

GOLGI FRACTIONS PREPARED FROM RAT LIVER HOMOGENATES

I. Isolation Procedure and Morphological Characterization

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ABSTRACT

In devising a new procedure for the isolation of Golgi fractions from rat liver homogenates, we have taken advantage of the overloading with very low density lipoprotein (VLDL) particles that occurs in the Golgi elements of hepatocytes ~90 min after ethanol is administered (0.6 g/100 g body weight) by stomach tube to the animals. The VLDLs act as morphological markers as well as density modifiers of these elements. The starting preparation is a total microsomal fraction prepared from liver homogenized (1:5) in 0.25 M sucrose. This fraction is resuspended in 1.15 M sucrose and loaded at the bottom of a discontinuous sucrose density gradient. Centrifugation at $\sim 13 \times 10^6$ *g*·min yields by flotation three Golgi fractions of density >1.041 and <1.173 . The light and intermediate fractions consist essentially of VLDL-loaded Golgi vacuoles and cisternae. Nearly empty, often collapsed, Golgi cisternae are the main component of the heavy fraction. A procedure which subjects the Golgi fractions to hypotonic shock and shearing in a French press at pH 8.5 allows the extraction of the content of the Golgi elements and the subsequent isolation of their membranes by differential centrifugation.

INTRODUCTION¹

The Golgi complex functions in the intracellular transport and subsequent processing of secretory proteins (2-5) including their terminal glycosylation (6-9); in addition, it is assumedly involved in either the production or conditioning of membrane for the cell surface (10-12). Reviews of the recent pertinent literature can be found in

references 13 and 14. A clear understanding of the role of the complex in all these functions requires the chemical and enzymic characterization of its membranes, which in turn depends on the isolation of a reasonably pure Golgi fraction from appropriate cell homogenates.

In the past, Golgi elements have been isolated together with fragments of the endoplasmic reticulum (ER) in the microsome fraction. The chemistry and enzymology of the latter is known in some detail at least for certain cell types,

¹ A partial, preliminary report on these findings was presented at the 11th Annual Meeting of the American Society for Cell Biology (1).

primarily mammalian hepatocytes (15, 16), but there were reasons, morphological (17, 18) as well as histochemical (19-21), to doubt that the ER and Golgi membranes are similar. Recently, Golgi-rich fractions or Golgi fractions have been isolated by density gradient centrifugation from liver homogenates (22-24) and found to contain glycosyltransferase activities in considerably higher concentrations than the microsomes (23-26). The identification of the components of these fractions as Golgi elements was based on a variety of criteria such as morphological features revealed by negative staining (22, 26), retention of the characteristic stacking into dictyosomes (22, 26), and metal (osmium) impregnation of the isolated vesicles (23). Since some of these criteria are not entirely satisfactory (cf. 27), and others are not satisfied by all the components of the fractions, the homogeneity of these preparations is still open to question. A reliable morphological marker capable of tabbing dispersed or isolated Golgi elements would be highly desirable.

The Golgi elements of mammalian hepatocytes often contain rounded particles 30-80 nm in diameter which have been identified as very low density lipoproteins (VLDL) (28-32, see also 33 and footnote 2). Such particles are conspicuously present in the hepatocytes of ethanol-treated animals (34) and may account in part for the substantial increase in the lipid content of their livers.

If Golgi elements were overloaded with VLDLs upon ethanol treatment, they would acquire the marker needed for their individual identification as well as a lower density which might facilitate their isolation. Based on this assumption, which proved valid, a procedure was worked out for preparing a series of three Golgi fractions of increasing density from homogenized livers of ethanol-treated rats. The lightest of these fractions is morphologically homogeneous. This paper

² VLDL particles have been isolated from Golgi-rich fractions prepared from rat liver homogenates and found to be immunologically alike and biochemically similar (but not identical) to VLDLs obtained from plasma (30-32) or liver perfusates (31). Differences recorded in lipid (30, 32) as well as in apoprotein (31) composition suggest that Golgi VLDLs undergo some changes either before or after discharge in the blood plasma.

describes our procedure; its companion presents a partial chemical and enzymic characterization of the fractions obtained.

MATERIALS AND METHODS

Preparation of Golgi Fractions

PREPARATION OF ANIMALS

All experiments were performed on young (150-250 g), male, Sprague-Dawley rats (Holtzman Co., Madison, Wis.) starved overnight. They were given 0.6 g ethanol/100 g body weight in 50% (wt:vol) solution by stomach tube (Intramedic Polyethylene Tubing no. PE 96, ID 0.034 inch; Clay-Adams, Inc., Parsippany, N. J.) and were sacrificed by decapitation 90 min later. When an easily assayable marker was needed for the intra-Golgi lipoprotein, 100 μ Ci [³H]palmitic-9,10-acid (New England Nuclear, Boston, Mass.) bound to bovine serum albumin (35; Armour Pharmaceutical Co., Chicago, Ill.) was injected intravenously 10 min before sacrifice (33).

CELL FRACTIONATION PROCEDURES

HOMOGENIZATION: The livers were collected in ice-cold 0.25 M sucrose, finely minced, forced through a tissue press, and then homogenized at $\sim 4^{\circ}\text{C}$ in 0.25 M sucrose with six to eight strokes of a motor-driven Teflon pestle in a Potter-Elvehjem homogenizer to give a 20% (wt:vol) homogenate. All subsequent operations were done at $\sim 4^{\circ}\text{C}$ and, unless otherwise specified, all separations were carried out with a Spinco L or L2-50 centrifuge using a no. 40 rotor (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.).

PREPARATION OF MICROSOMAL AND GOLGI FRACTIONS: The homogenate was cleared of cell debris, nuclei, and mitochondria by centrifuging for 10 min at 10,000 g_{av} . The first supernate was diluted back to the original volume with 0.25 M sucrose and spun for 90 min at 105,000 g_{av} . The resulting microsomal pellets were resuspended in 0.25 M sucrose by means of three to five strokes of a loosely fitting, motor-driven Teflon pestle and the ensuing suspension was diluted with 2.0 M sucrose to give a final concentration of 1 g (wet weight) tissue equivalent per 1.5 ml of 1.15 M sucrose. A small sample of this preparation was diluted with water to a sucrose concentration of 0.25 M and spun for 60 min at 105,000 g_{av} . The resulting pellet, resuspended in water or buffer, is referred to as the "total microsomal fraction." The remainder of this fraction was loaded under a discontinuous sucrose density gradient and spun in an SW 25.1 or SW 25.2 rotor to

obtain the final Golgi fractions. Details of the procedure are given in the Results section.

Electron Microscopy

0.5–1.0 ml-aliqouts of all the fractions of interest were mixed with an equal volume of 2% OsO₄ in water and placed in the bottom of a centrifuge tube. The remainder of the tube was filled with water and the tube spun for 1 h at 105,000 *g*_{av}. The supernate was discarded and the pellet was dehydrated *in situ* and then cut into strips which were embedded in Epon oriented in such a way that thin sections could be cut through the entire depth of the pellet. In some cases the pellets were stained *in toto* with uranyl acetate before dehydration.

Samples of liver tissue from normal and ethanol-treated rats were fixed in 1–4% OsO₄ in 0.1 M phosphate or cacodylate buffer, pH 7.4, for 2 h. The tissue was dehydrated and embedded in Epon as above.

Thin sections were cut on a Porter-Blum Servall Mt2 microtome with a Dupont diamond knife, and mounted on Formvar (Monsanto Co., St. Louis, Mo.) and carbon-coated grids. The sections were stained for 1 min in ethanolic uranyl acetate (36) and then either in alkaline lead citrate (37) for 5 min or in lead hydroxide (38) for 5–20 min. They were examined with a Siemens Elmiskop I or 101 electron microscope operated at 80 or 60 kV or a Hitachi 11C electron microscope operated at 75 kV. High concentrations of OsO₄, long staining in lead hydroxide, and microscopy at 60 kV were needed to enhance the density of VLDL particles, which is low in normally processed, Epon-embedded specimens.

Golgi Complex of Hepatocytes

NORMAL RATS

The general structure of the Golgi complex of the rat hepatocyte has been described by Bruni and Porter (39) and by Novikoff and Shin (40); its relationship with the ER as well as its involvement in VLDL processing has been studied in detail by Claude (41, 42).

In the rat hepatocyte, the complex is less orderly constructed and less clearly polarized than in other protein-secreting cells. The stacks of cisternae (saccules) and the vacuoles located on one of their sides have usual Golgi features and are accordingly easily recognized, but transitional elements of the type regularly found on the opposite side of the stacks in other secretory cells (3, 43) and in oocytes (44) are rarely encountered: their place is usually taken by irregularly dis-

tributed smooth ER elements. In addition, there are relatively few smooth-surfaced vesicles at the periphery of the complex and, according to evidence obtained on sectioned cells (42) as well as on negatively stained samples of isolated Golgi elements (22), smooth-surfaced tubules (rather than vesicles) connect the Golgi vacuoles and Golgi saccules with the ER.

The shape of the cisternal stacks varies so much that the convex and concave surfaces usually found in other cell types are rarely evident. The location of the complex close to the bile capillaries is quite constant, but its orientation is highly variable: often the whole structure is turned with its vacuolar side towards the bile capillary, but any other orientation, including the opposite one (Figs. 1 and 2), can be found. Because of these peculiarities, the terms used so far in describing the complex in other cells (13, 14) are not easily applicable to the hepatocyte.³ To avoid confusion and inconsistencies, we shall use in the following description the terms “cis” (located) and “trans” (located) to designate the location of a given structure within or near a Golgi stack, “trans” applying to elements on the vacuolar or secretory side and “cis” to elements on the opposite side of the stack.

The Golgi apparatus of the hepatocytes of normal, starved rats contains relatively few VLDLs. They occur usually in the large vacuoles, rarely in the last trans-Golgi cisternae (Fig. 1), and only occasionally in cis-Golgi saccules, their associated vesicles, and the adjacent smooth and rough ER elements.

EFFECTS OF ETHANOL TREATMENT

In ethanol-treated animals, VLDLs are found in increased numbers throughout most Golgi elements (Fig. 2). The extent of filling varies, but occasionally nearly all Golgi components, especially all stacked saccules, are heavily loaded (Figs. 3 and 4). More often only the rims of the stacked cisternae are filled while their empty

³ The Golgi complexes of the hepatocytes only rarely have the morphology and orientation implied by such terms as convex-concave and proximal-distal. The terms forming vs. mature face imply much more than we actually know about points of entry of products into, or flow of membrane and products across, the Golgi complex.

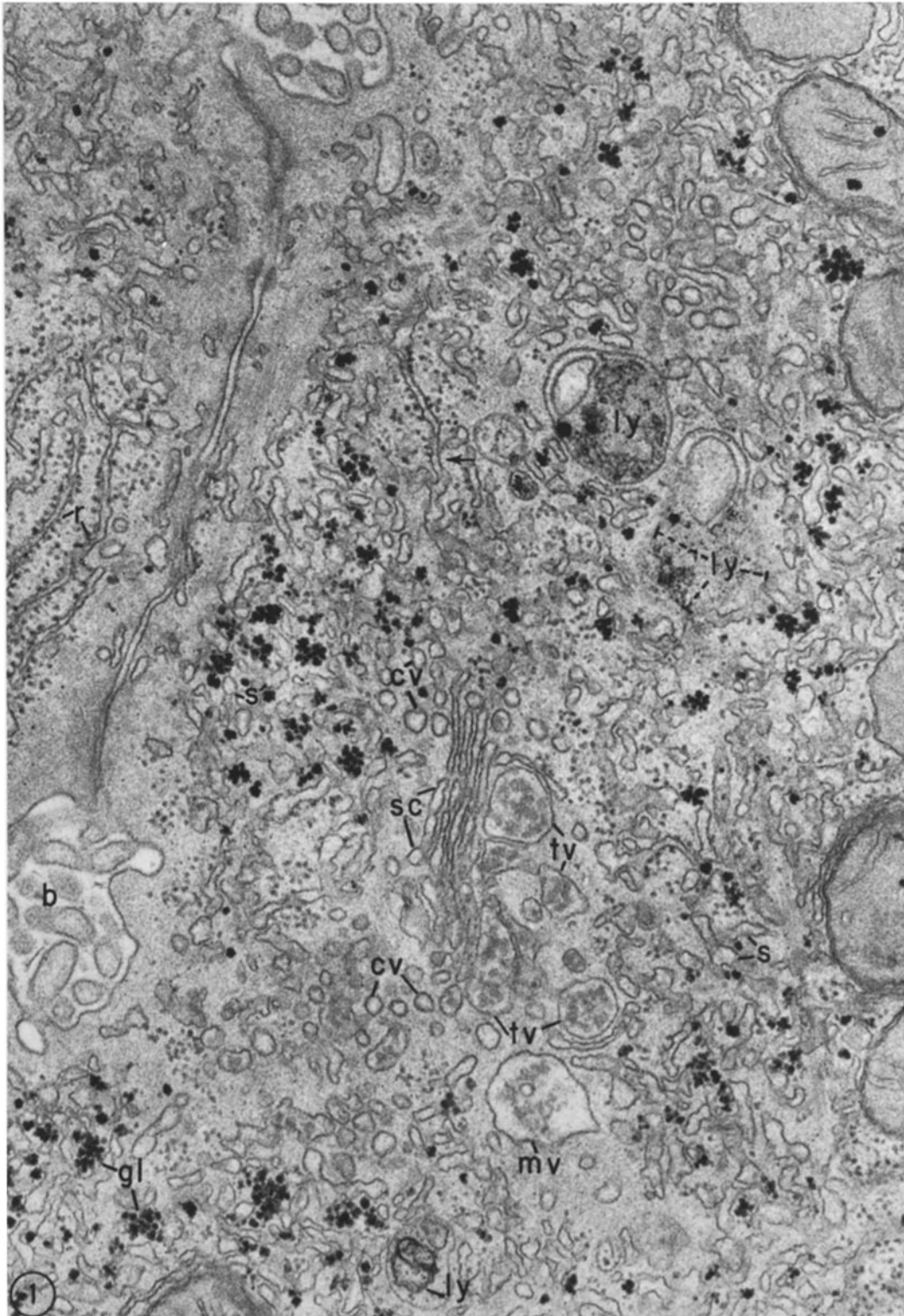


FIGURE 1 Hepatocyte of control rat. A Golgi complex with a "reversed" orientation appears in the vicinity of the bile capillary (*b*); VLDLs mark a number of trans-Golgi vacuoles (*tv*), but are absent from the stacked cisternae (*sc*) and most cis-Golgi vesicles (*cv*). The Golgi complex is surrounded by ER masses, primarily of the smooth type (*s*) with associated glycogen particles (*gl*). The arrow marks a clear example of continuity of rough to smooth ER. Lysosomes are marked *ly*, a multivesicular body *mv*, and rough ER elements *r*. $\times 60,000$.

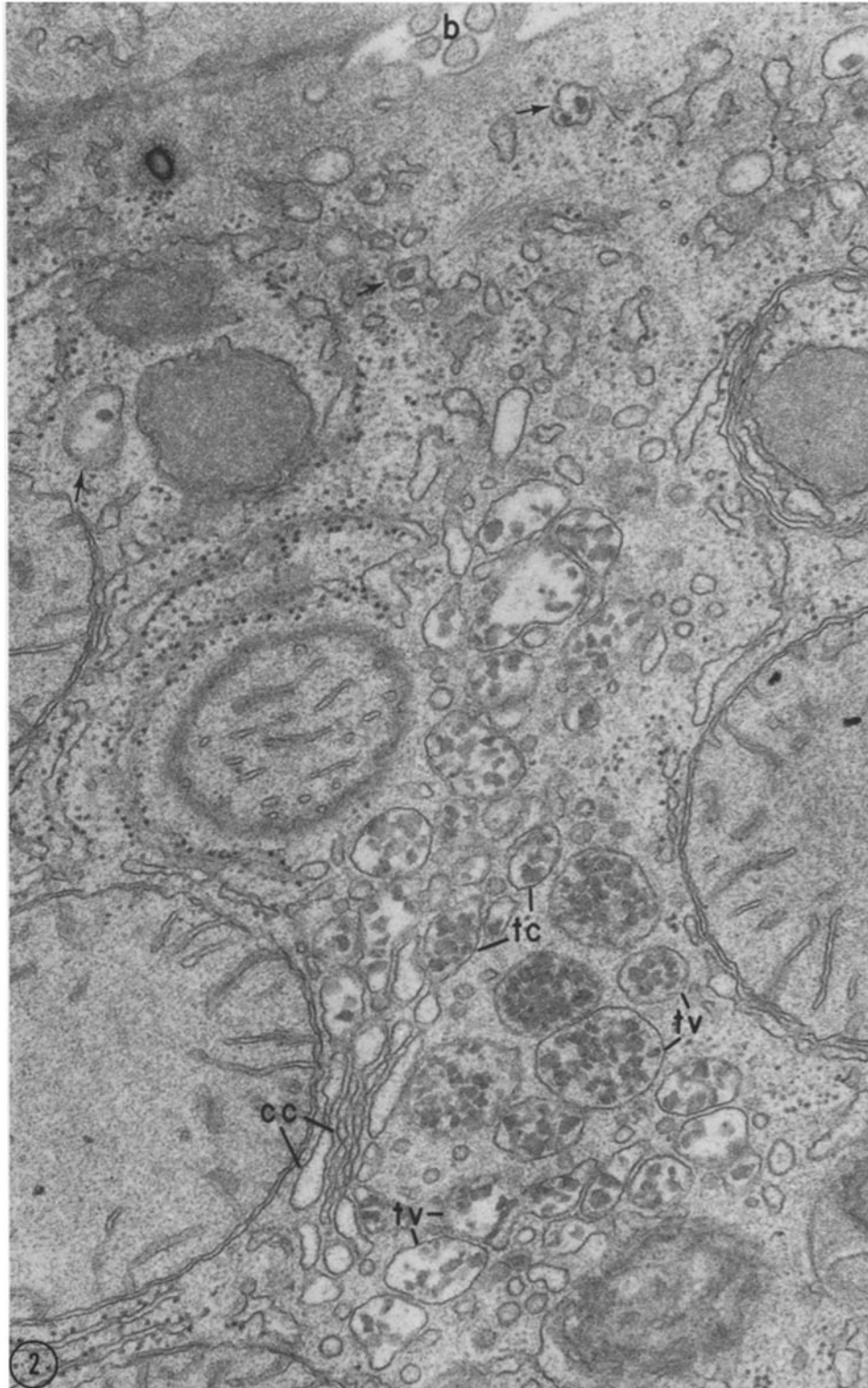


FIGURE 2 Hepatocyte of ethanol-treated rat. Practically all the elements of the Golgi complex, especially the trans-Golgi vacuoles (*tv*) and cisternae (*tc*) are loaded with VLDLs; only the cis-Golgi cisternae (*cc*) are partially empty (upper part loaded, lower half empty). VLDLs, single or in pairs, also mark elements of the smooth ER (arrows). Part of the lumen of a bile capillary appears at *b*; note that in this case the Golgi complex has a half "reversed" orientation: its main axis is nearly perpendicular to the bile capillary. $\times 60,000$.

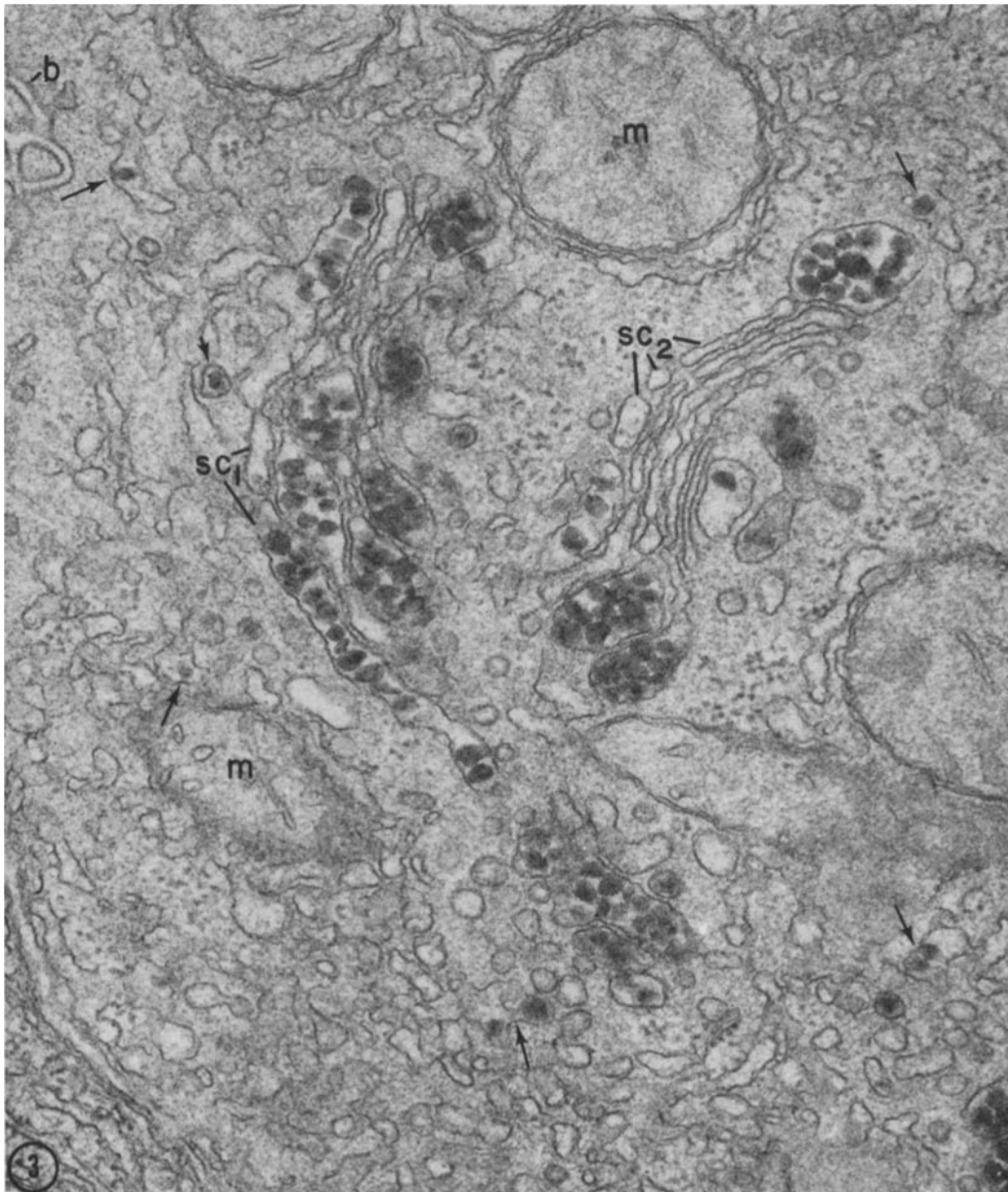


FIGURE 3 Hepatocyte of ethanol-treated rat. The field includes two stacks of Golgi cisternae. In stack sc_1 , practically all cisternal profiles are loaded from end to end with VLDLs, while in stack sc_2 , the central part of the profiles is empty and only their ends are distended by enclosed particles. The short arrow marks a cis-Golgi vesicle containing a single VLDL particle; the long arrows point out elements of the smooth ER with a similar content. (*b*) bile capillary, (*m*) mitochondrion. $\times 50,000$.

central parts appear flattened or collapsed (Fig. 3). Within each stack, the load generally decreases in the cis direction (Fig. 2), yet VLDLs are often encountered in vesicles and smooth ER

elements located on the cis side of the complexes (Fig. 5). By contrast, smooth ER elements with a trans-Golgi location usually contain few particles. These elements have been designated

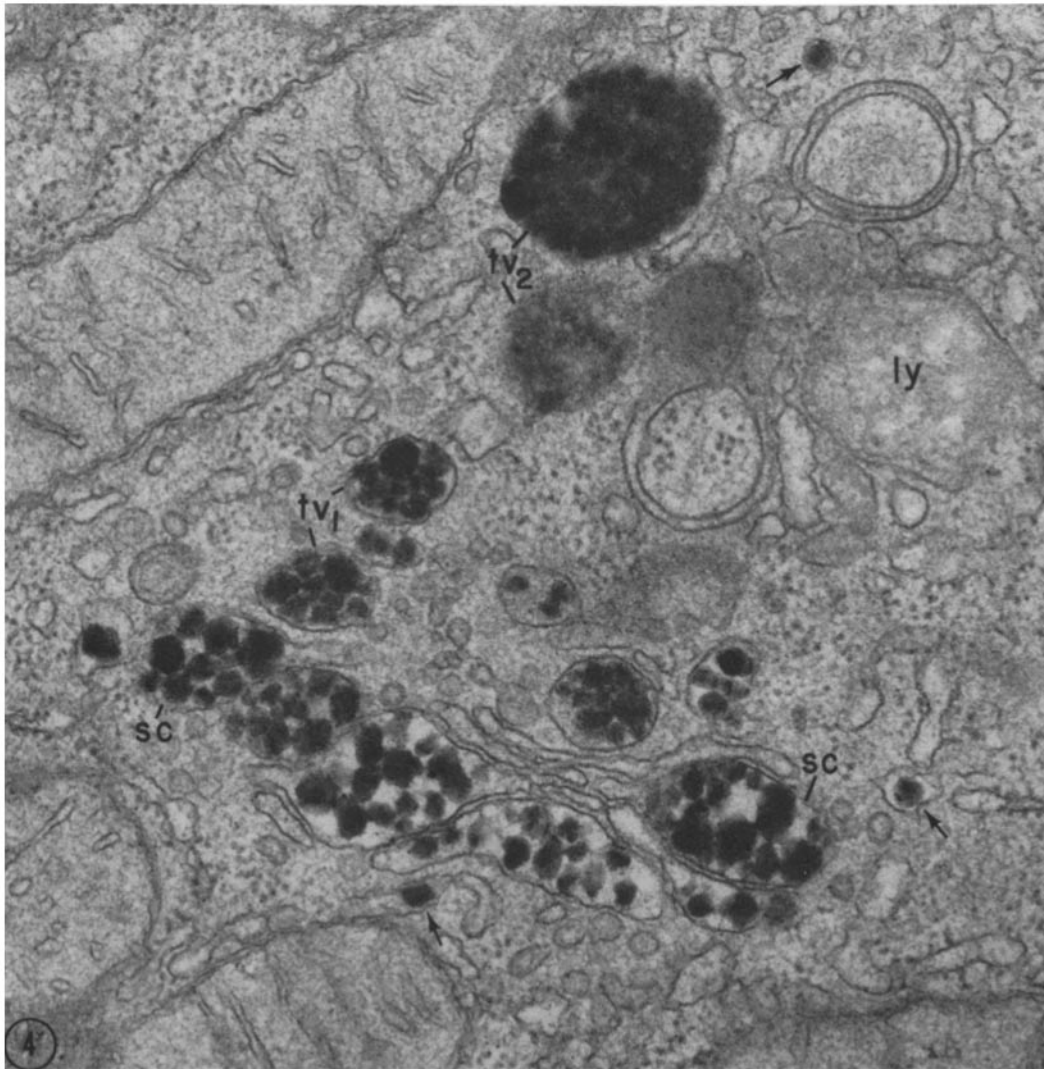


FIGURE 4 Hepatocyte of ethanol-treated rat. Practically all stacked cisternae (*sc*) are distended and loaded with VLDLs. Most of the trans-Golgi vacuoles (*tv*₁) are of the usual appearance, but in two of them (*tv*₂) the VLDLs appear compacted and partially fused. The arrows point to smooth ER elements marked by single VLDL particles. One of the lysosomes in the field is marked *ly*. $\times 50,000$.

GERL⁴ by Novikoff (46) and Novikoff et al. (45), who consider them as a specialized ER region

⁴ In hepatocytes, GERL has been described as a network of smooth surfaced tubules with associated vesicles (some of them of the coated variety). The complex is considered part of the ER and appears to be clearly differentiated from the adjacent saccules of the Golgi stacks (45). A similar situation has been described in neurons (46, 47), but in other cells, especially in tumorous melanocytes (48), the distinction is less clear: GERL has at least in

interposed between the Golgi apparatus and the rest of the system and involved in the formation of lysosomes. The concentration of VLDLs within individual Golgi elements varies considerably. In some vacuoles the relatively abundant, light matrix which usually surrounds and separates the particles is reduced in volume and increased in density to the point that, in extreme cases, the part the appearance and location of trans-Golgi saccule(s).

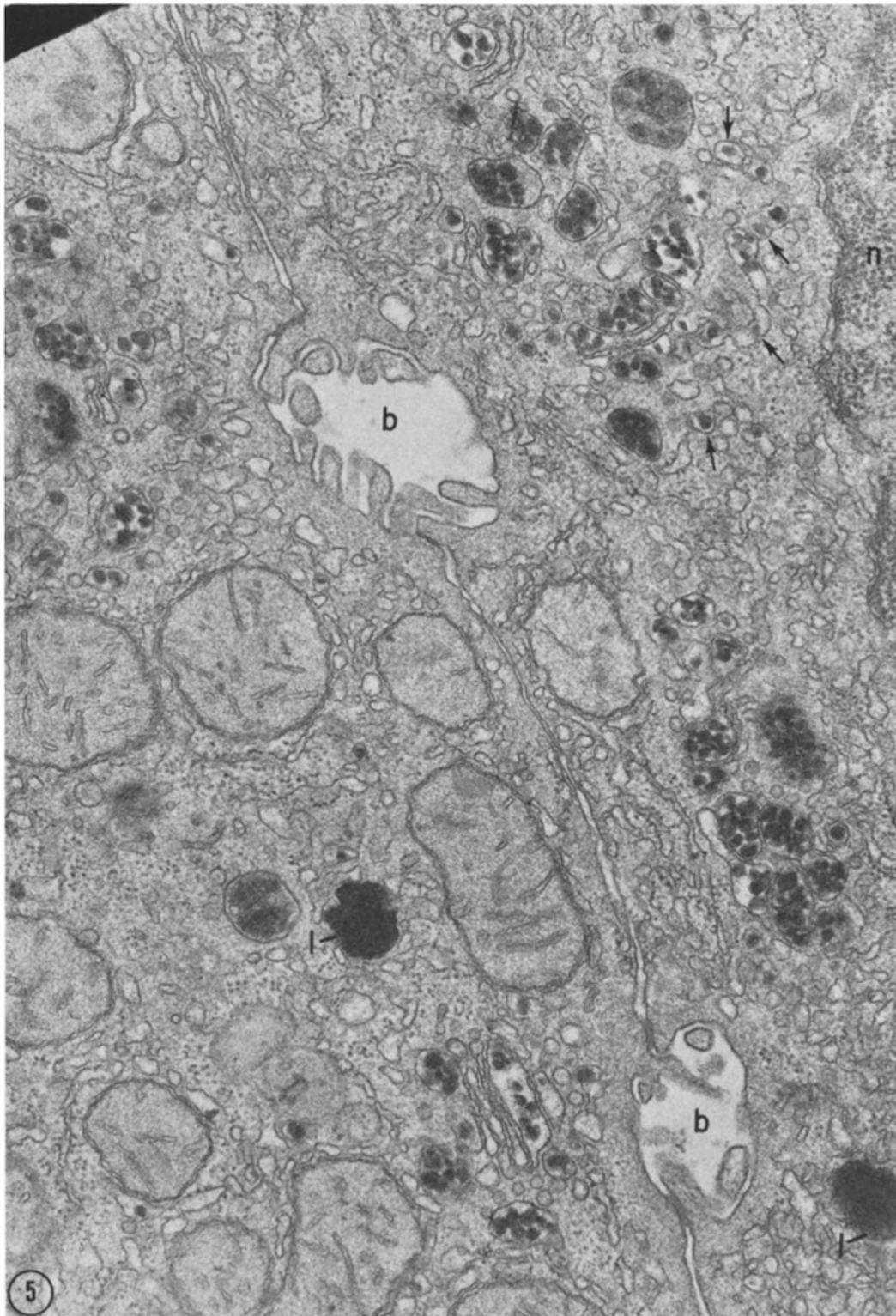


FIGURE 5 Two adjacent hepatocytes in the liver of an ethanol-treated rat. The field shows two profiles of bile capillaries (*b*) and four Golgi complexes, all extensively marked by VLDLs. The arrows point to convoluted elements, presumably smooth ER tubules located at the periphery of one of the complexes and loaded with particles, singly or in rows. Lipid droplets free in the cytoplasmic matrix are marked *l*, and part of a nucleus is marked *n*. $\times 36,000$.

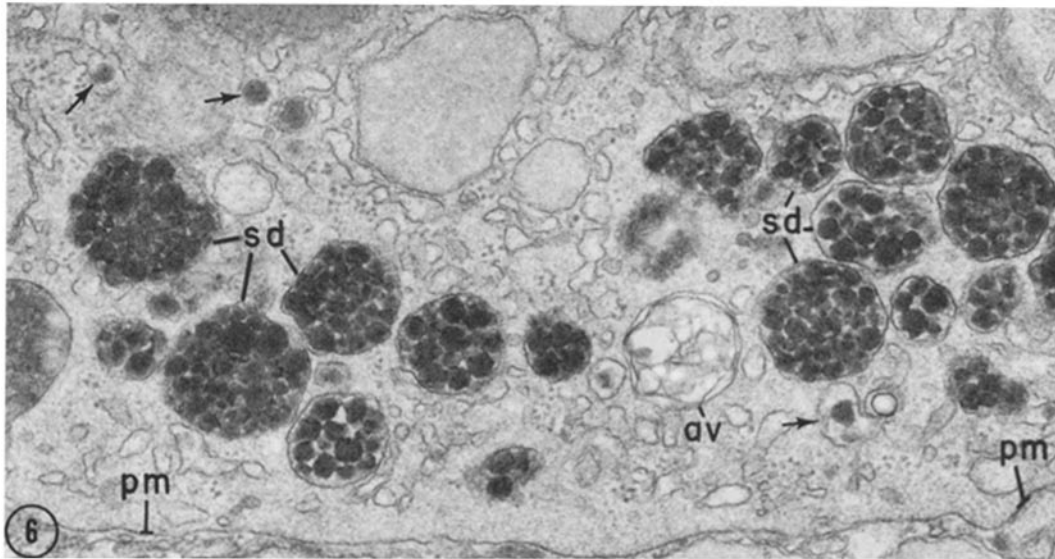


FIGURE 6 Hepatocyte of ethanol-treated rat. Accumulation of secretion droplets (*sd*) loaded with VLDLs at the periphery of the cell in the vicinity of the plasmalemma (*pm*). They are probably trans-Golgi vacuoles on their way to discharging sites. The arrows mark VLDL-containing elements of the smooth ER. An autophagic vacuole appears at *av*. $\times 36,000$.

particles appear compacted and possibly fused (Fig. 4). Such vacuoles occasionally contain membranous residues (in addition to still recognizable VLDLs), a feature which suggests either conversion into, or interaction with, lysosomes (cf. 49). The overloading of Golgi complexes with VLDLs affects practically all hepatocytes (Fig. 5), but the degree of VLDL accumulation varies widely enough from cell to cell and complex to complex to give a load distribution that overlaps extensively with that found in the hepatocytes of the controls.

In addition to those found in Golgi complexes, occasional VLDLs are seen in vesicles and in tubules of the smooth and rough ER throughout the cytoplasm in control as well as in ethanol-treated animals, but nothing like the accumulation seen in Golgi elements is encountered elsewhere. The only exception is some large vacuoles, loaded with particles and similar to those usually seen in close association with the complex, but found at some distance from it, often in the vicinity of the plasmalemma of the sinusoidal front of the cell (Figs. 6 and 7). Such vacuoles occasionally are "caught" discharging (Fig. 7). We assume that they are the equivalents of secretion droplets (or granules), post-Golgi membrane-bound compartments in which lipoprotein par-

ticles are sequestered and stored until such time as they are released into the blood (28, 29, 33).

Appearances suggesting transport and discharge of single particles or small clusters of particles by small, coated vesicles are more frequently encountered but more difficult to interpret (Fig. 7). There is evidence that coated vesicles operate in uptake (rather than discharge) of macromolecules (50-53), but in our material similar "coats" are found on part of the surface of large vacuoles loaded with particles and opened to the extracellular space (Fig. 7), i.e., in instances in which discharge is the most probable interpretation.

These results show that we can cause the Golgi vacuoles, saccules, and vesicles to become loaded with VLDLs by intoxicating the animals with ethanol. Since the treatment increases the proportion and diversifies the type of Golgi elements which contain such particles, its overall effect is expected to be a shift in the density distribution of the total population of Golgi elements toward lower densities. With such a shift, it may be possible to isolate Golgi-derived vesicles, perhaps as homogeneous populations, from total microsomal fractions. Clusters of 30-80-nm particles can serve as a characteristic marker for vesicles derived from the Golgi complex since there is no other

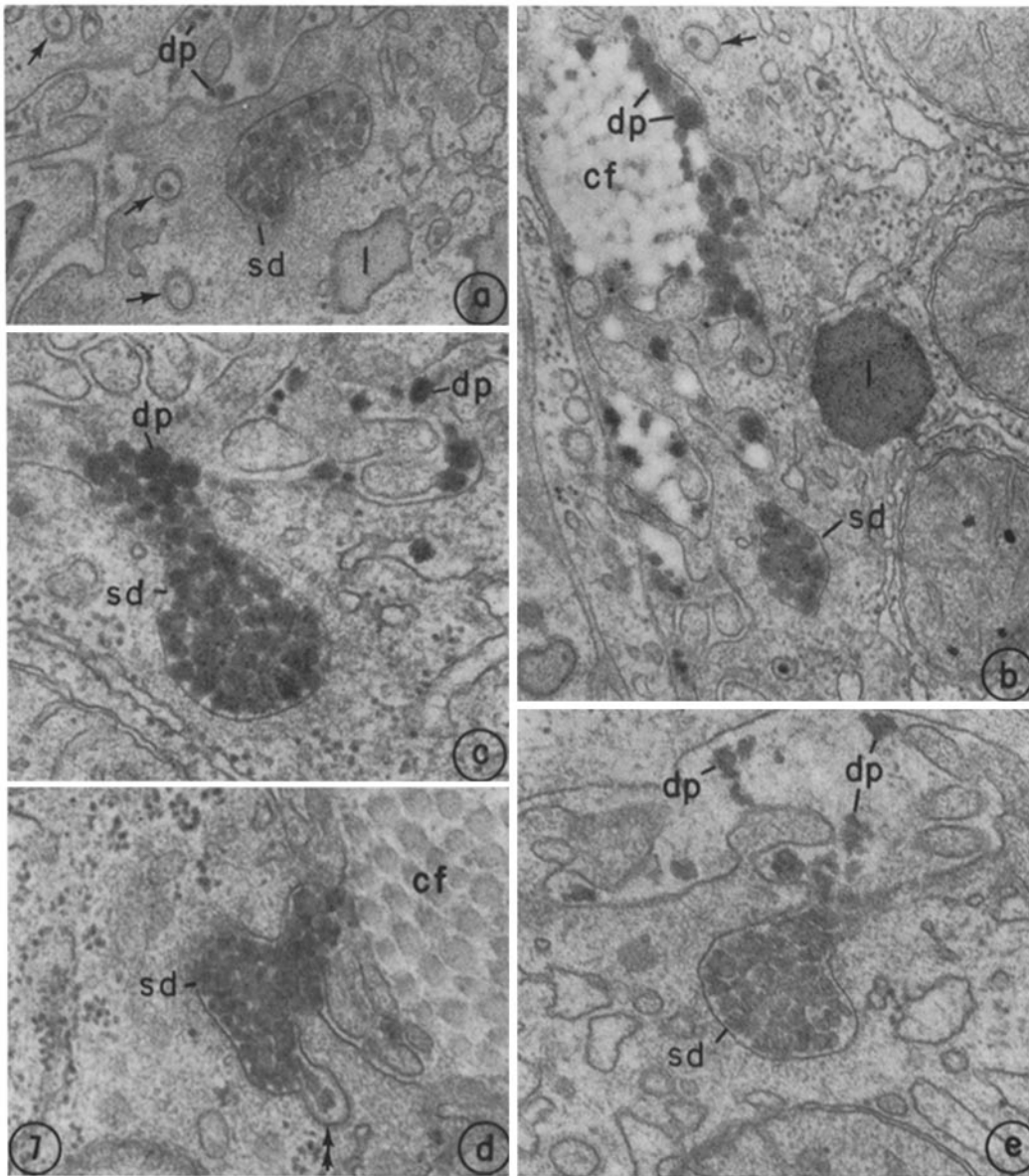


FIGURE 7 Hepatocytes of ethanol-treated rats. *a* and *b* show secretion droplets (*sd*) loaded with VLDLs in the immediate vicinity of the plasmalemma on the sinusoidal front of the cells, coated vesicles marked by single VLDLs (arrows), discharged VLDLs (*dp*) in the space of Disse, and lipid droplets (*l*) in the cytoplasmic matrix. *c-e* show secretion droplets (*sd*) in the process of discharging VLDLs in the space of Disse. Note that the discharging droplet in *d* is provided with a coat on part of its perimeter (double arrowhead). Bundles of collagen fibrils are marked *cf*. (*a*) $\times 54,000$, (*b*) $\times 46,000$, (*c*) $\times 66,000$; (*d*) $\times 70,000$, (*e*) $\times 60,000$.

quantitatively significant source of similarly marked vesicles in hepatocytes except for the secretion droplets already mentioned. We can thus attempt to purify the elements of the Golgi

complex by using some of their characteristic morphological features (such as type of membrane and especially type of content) as a necessary and sufficient means of identification after isola-

tion, without depending on the preservation of the stacks (dictyosomes) as several previous investigators have done in the past (54, 55) or more recently (22, 26). Assuming that the fractions thus obtained are reasonably homogeneous, their biochemical characterization could be based on the morphological identification of their components.

Isolation of Golgi Fractions from Microsomes

PRELIMINARY SUBFRACTIONATION OF MICROSOMES

A preliminary subfractionation of the total microsomal fraction was carried out to find out whether we could obtain Golgi-enriched fractions and to explore the range of densities into which Golgi-derived vesicles would fall. The microsomes were resuspended in 1.15 M sucrose and loaded into the bottom of a centrifuge tube of cellulose nitrate. Discontinuous sucrose gradients were constructed above the load and centrifuged for 3 h at 25,000 rpm in a Spinco SW 25.1 rotor (63,500 g_{av}). The material which accumulated at the various interfaces was collected and processed for electron microscopy. The interfaces surveyed were: (a) water/0.3 M, (b) water/0.4 M, (c) water/0.5 M, (d) 0.25 M/0.75 M, (e) 0.3 M/0.5 M, (f) 0.4 M/0.6 M, (g) 0.5 M/0.75 M, (h) 0.6 M/0.86 M, and (i) 0.86 M/1.15 M sucrose.

The results showed that the subcellular components collected at gradient densities ranging from ~ 1.000 (water) to ~ 1.173 (1.15 M sucrose) could be divided into four groups. The lightest group, which accumulated at $\rho < 1.041$ (0.30 M sucrose), consisted primarily of free, relatively large, lipid droplets (diameter $\approx 0.5 \mu\text{m}$) and contained only few Golgi vesicles. The second group, collected at $\rho \approx 1.041$ to 1.081 (0.30–0.60 M sucrose), was made almost exclusively of vesicles derived from the Golgi complex as shown by their enclosed VLDL markers. The third group, accumulated at $\rho \approx 1.081$ to 1.124 (0.60–0.86 M sucrose), consisted mainly of Golgi vesicles but a variable fraction of it (up to one-fourth) was made up of large, empty, often collapsed, and apparently VLDL-free vacuoles. The intracellular origin of the latter was not entirely certain, although most of them appeared to be empty Golgi cisternae. The fourth group, collected at $\rho > 1.124$ but < 1.173 (0.86–1.15 M sucrose), consisted of small vesicular, tubular,

and cisternal elements marked only in part by VLDLs; its cisternal elements had a characteristic appearance with collapsed centers and slightly distended, torus-shaped rims.

ISOLATION OF MORPHOLOGICALLY HOMOGENEOUS, LIGHT, GOLGI FRACTION

To isolate the purest fraction possible, even at the price of low yield, we designed a gradient which could eliminate the fat droplets of the light range and the large collapsed vacuoles of the heavy range while giving an acceptable yield of vesicles marked by VLDLs. The density range we chose to try extended from 1.034 to 1.081 (0.25–0.6 M sucrose).

The final gradient was constructed by layering 10 ml of 0.6 M sucrose over the load (8–12 ml, i.e., 5.3–8.0 g wet tissue equivalents) and filling the remainder of the centrifuge tube above 0.6 M sucrose with 0.25 M sucrose (10–14 ml). The gradient was spun for 3.5 h at 25,000 rpm in a Spinco SW 25.1 or for 3 h at 25,000 rpm in an SW 25.2 (footnote 5) rotor ($\sim 13 \times 10^6 g \times \text{min}$ at the 0.25 M/0.6 M interface in either case). The fluid above the 0.25 M/0.6 M interface was drawn off with a syringe and discarded. The cloudy band at the interface was collected and processed for electron microscopy as indicated under Materials and Methods.

MORPHOLOGY OF LIGHT GOLGI FRACTION

The pellet obtained from the band at the 0.25 M/0.6 M sucrose interface is shown in Figs. 8–10. It consists primarily of vesicles ranging in size from 50 to 500 nm, all limited by a unit membrane ~ 7.5 –9.0-nm thick and practically all filled with 30–80-nm dense particles.

The form of the vesicles varies considerably from predominantly spherical or ellipsoidal at the bottom⁵ of the pellet (Fig. 8 a) to elongated, discoidal, or possibly tubular in the upper layers and finally to fine tubular and small spherical at its

⁵ In the tubes of the SW 25.2 rotor the load was usually increased to 15–20 ml, the 0.6 M sucrose layer to 15 ml, and the 0.25 M sucrose layer to 25–30 ml.

⁶ Since the pellet was obtained from an OsO_4 -treated suspension of vesicles, position in the pellet does not necessarily reflect the native sedimentation characteristics of the vesicles.

top (Fig. 8 *b*). In addition, ring-, dumbbell-, and barbell-shaped profiles are frequently encountered (Figs. 9 and 10), and occasionally more complicated profiles are seen (Fig. 10) which apparently represent sections at various levels and angles through Golgi cisternae filled with lipoprotein particles along their rim, but buckled and partially or completely collapsed in their center (see Figs. 9 and 10 for more detailed explanations).

In size and shape as well as in the characteristics of their contained particles, the isolated vesicles generally resemble the Golgi vacuoles and saccules seen *in situ* in intact hepatocytes. In the isolated vesicles, however, the VLDLs appear more closely packed, and the matrix around them is greatly decreased in volume and increased in density to the point that the density pattern seen *in situ* seems to be reversed: although the VLDLs remain dense in the absolute sense, they appear light against the denser background of the highly condensed surrounding matrix. The limiting membrane of the vesicles is usually tightly applied on the compacted masses of particles and matrix. Finally, the frequency of large, spherical vesicles and the number of VLDLs contained by an average vesicle appear to be higher in the isolated fraction than *in situ*. All these differences could be explained by fragmentation followed by rounding up of some Golgi cisternae and by extensive compaction because of water loss undergone by all isolated Golgi elements during the long period in which they are exposed to hypertonic sucrose. We must postulate, however, that these changes are irreversible under our experimental conditions.

Notwithstanding the structural changes mentioned, the morphology of the pellets indicates that most Golgi components, especially the large spherical vacuoles as well as the flattened or partially distended saccules, are represented in our light

Golgi fraction. The only elements definitely present in smaller relative concentration than *in situ* are the small vesicles and fine tubules which in the intact cell have a cis-Golgi location and contain either a single or only a few VLDLs. Few such elements are found at the top of the pellet, but most of them may be too heavy for isolation in this fraction. Accordingly, the preparation described contains the main Golgi components but in a mix which appears to be definitely biased in favor of trans-Golgi elements.

The light Golgi fraction comprises only a few contaminants, namely: (*a*) a few bodies with dense, heterogeneous content, presumably lysosomes, which represent less than 1% of its components and are concentrated at the bottom of the pellets; these lysosomes apparently contain a large amount of lipid residues, (*b*) small "empty" vesicles, many of which appear to be connected with Golgi saccules (Figs. 8 and 9); some of them, however, may be derived from other intracellular sources, (*c*) some of the few small vesicles containing a single or a few VLDLs (Fig. 8) could be derived from the smooth ER rather than the Golgi complex; the electron microscopy of the pellets cannot solve the problem of their origin, but enzymic assays, to be reported in the companion paper (56), indicate that such contamination is either absent or negligible.

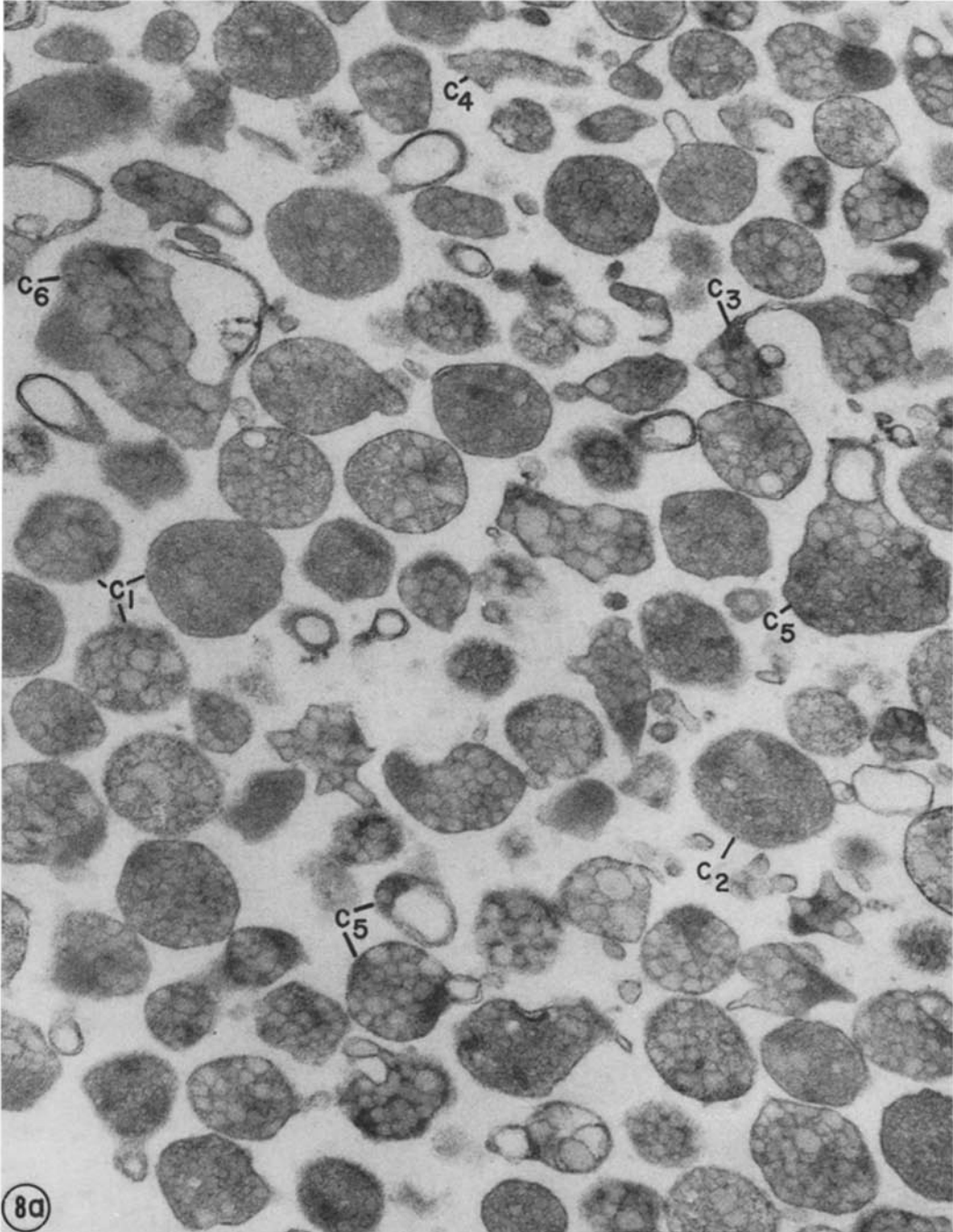
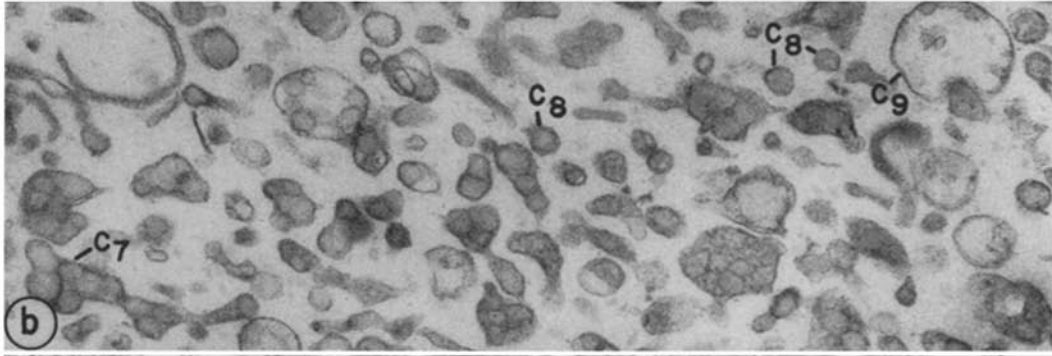
We can conclude that the preparation obtained is a reasonably representative and, on morphological grounds, highly purified Golgi fraction; we will refer to it hereafter as GF₁ or light Golgi fraction.

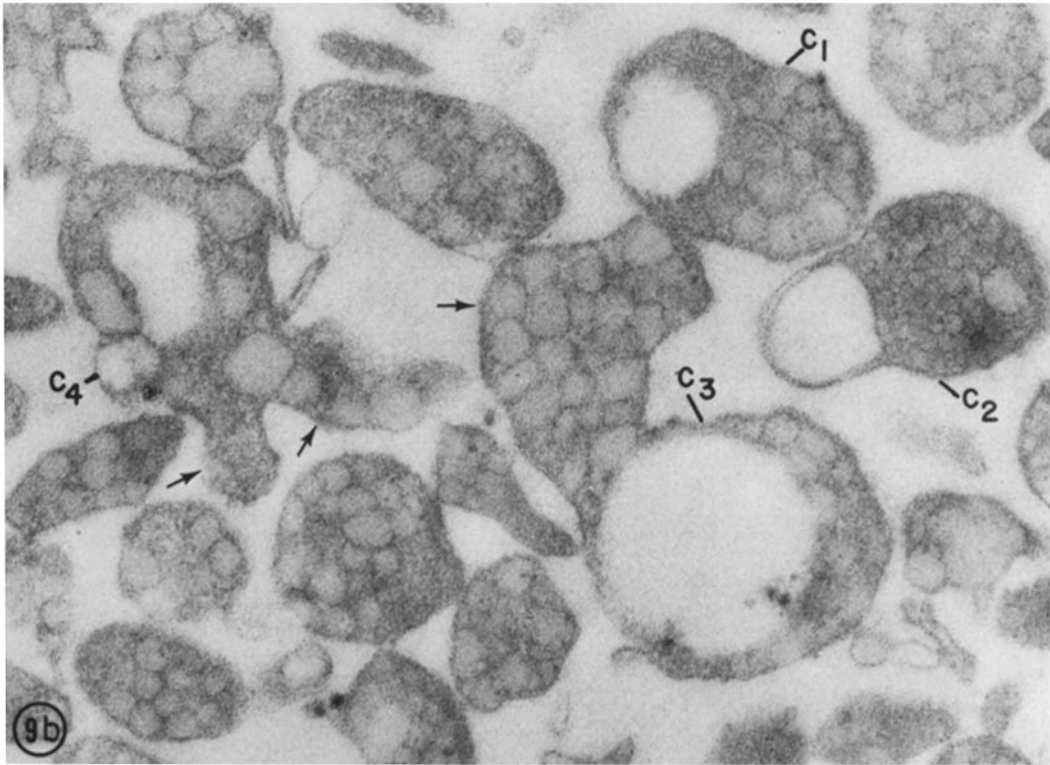
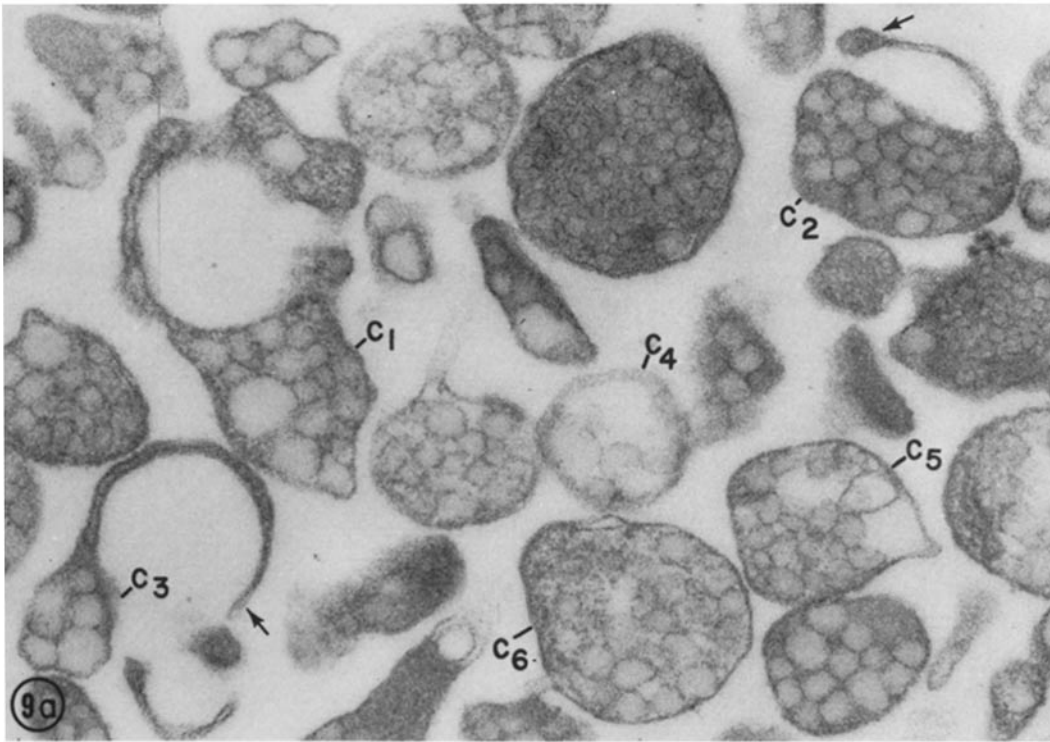
ATTEMPTS TO IMPROVE YIELD OF GOLGI ELEMENTS: ISOLATION OF SERIES OF THREE GOLGI FRACTIONS

Since little of the protein and phospholipid of the original microsomal preparation was recovered in

FIGURES 8-10 Light Golgi Fraction (GF₁), sectioned pellets.

FIGURE 8 (*a*) Field at the bottom of the pellet showing that the fraction consists essentially of VLDL-loaded Golgi elements. Most of their profiles are rounded (*c*₁) or oval (*c*₂) but dumbbell- (*c*₃), barbell- (*c*₄), ring-shaped (*c*₅), and highly irregular profiles (*c*₆) are also encountered (see legends of Figs. 9 and 10 for a detailed explanation of their derivation from Golgi cisternae). $\times 36,000$. (*b*) Field near the top of the pellet showing that, in addition to some of the components seen in *a*, the fraction contains at this level elongated (*c*₇) and small circular profiles (*c*₈) marked by single VLDL particles. The latter types of profiles, which mainly represent small Golgi vesicles, predominate at the very top of the pellet. Note the large circular or oval profiles which have lost part of their VLDL content (*c*₉). $\times 36,000$.





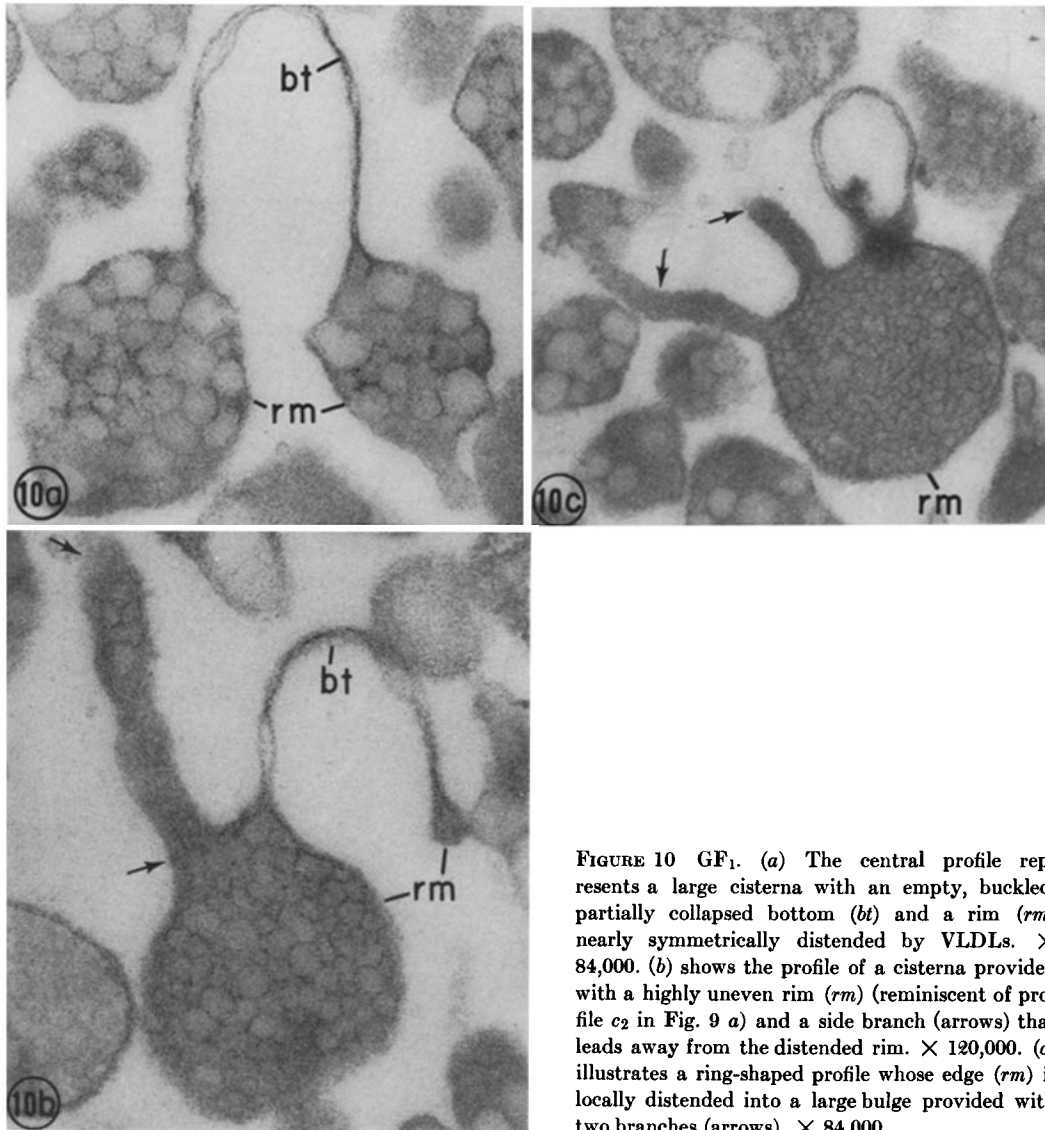
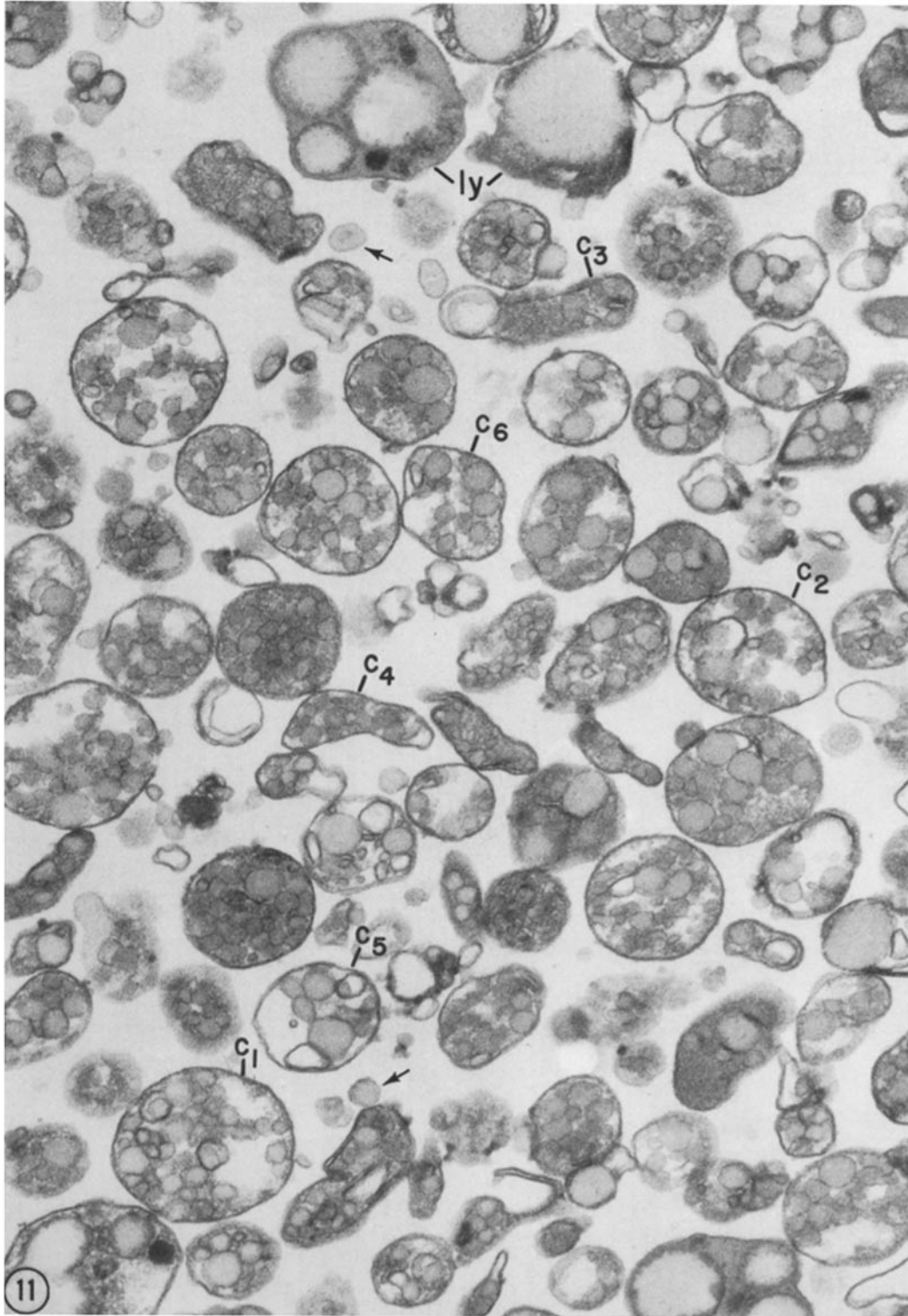


FIGURE 10 GF₁. (a) The central profile represents a large cisterna with an empty, buckled, partially collapsed bottom (*bt*) and a rim (*rm*) nearly symmetrically distended by VLDLs. $\times 84,000$. (b) shows the profile of a cisterna provided with a highly uneven rim (*rm*) (reminiscent of profile *c*₂ in Fig. 9 a) and a side branch (arrows) that leads away from the distended rim. $\times 120,000$. (c) illustrates a ring-shaped profile whose edge (*rm*) is locally distended into a large bulge provided with two branches (arrows). $\times 84,000$.

FIGURES 9 and 10 GF₁. Sections through the middle region of the pellet.

FIGURE 9 (a) Profile *c*₁ represents a buckled cisterna with an empty bottom and an irregularly distended rim filled with VLDLs. In profile *c*₂ the rim is very thin on one side (arrow), and in profile *c*₃ it is completely missing on the right (arrow). Such profiles may result from a pinching off of parts of the dilated rim either in vivo or during tissue homogenization. Most of the other profiles (*c*₄-*c*₆) are sections through rounded outpocketings of dilated rims or through vacuoles detached therefrom. Some of the profiles (*c*₄ and *c*₅) have lost part of their contained VLDLs. $\times 80,000$. (b) The irregular ring-shaped profiles marked *c*₁ and *c*₂ represent oblique sections cutting in part through the VLDL-filled rim and in part through the empty, collapsed bottom of Golgi cisternae. The ring profile at *c*₃ has a large outpocketing of its rim filled with VLDLs (arrow). At *c*₄, the ring profile has two branches (arrows) which may represent connecting tubules with either the ER or other Golgi elements. $\times 87,000$.



FIGURES 11 and 12 Intermediate Golgi Fraction (GF₂), sectioned pellets.

FIGURE 11 Field at the bottom of the pellet. Most profiles resemble those found in GF₁ except for the relatively high frequency of partially emptied vacuoles (*c*₁ and *c*₂). Elongated profiles (*c*₃ and *c*₄) filled with VLDL are quite numerous. Note the frequency of membrane-bound vesicles and membrane fragments in VLDL marked Golgi vacuoles (*c*₂, *c*₅, and *c*₆). Some VLDLs have been displaced from the interior of the vesicles into the surrounding medium, most probably during the collection of the fraction before fixation for electron microscopy (arrows). Lysosomes are marked *ly*. × 36,000.

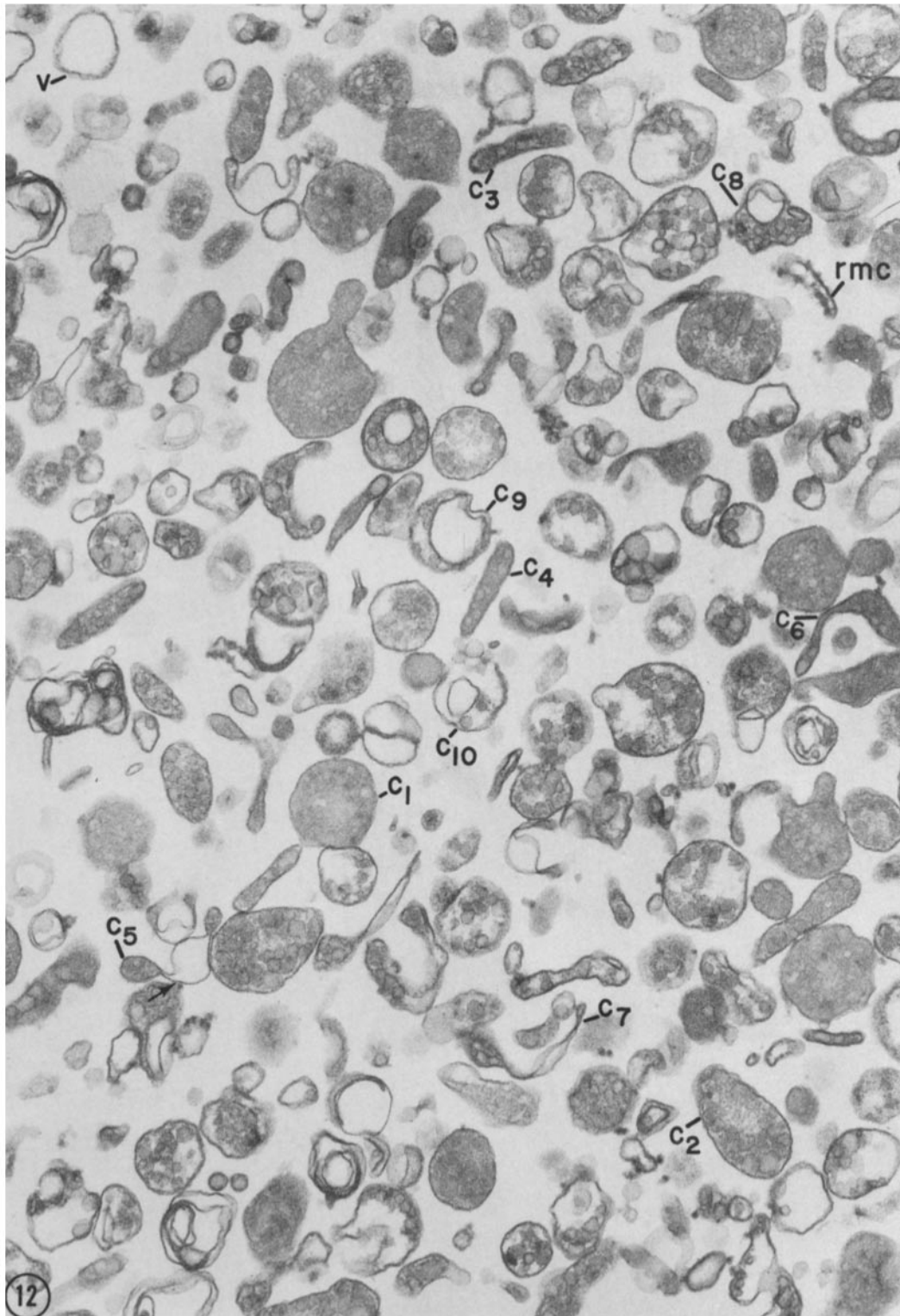


FIGURE 12 Field in the upper half of the pellet. The frequency of circular (c_1) and oval (c_2) profiles is lower, and that of elongated profiles (c_3 and c_4) is higher, than at the bottom of the pellet (Fig. 11). Normal or oblique sections through Golgi cisternae are seen at c_5 - c_7 . Ring-shaped profiles, partially emptied, appear at c_8 - c_{10} . The large empty vacuoles marked v may represent sections through the dilated and emptied bottoms of the type of cisterna seen at c_5 (the arrow indicates the probable plane of section). (rmc) contaminating rough microsomes. $\times 36,000$.

GF₁ (1, 56), and since a large number of VLDL-containing vesicles are still present among the remnant microsomes, an attempt was made to improve the recovery of Golgi components by collecting vesicles of increasingly higher density. To this intent, discontinuous density gradients were used and in each of them the load, i.e. 15 ml of total microsomes resuspended in 1.15 M sucrose, was overlaid with three successive 15-ml layers of 0.86, 0.6, and 0.25 M sucrose. Upon centrifugation in a SW 25.2 Spinco rotor at 75,000 g_{av} for 3 h (with the brake off) fluffy bands appeared at each interface. The bands were collected and processed for electron microscopy as described.

The material banded at the 0.25 M/0.6 M sucrose interface was identical to the light Golgi fraction (GF₁). The band at the 0.6 M/0.86 M interface is referred to as the intermediary Golgi fraction, or GF₂; it was found to consist of Golgi elements similar in morphology to those seen in GF₁, except for their generally smaller size, partial loss of content, and higher frequency of vesicular, tubular, and small cisternal elements (Figs. 11 and 12). Most of these elements were marked by VLDLs, but many of them appeared partly emptied of their lipoprotein load, probably as a result of rupture and resealing during homogenization. The fraction also contained a variable amount of large empty or collapsed cisternae, many of which could be identified as of Golgi origin by their occasional continuity with VLDL-loaded vesicles. In addition to the actual or possible contaminants described in GF₁, GF₂ contained a few rough microsomes probably floated to the density of the band by adsorption to, or aggregation with, Golgi elements.

By morphological criteria, especially by the VLDL content of its vesicles, the band at the 0.6 M/0.86 M sucrose interface can be recognized as a Golgi fraction of relatively high homogeneity

only slightly more contaminated by other subcellular components than GF₁. In addition, it appears to be a representative preparation since it includes all types of Golgi components although in a mix biased in favor of elements of medium to small size.

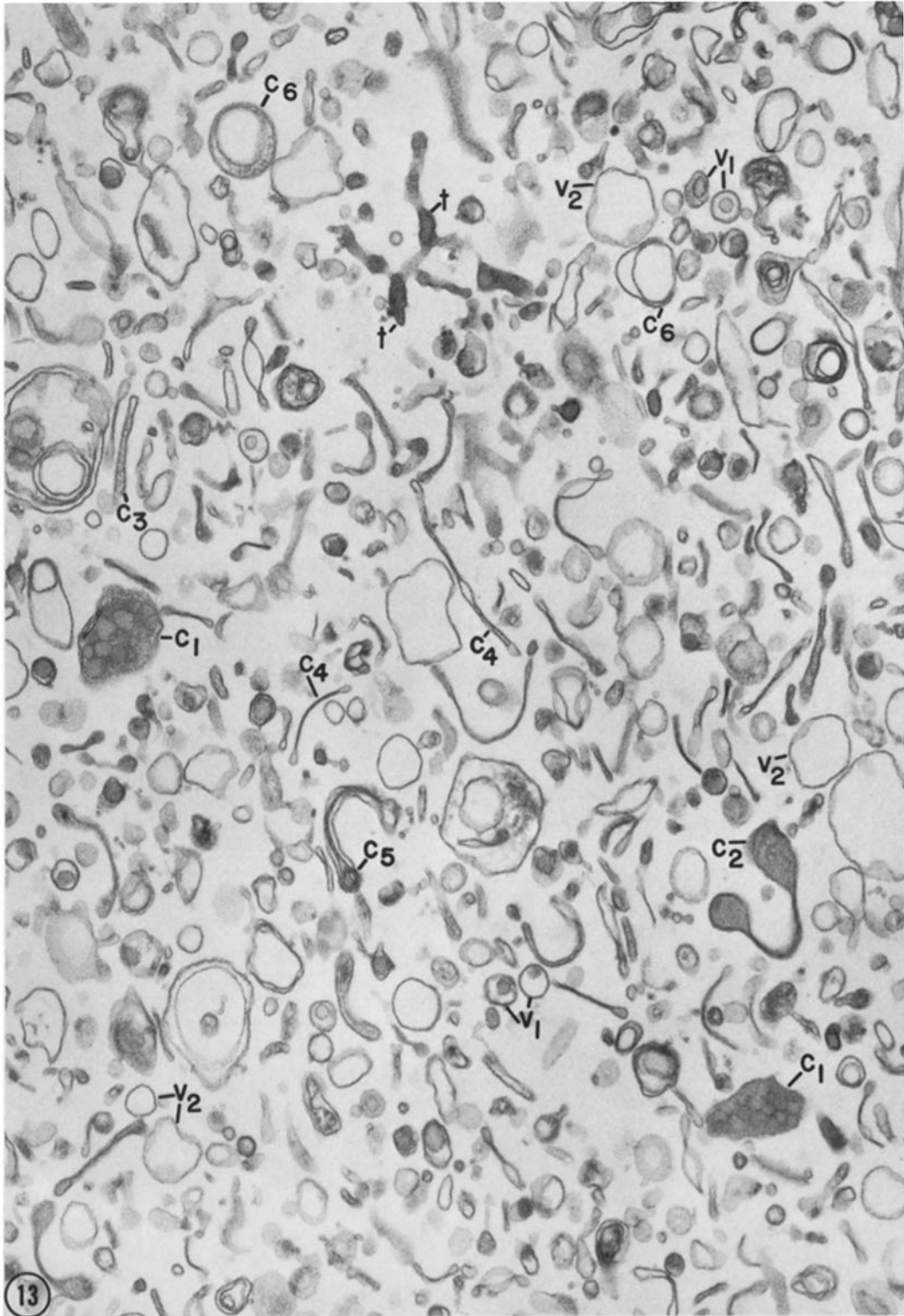
The material banded at the 0.86 M/1.15 M sucrose interface is referred to as the heavy Golgi fraction, or GF₃. It is comprised primarily of small vesicles, tubules, and small flattened cisternae, the latter characteristically showing slightly dilated rims and collapsed and fused central areas (Figs. 13–15). Practically all the elements of the fraction are bounded by smooth membranes and many of them are marked by their content of VLDL particles which occur singly or in small clusters. Many elements, however, especially many tubules and cisternae, contain only a dense, homogeneous matrix. The relatively high frequency of recognizable but VLDL-free cisternal elements in this fraction suggests that many Golgi cisternae lose during homogenization most of their VLDL-loaded rims which, converted to “vacuoles,” float to the lighter fractions, i.e. GF₁ and GF₂, while the partly empty and collapsed center pieces float only to GF₃. In addition to the elements mentioned, the band includes a relatively large population of medium sized (~100 nm) vesicles either completely empty or containing only a few VLDLs. A few rough microsomes are among its identifiable contaminants.

On account of their morphology and content, a large proportion of the elements of GF₃ can be identified as of Golgi derivation but the origin of the unmarked, noncisternal elements remains undecided: (a) they may represent Golgi elements which have not acquired or have lost their VLDLs, (b) they may derive from other cell structures, such as the smooth ER or the plasmalemma, or (c) they

FIGURES 13–15 Heavy Golgi Fraction (GF₃), sectioned pellets.

FIGURE 13 Field in the middle of the pellet showing the variety of Golgi elements present in this fraction. Among them, there are profiles of Golgi vacuoles (*c*₁) and cisternae (*c*₂) marked by VLDL clusters, small circular profiles representing vesicles or tubules (*v*₁) marked by single particles as well as elongated profiles (tubules) occasionally organized in networks (*t*) and similarly labeled.

Cisternal elements of varied sizes, partially (*c*₃) or extensively (*c*₄) collapsed, represent an important and characteristic component of this fraction; occasionally such cisternae occur in pairs (*c*₅). The ring profiles marked *c*₆ are sections through the empty bottoms of buckled cisternae. Finally, the fraction comprises a relatively large number of empty vesicles (*v*₂) of different sizes limited by a single membrane; their origin is uncertain, but occasionally they are found in continuity with recognizable Golgi elements. For further details see the legends for Figs. 14 and 15. $\times 36,000$.



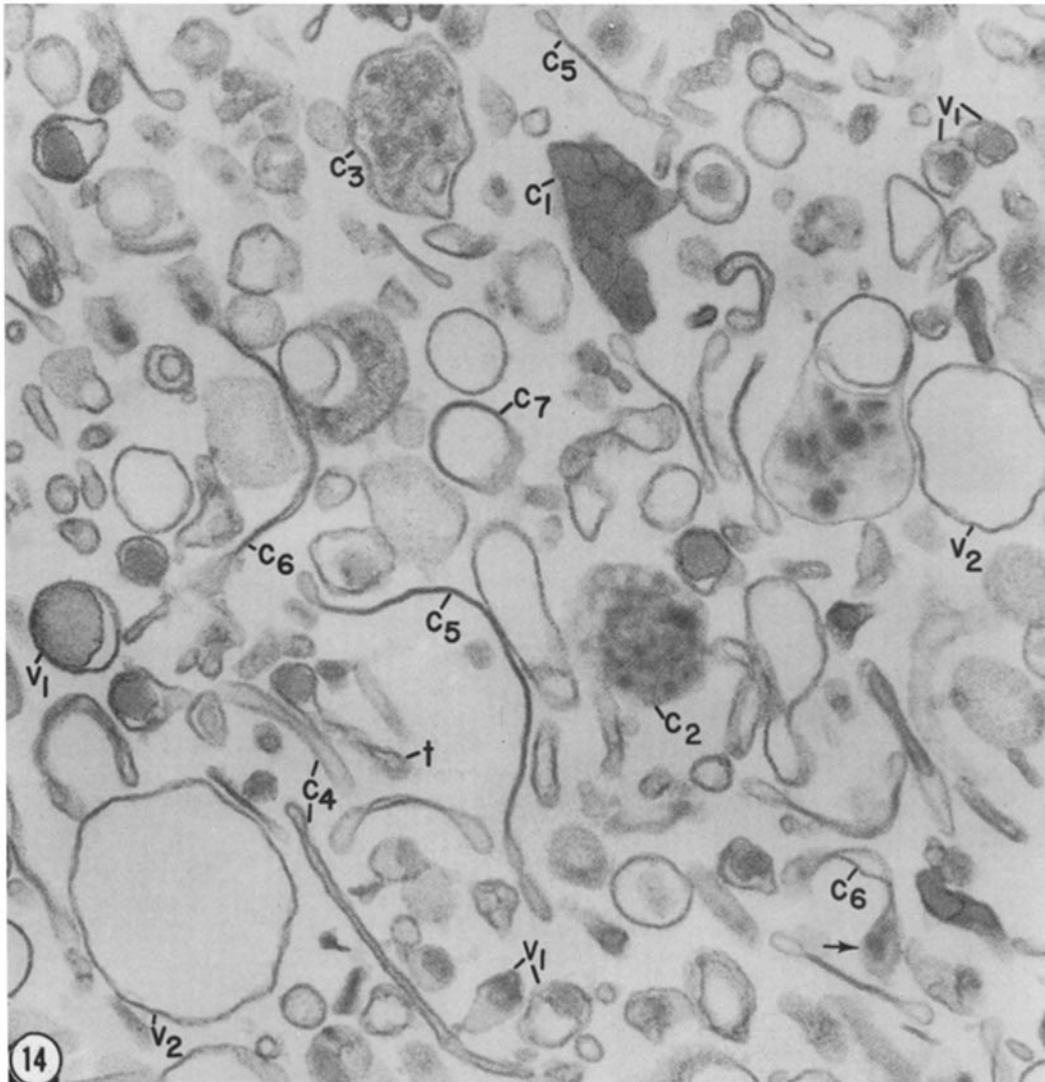


FIGURE 14 GF₃. Field in the middle region of the pellet showing at higher magnification and in further detail the components of the fraction. *c*₁-*c*₃ indicate Golgi vacuoles loaded with either usual VLDL particles (*c*₁), or altered, fused particles (*c*₂) or altered particles mixed with membranous residues (*c*₃). Circular and elongated profiles marked by single VLDL particles are seen at *v*₁ and *t* and profiles of cisternal elements of different sizes and varied degrees of collapse at *c*₄-*c*₆. Those at *c*₄ still have a visible lumen, whereas those at *c*₅ and *c*₆ have their lumina largely obliterated by membrane fusion. In most of them, only the rims remain open (*c*₅), while in others (*c*₆) open pockets occur also in the central part of the cisternae. The rims of collapsed and obliterated cisternae are occasionally marked by VLDL particles (arrow). A ring profile whose lumen is obliterated by membrane fusion appears at *c*₇. Empty vesicles (*v*₂) of uncertain origin are scattered among the other components of the fractions. × 78,000.

may be a mixture of elements originating from both sources.

GF₃ is the least homogeneous of the Golgi fractions obtained and also the least representative being heavily biased towards small cis-Golgi ele-

ments and probably towards collapsed center pieces of stacked cisternae.

Examination of the pellet of residual microsomes showed that practically all recognizable Golgi elements have been removed from the origi-

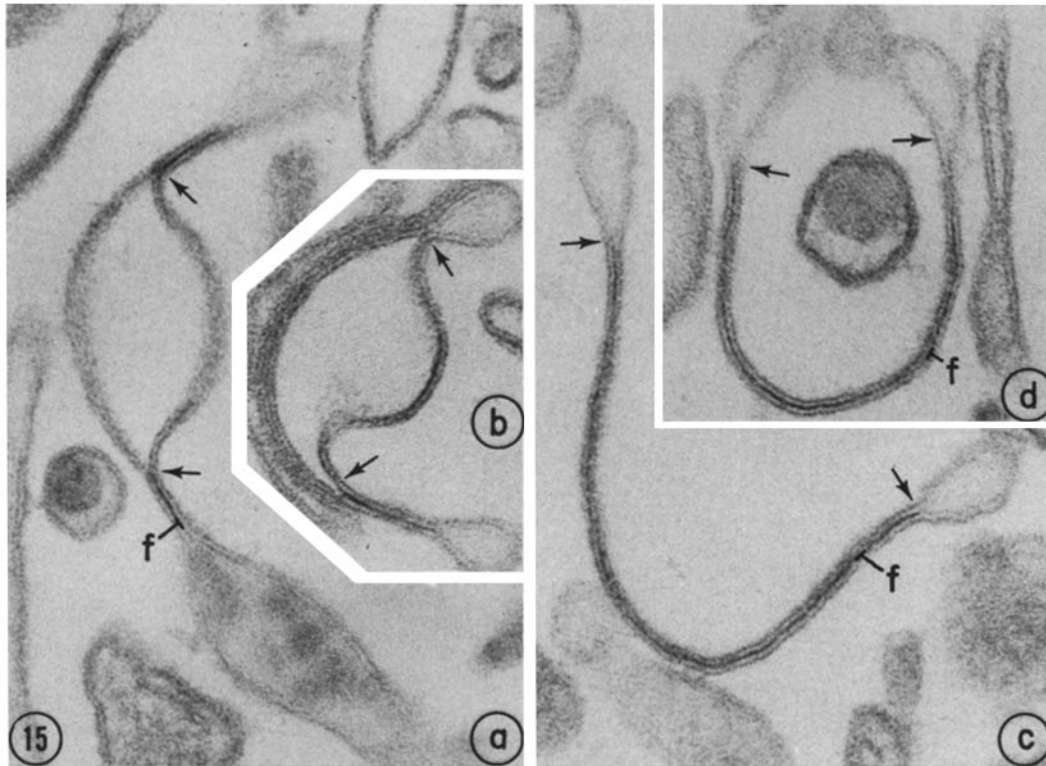


FIGURE 15 GF₃. Details of collapsed Golgi cisternae with lumina partially obliterated by membrane fusion. The fusion line (*f*) is clearly visible wherever the fused membranes are normally sectioned. (a) Large cisterna with open rims and an open excentric pocket (between arrows). The lower part of the rim is marked by VLDL particles. (b) Two stacked cisternae, one of which shows an area of fusion between the rim and a central pocket (between arrows). (c and d) Buckled cisternae with lumina almost completely obliterated by fusion; only their thin, flattened rims remain open (arrows). (a-d) $\times 180,000$.

nal microsomal fraction by the isolation of the three Golgi fractions mentioned above. This is confirmed by the biochemical data presented in the companion paper which indicate that the galactosyltransferase activity of the starting microsomal preparation is almost fully recovered in the three Golgi fractions derived therefrom (56).

ISOLATION OF HEPATIC GOLGI FRACTIONS FROM RATS NOT TREATED WITH ETHANOL

Since the Golgi elements of the hepatocytes of control rats contain a small and variable amount of VLDLs, an attempt was made to separate Golgi fractions from the liver homogenates of animals without previous ethanol treatment. The protocol described above for the isolation of a set of three Golgi fractions was used but the bands obtained were different: GF₁ was either absent or extremely small, GF₂ was much smaller, and GF₃ smaller

than after ethanol treatment (Fig. 16). The morphology of the fractions was similar to that already described for ethanol-treated animals except for a trend towards a smaller size of their elements and a lighter load of VLDL particles. As will be shown in the companion article (56), recovery of galactosyltransferase activity is incomplete in control animals.

SUBFRACTIONATION OF GOLGI FRACTIONS: ISOLATION OF GOLGI MEMBRANES

Since Golgi fractions, especially GF₁ and GF₂, consist of membrane-bound vesicles loaded with lipoprotein particles, it was obviously desirable to obtain the Golgi membranes alone, free of the lipoprotein content. Most of the experiments designed to achieve this goal were carried out with GF₁ but the results obtained were comparable for all three fractions.

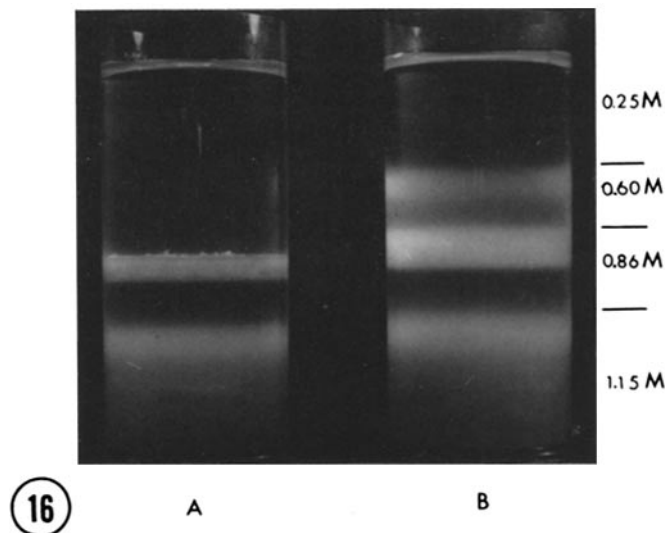


FIGURE 16 Isolation of GF₁, GF₂, and GF₃ by flotation in a discontinuous density gradient. The starting preparation (resuspended microsomal fraction) was loaded at the bottom of the tubes. The gradients were developed by centrifuging for 3.5 h at 25,000 rpm (63,500 g_{av}) in a SW-25.1 rotor. (A) control rat, (B) ethanol-treated rat. Note that in A (control) the upper band (GF₁) is virtually absent and that the middle (GF₂) and lower (GF₃) bands are reduced in width. In this experiment the volumes of the layers were (top to bottom): 9 ml, 5 ml, and 8 ml over a 10-ml load.

To disrupt Golgi-derived vesicles and liberate their contents, the means listed in Table I were used. After the treatment, the sucrose concentration was adjusted to 0.15 M or 0.25 M and the suspension centrifuged for 60 min at 105,000 g_{av} . This field was expected to be strong enough to sediment empty vesicles since the starting preparation (total microsomal fraction) had been isolated in 0.25 M sucrose. Liberated VLDLs should remain in the supernate or float to the meniscus since their density is ≤ 1.006 (57).

The results of the disruption-separation procedures were monitored by administering [³H]-palmitate to the animals 10 min before decapitation and by following the distribution of radioactivity between the pellet and supernate in which the treated Golgi fractions were resolved. Most of the radioactivity was expected to be incorporated in VLDLs and hence remain in the supernate in the case of successful disruption of lipoprotein-loaded Golgi vesicles. The most promising results were further checked by electron microscopy. To this intent, the pellet as well as aliquots of the supernate were fixed and processed as indicated under Materials and Methods.

As a result of these experiments, the procedure finally adopted for the separation of Golgi mem-

brane fractions was as follows. The Golgi fraction (GF₁, or GF₂, or GF₃) was resuspended in 0.04 M sodium Veronal-HCl buffer, pH 8.5, and the suspension was passed twice through a French press under a pressure of 4,000 lb/in². 3 ml of 1.0 M sucrose were added to 9 ml of the disrupted suspension and the mixture centrifuged for 1 h at 105,000 g_{av} . The resulting supernate was taken to represent the content, and the pellet the membranes of the corresponding Golgi fraction. In the case of GF₁, the membranes obtained in this way contain 15–25% of the lipid label of the starting fraction.

The entire procedure, from ethanol treatment of the animals to separation of Golgi membranes, is summarized in the flow sheet in Fig. 17.

MORPHOLOGY OF GOLGI MEMBRANE FRACTION: Fig. 18 shows the membrane pellet obtained from GF₁ as described above. It consists almost entirely of empty, closed, or ruptured vesicles and small membrane fragments. Only at the bottom of the pellet are there a few vesicles which still contain dense 30–80-nm particles. The morphology of the preparation is consistent with the radiochemical results which indicate that 75–85% of the label is lost to the supernate. Since some of the remaining label may be associated with the membrane rather than the content of the vesicles,

TABLE I
Effectiveness of Procedures for Disrupting Golgi Vesicles

Golgi fractions from animals which had been injected with [³H]palmitate 10 min before decapitation were treated according to the procedures listed. Then sucrose was added to the suspension, the mixture was centrifuged, and the distribution of label between the ensuing pellet and the supernate was assayed.

Series*	Procedure	Percent of label released to supernate
I	Freezing and thawing, 10 times	10
	Hypotonic shock †	5
	Freezing and thawing, 6 times in hypotonic medium	6
	Sonication §, 20 s in hypotonic medium	41
	Sonication, 50 s in hypotonic medium	55
	Freezing and thawing, 6 times in hypotonic medium, followed by sonication, 20 s in hypotonic medium	53
II	Alkaline, hypotonic shock (Tris, pH 8.0)	38
	Alkaline, hypotonic shock (Tris, pH 8.0) followed by sonication, 20 s, followed by passage through French press ¶	79
III	Neutral or alkaline hypotonic shock** followed by passage through French press 1 time	
	Veronal, pH 7.0	51
	“ “ pH 7.5	66
	“ “ pH 8.0	74
	“ “ pH 8.5	84
	Alkaline hypotonic shock followed by passage through French press 2 times	
	Veronal, pH 8.0	79
“ “ pH 8.5	84	

* In series I the Golgi fraction used was the material from the 0.25/0.75 M sucrose interface. The final sucrose concentration was 0.15 M, and the centrifugation was for 1 h at 40,000 rpm in a Spinco no. 40.3 rotor. In series II the Golgi fraction used was the same as in series I. Final sucrose concentration was 0.25 M, and the centrifugation was the same as for series I. In series III the Golgi fraction used was GF₁. Final sucrose concentra-

tion was 0.25 M, and the centrifugation was the same as for series I. The Golgi membrane fraction appears to be sufficiently freed of content to warrant chemical and enzymological studies. Since the vesicles of the membrane subfractions are considerably smaller than the Golgi elements of the starting preparations, it follows that extensive fragmentation and resealing of vesicles occurs during this procedure. The membrane fraction obtained from GF₂ is comparable to the one described above; that prepared from GF₃ still shows a large number of flattened cisternae which appear to retain a good part of their content.

MORPHOLOGY OF GOLGI CONTENT FRACTION: The morphology of the content subfraction is illustrated in Fig. 19 for GF₁. The preparation was found to consist primarily of small, dense particles 30–80 nm in diameter (a few were larger) which, on account of their size and density, could be identified as VLDLs.

DISCUSSION

Our results show that it is possible to overload the Golgi elements of rat hepatocytes with VLDL particles by acute intoxication with ethanol. VLDL overloading provides an inner morphological marker for individually dispersed Golgi elements and perturbs their density to an extent that facilitates their separation. In general, this density-perturbing procedure is reminiscent of the technique used by Wattiaux et al. (58) and Leighton et al. (59) to change the density of lysosomes by overloading them with a light, nondegradable detergent (Triton WR-1339) as a prerequisite for their

tion was 0.25 M, and the centrifugation was the same as for series I.

† In series I the Golgi fraction originally suspended in 0.25 M sucrose was diluted to 0.08 M sucrose with water.

§ Sonication was performed with a Branson probe sonicator, Model LS75, at maximum power output (Branson Sonic Power, Co., Danbury, Conn.).

|| In series II, the Golgi fraction was resuspended in water, and Tris-HCl buffer was added to a final concentration of 0.04 M Tris of the pH indicated.

¶ The material in the hypotonic buffer was passed through the French press under a pressure of 4,000 lb/in².

** In series III, the Golgi fraction was resuspended in water, and Veronal-HCl buffer was added to a final concentration of 0.04 M sodium Veronal of the pH indicated.

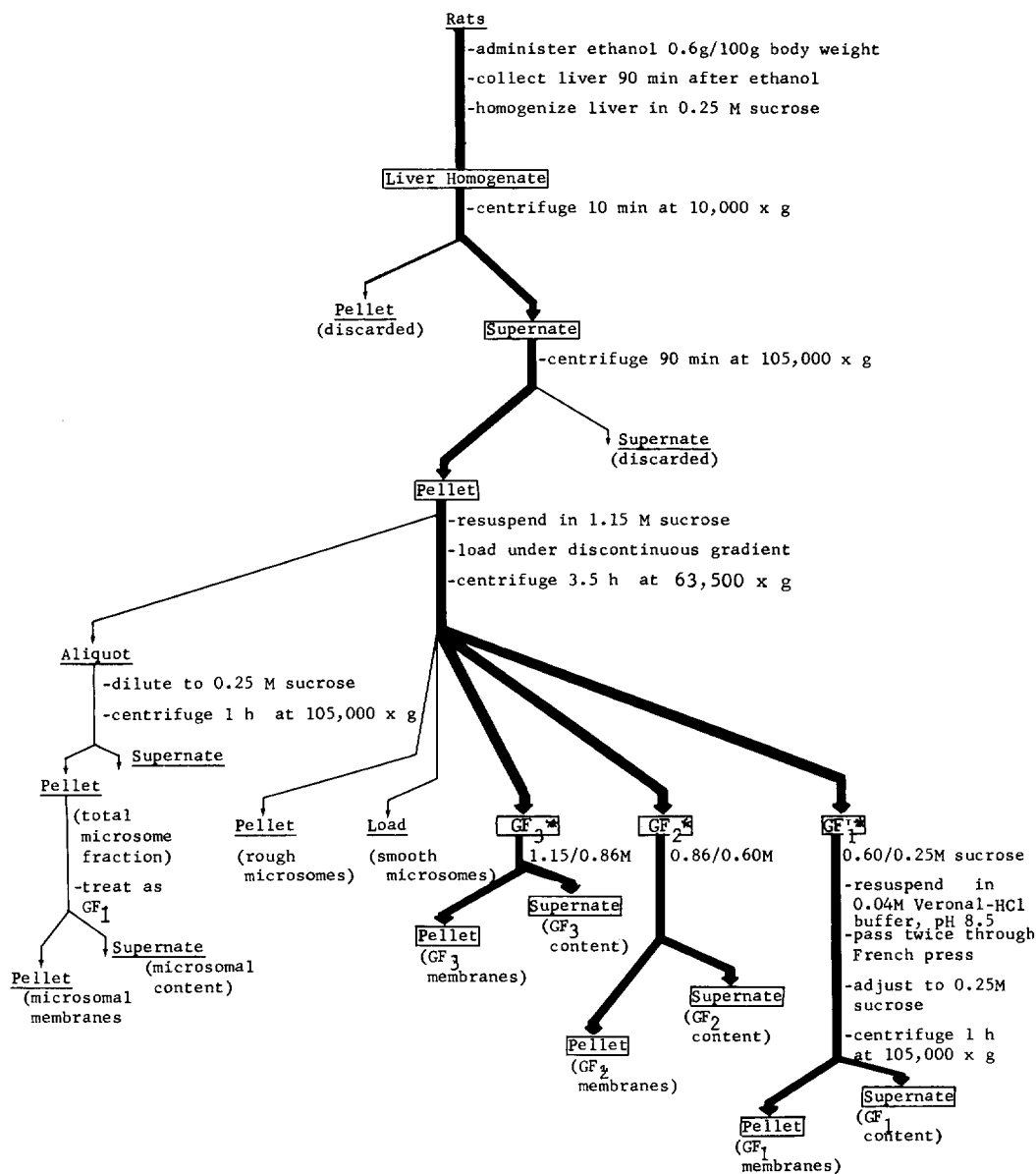


FIGURE 17 Flow diagram of the procedure for isolating Golgi fractions and Golgi subfractions (membranes and content) from rat liver homogenates. The times and centrifugal fields given apply for the combination of Spinco rotors type 40 and SW-25.1. When larger amounts of homogenates and microsomes were processed, the rotors used were type 30 and SW-25.2. In this case, the durations of the first and second runs were not changed, but the fields used were 8,800 g_{av} and 78,500 g_{av} , respectively; for the third run the time was 3 h and the field 75,000 g_{av} . At the end of the third run, samples of the load and pellet were processed for electron microscopy as given under Materials and Methods and found to consist essentially of smooth and rough microsomes, respectively. GF₁, light Golgi fraction; GF₂, intermediate Golgi fraction; GF₃, heavy Golgi fraction.

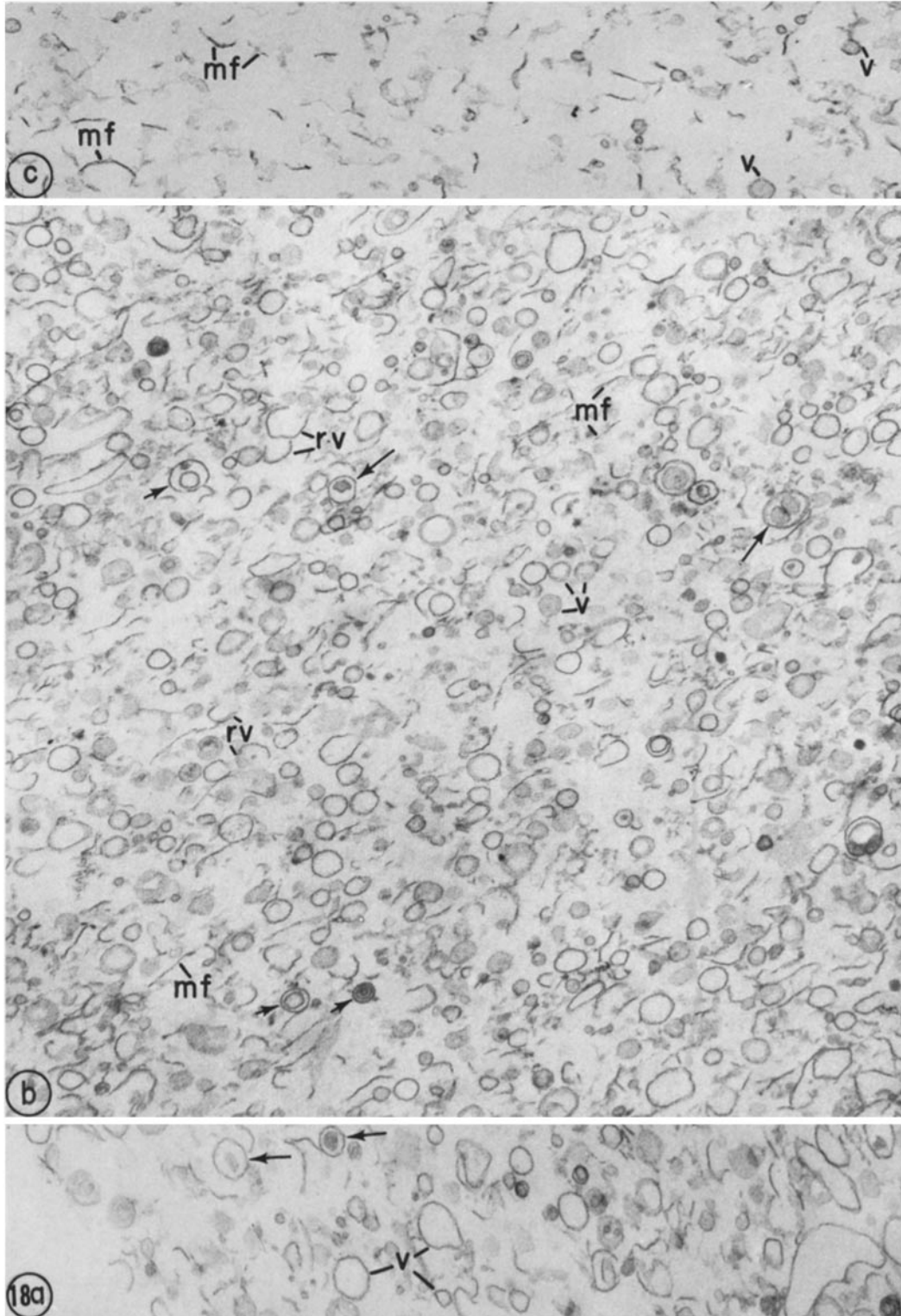


FIGURE 18 Golgi membrane fraction prepared from GF₁ as given in the text. *a*, *b* and *c* show the bottom, the middle, and the top of the membrane pellet, respectively. The preparation consists essentially of closed empty vesicles (*v*), ruptured vesicles (*rv*), and membrane fragments (*mf*). The frequency of the latter increases markedly at the top (*c*). Few vesicles retain VLDL particles in their cavities (arrows). The small size of the vesicles, the frequency of membrane fragments, and the presence of concentric vesicles (arrows) suggest that extensive reorganization of the Golgi membranes occurs during the VLDL extraction procedure. $\times 42,000$.

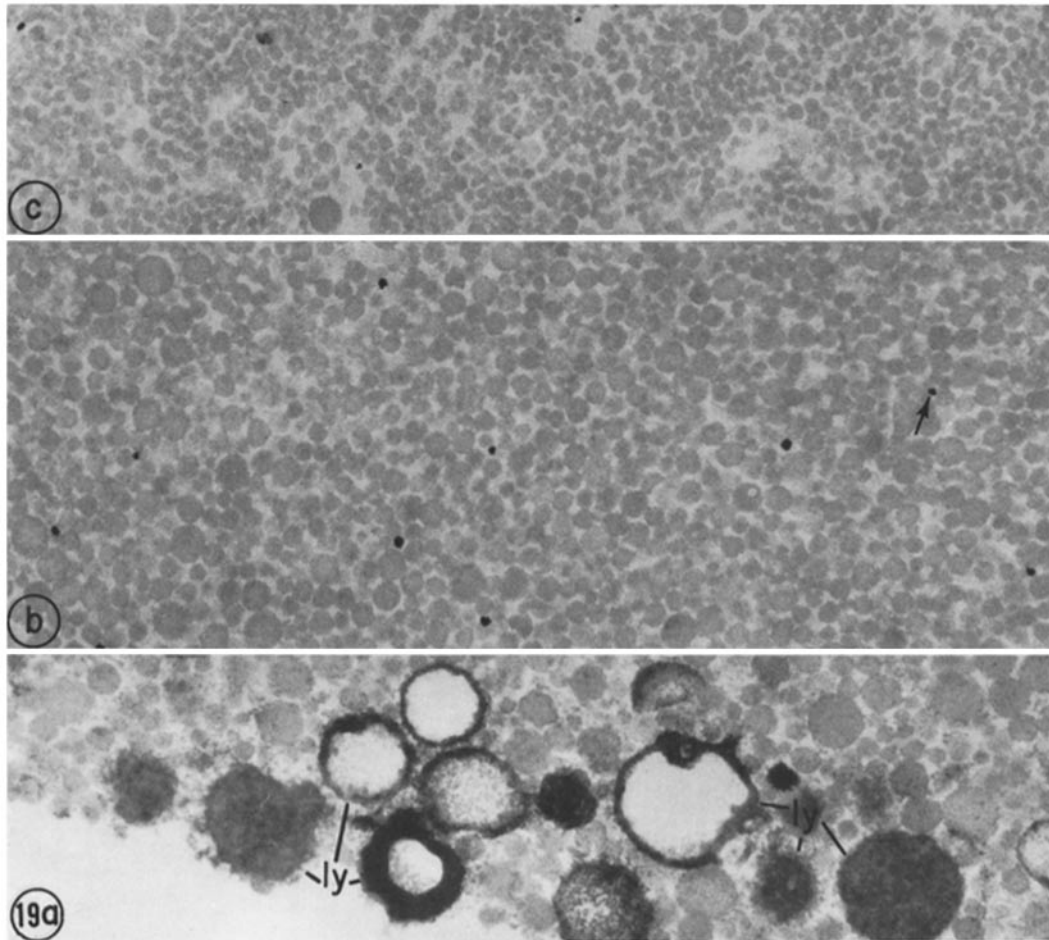


FIGURE 19 Content of GF₁. The preparation, processed as given in the text, consists essentially of VLDL particles which decrease in size from the bottom (a) to the top (c) of the pellet. The bodies with a content of heterogeneous density (*ly*) at the bottom of the pellet are lysosome cores. The very dense small bodies (arrows) are probably dust particles. $\times 42,000$.

separation from other subcellular components. In our case, the density perturbant has the advantage of being an endogenous product; its accumulation is reversible and seems to have no lasting untoward effects on the cell.

Our procedure relies on morphological criteria for monitoring the fractionation and for assessing the quality of the fractions obtained. Such criteria are sufficient for ascertaining the Golgi origin of GF₁ and GF₂ but are only partly useful in the case of GF₃. For the latter, morphological observations must be supplemented by enzymic assays.

Our procedure gives a light fraction (GF₁) of high purity but low yield which could be used as a satisfactory source for biochemical studies of Golgi elements; in addition, it achieves high yield by

providing for the separation of a series of three Golgi fractions of increasing density. The series should prove useful in studying functional differentiations within the Golgi complex since the ratio trans-Golgi:cis-Golgi elements varies considerably from GF₁ to GF₃. Finally, the procedure provides for the separation of the membrane from the content (i.e., VLDLs and surrounding matrix) of Golgi elements and makes available for study the membranes themselves.

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