discrete, size-limited granules, 300–400 A in diameter.[3] Rich in unsaturated fatty acids, they react readily with osmium fixatives and subsequent electron stains, so that they may serve as their own markers and may be easily detected and identified in thin sections of liver by electron microscopy. Such osmiophilic bodies, assumed to contain triglycerides, were observed in rat liver and the endoplasmic reticulum, especially after treatment with orotic acid, by Novikoff and collaborators in 1964 (37, 38), and following treatment with ethionine and adenine by Baglio and Farber in 1965 (2). Since then, their chemical constitution has been ascertained by analysis conducted on the granules isolated by differential centrifugation, by Schlunk and Lombardi (45, 46). Ashworth et al. (1), Hamilton et al. (16, 17, 29), Jones, Ruderman, and collaborators (20–22, 42), Lombardi (25–29, 50), and O. and Y. Stein (47–49) have extensively investigated the nature of the lipoprotein granules and their synthesis and transport in liver cells, by various techniques, including chemical extraction and analysis, radioisotope tracers, radioautography, and electron microscopy. From concordant results, a number of points of interest to the present study seem to be well established: (*a*) the osmiophilic granules seen in liver cells consist predominantly of triglycerides, in association with relatively small proportions of phospholipids and proteins (45); (*b*) under timed, experimental conditions, such as those of Jones et al. (22), using isolated, perfused rat liver and radioactive precursors, and those of O. and Y. Stein (47) by means of electron microscope autoradiography, the lipoprotein granules appear first wihtin cavities of the endoplasmic reticulum, especially in the channels of the smooth (agranular) ER; (*c*) they are found next in Golgi cisternae and vesicles, indicating that the latter are involved in the transport of the granules, without affecting appreciably their sizes and density; (*d*) the synthesis and transport of lipoprotin granules are a relatively rapid process which may be completed within ~5–15 min from the uptake of fatty acids at the cell border to the discharge of the granules in the space of Disse, including their transport from the site of synthesis and their transit through smooth ER and Golgi cisternae. These values of relatively rapid turnover and transit of lipoprotein

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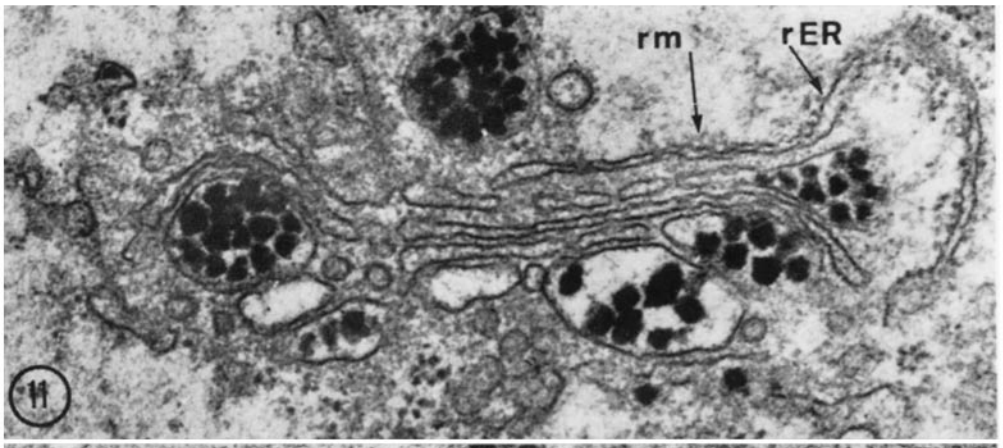
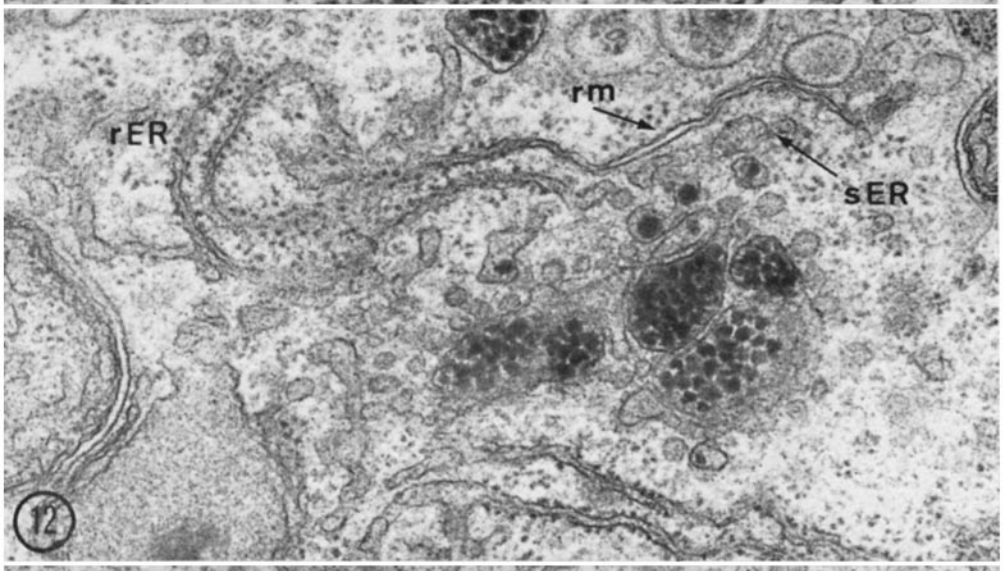
FIGURES 11, 12 and 13 These micrographs demonstrate differentiated smooth ER lamellae, similar to those described in Figs. 9 and 10, and encountered in the vicinity of Golgi complexes. In cross-sections the profiles of these lamellae appear sharply defined, and assume relatively straight and somewhat rigid configurations (*rm*, in Figs. 9–10, and 11–13) which seem typical for this type of structure.

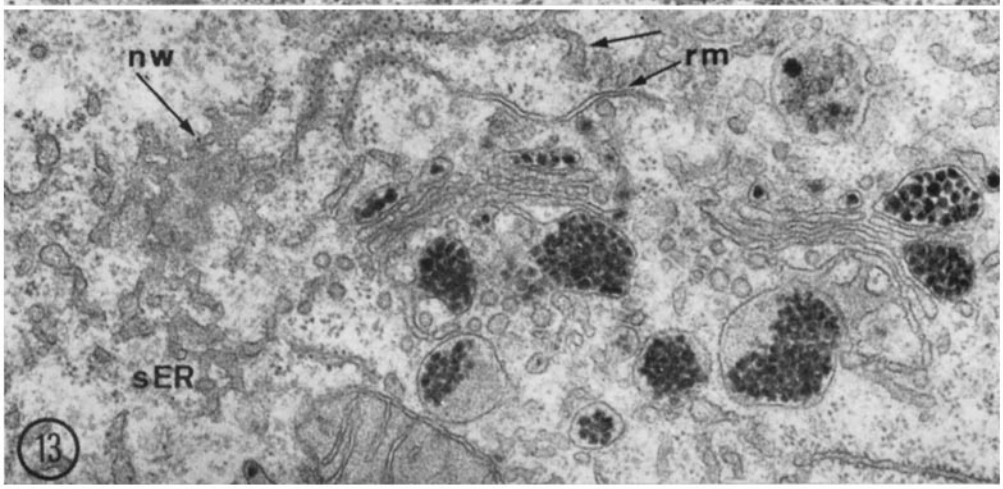
FIGURE 11 In this micrograph, a smooth-surfaced lamella (*rm*) is found disposed in parallel alongside typical flattened sacs of the Golgi apparatus, and appears to be closely associated with them, being embedded in the same, dense, finely granular cytoplasmic matrix. The straight smooth membrane is still in direct communication with the original rough ER lamella (*rER*) through a sharply bent junction. The observations indicate that new smooth lamellar components of the endoplasmic reticulum system may be introduced into the Golgi apparatus, even at the level of what appears to be the mature stage of the Golgi structures. × 54,500.

FIGURE 12 One of these lamellae (rm) is found disposed alongside tubular elements of the smooth ER network (*sER*), the latter being obviously engaged in lipoprotein synthesis. By comparison, the elements of the smooth ER appear more flexuous and more irregular in shape. At the left, both types of membrane are connected with the same group of rough ER lamellae (*rER*). × 47,000.

FIGURE 13 In this micrograph, a rigid lamella (*rm* and arrow) appears interposed between smooth ER extensions (arrow) and components of the Golgi apparatus. At the left, the opposite ends of the rough ER lamellae seem to be associated with elements of an elaborate smooth ER network (*sER* and *nw*). × 31,000.


rm
rER
11


rER
rm
sER
12


nw
rm
sER
13

granules in liver cells are based on data obtained by Hamilton et al. (16, 17), and by the authors just mentioned (22, 47).

Our results confirm their findings, with emphasis on the following facts: (*a*) that the electron-opaque granules are never seen within cisternae of the rough ER, but are exclusively found in the tubular channels of smooth endoplasmic reticulum; and (*b*) that they are first detected immediately on the smooth side of rough-smooth ER junctions (Fig. 1). These observations, provided by electron microscopy, strongly suggest that triglycerside synthesis may be restricted to smooth membrane compartment of the endoplasmic reticulum, the protein and possibly the phospholipid moieties being synthesized in the rough ER compartment, or else, that a factor yet unknown and necessary to pack triglycerides into the shape and size of the granules is likewise restricted to the same smooth ER compartment. It is not known, however, whether the glycerides in the granules are actually complexed or combined with the phospholipids and proteins found associated with them, or whether they are in the form of lipid droplets enveloped in a limiting membrane, a situation which would also account for the presence of a certain proportion of phospholipids and proteins. Close examination of the electron micrograph of lipoprotein granules (liposomes) isolated by Schlunk and Lombardi (1967, Fig. 2) suggests that many of the granules shown in the illustration may actually possess two membranes, as expressed by a boundary of greater density outlining the surface of the granules or droplets, and a looser and wider membrane, which very likely represents a microsome-like portion of the smooth ER carried over with the granules at the time of homogenization. The way that triglycerides are conditioned, i.e. complexed or chemically combined with phospholipids and proteins, or in the form of lipid droplets enclosed in a membrane, is of importance in several respects: (*a*) in understanding what mechanism may prevent the lipoprotein granules from fusing together while in the smooth ER channels or in the Golgi compartments, especially when they are brought close together in the concentrating vesicles; (*b*) in learning what chemical or structural modifications, not clearly reflected in their morphology, they may undergo, if any, as a result of their passage through the Golgi compartments; and (*c*) in finding out to what extent they resemble physically and chemically the very low density lipoprotein of plasma, to which they have been assumed to be related.

Elaboration and Differentiation of the Golgi Apparatus

FENESTRATED PLATES: Evidence has been presented which indicates that the smooth ER network transporting the product of synthesis, i.e. lipoprotein granules, becomes denser at the forming face of the Golgi apparatus and that, through partial fusion of elements of the network, a structure is being elaborated which has the appearance of a fenestrated plate (Fig. 9). Further, complete coalescence may lead to the production of solid membrane regions, as illustrated in the upper part of the fenestrated structure in Fig. 9 (*G2*) and, more extensively, in the corresponding region of Fig. 10 (*G3*). It may be expected that, concurrently with this lateral fusion of tubules, the inner space of the new structure so produced will widen accordingly, resulting in the formation of a common flat cisterna of a size and shape determined by the progressively expanding boundary. The structures illustrated in Figs. 9, 10, and 8, respectively, may correspond to successive stages in this process of solid membrane elaboration.

Large fenestrated membranes and reticular extensions having unequivocal connections with Golgi cisternae have been reported as early as 1960 by Manton (30) in the case of the plant cells, and by Mollenhauer and Zebrun (33) in cells of mammalian testis. Kessel and Beams (23), in cells of the ovary of a tunicate, observed extensive branching of the Golgi lamellae, resulting in a honeycomb or lattice-like arrangement, radiating from the Golgi cisternae. Fenestrated membrane structures have also been reported by Lane (24) in ganglionic neurons of the grasshopper, and by Flickinger (14, 15) as being associated with Golgi cisternae of rat and mice epipidymis.

Isolation of Golgi structures, by differential and sucrose gradient centrifugation, from plant cells by Cunningham et al. (10) and from liver by Fleischer et al. (13), and their examination by negative staining, seems to support and extend the observations derived from the study of thin sections. On the basis of these findings, Mollenhauer and Morré (33) and Flickinger (14, 15) have proposed Golgi models representing the elements of the stack as composed of a central disc, corre-

sponding to the classical Golgi flattened sac, communicating at ist periphery with an expanding system of branching and anastomosing tubules (Mollenhauer and Morré), or of an expanding, perforated lamella (Flickinger); in both cases, the direction of growth is assumed to be from the center to the periphery, and to be the expression of the maturation of the Golgi structures, or of their specific activity.

The observations reported in the present study lead one to arrive at a different conclusion, namely, that the development of membranes, and, hence the growth and differentiation of the Golgi structures, proceeds, from an opposite direction, i.e., through the successive stages of tubular smooth ER network, intermediate fenestrated plate, and solid membrane Golgi cisternae, ending at the boundaries of concentrating vesicles.

"RIGID" SMOOTH MEMBRANES: Another type of smooth membrane frequently found in the vicinity of the Golgi apparatus, and occasionally in association with it, must also be considered in relation to the development of the Golgi structures, although the elucidation of their possible role in this respect may require further studies. These smooth lamellae, which may attain the size of Golgi sacs in area, have been described in detail in the text, and in the legends of Figs. 9–13. Their outlines are characterized by a relatively rigid, somewhat angular appearance, and these smooth lamellae are always observed attached to, and in continuity with rough ER lamellae. Occasionally, they are found to be continuous, at both ends of their smooth-surfaced profile, with segments of rough ER lamellae, as illustrated in Fig. 10 (*rm* right and left of upper profile), a relation which suggests that they are part, or a product, of the rough ER system. Likewise, the fact that this type of lamella has been found in close association with the flattened elements of Golgi stacks, as shown in Fig. 11, indicates that they may be part of the apparatus, and play a role in its differentiation and growth.

Turnover of Smooth ER and Golgi Membranes

According to the observations of Jones et al., (22) and O. and Y. Stein (47), the turnover of lipoprotein complexes is relatively rapid, the lipoprotein granules appearing in endoplasmic reticulum cisternae as early as 2 min following time-limited administration of triglyceride precursors either by liver perfusion *in situ* (22) or by intravenous inoculations (47), no free fatty acids being recovered from liver after that time. Lipoprotein granules were seen to reach the Golgi region and the cell border during the next 5–10 min and they continued to accumulate in Golgi cisternae during the 5–20 min period, indicating that the transport of lipoprotein granules, from their site of synthesis to their discharge in the space of Disse, may be accomplished within 5–15 min and possibly less.

The present studies, and observations to appear in subsequent papers,[4] demonstrate that during the lipoprotein synthesis cycle a transfer of membranes is taking place, as evidenced, first, by smooth ER membrane fusion and incorporation at the forming face of the Golgi apparatus, and, second, by consumption of Golgi membranes during the complex process of enlargement of concentrating vesicles, the Golgi sacs, their detachment from and their extrusion into the cytoplasm (together with the lipoprotein granules they contain) in the form of "secretory granules." Thus, the discharge of lipoprotein granules is accompanied by a loss of Golgi membranes, those of the secretory granules in turn being transformed and ultimately discarded in lysosome-like bodies.[4]

The actual rate of turnover of the Golgi membranes is not known, but one may assume that it is, as just indicated, proportional to the rate of synthesis and disposal of the lipoprotein granules. Since the observed time of transport of the latter, from their site of synthesis (rough and smooth ER) to the concentrating vesicles, is relatively short, of the order of 5–10 min (22, 47), the turnover of the Golgi membranes may prove to be appreciably faster than the turnover reported for the endoplasmic reticulum as a whole, with a total, membrane protein half-life of 2–3 days. There may not be incompatibility between these two sets of values, however, since it is becoming more and more apparent that the endoplasmic reticulum is far from being homogeneous in its makeup and in the synthesis and degradation rates of its various components, a point illustrated and discussed in a paper by Schimke et al. (44).

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[4] Preliminary note on: Disposal of lipoprotein granules in liver cells. In *Proceedings of the VIIth International Congress for Electron Microscopy*. Grenoble, France, Aug. 30–Sept. 5, 1970. 3:85.

Golgi complexes in liver cells appear to be exclusively concerned with lipoprotein granule synthesis and segregation, no other functions, possibly associated with it, having been detected thus far. They present themselves in a variety of sizes and shapes, and may vary in number with time, according to changes in general lipid metabolism and diet. A rough evaluation of their occurrence indicates that, in a given time, there may be at least 50 Golgi complexes per liver cell, representing a total, relative volume of about 2% of the whole cytoplasm, and probably less than 8% of the total microsomal fraction. Methods now available for the separation of Golgi structures from liver (Cunningham [10], Fleischer [13], and Morré et al.) may permit the determination of the respective turnover rates of the Golgi structures proper, and of the particular portion of the endoplasmic reticulum specifically associated with it.

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