CYTOCHEMISTRY OF GOLGI FRACTIONS PREPARED FROM RAT LIVER

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ABSTRACT

Cytochemical tests for several marker enzymes were applied to liver tissue and to the three Golgi fractions (GF_1, GF_2, GF_3) separated by the procedure of Ehrenreich et al. from liver homogenates of alcohol-treated rats. 5'-Nucleotidase (AMPase) reaction product was found in all three fractions but in different locations: It occurred along the inside of the membrane of VLDL-filled vacuoles in GF_1 and GF_2, and along the outside of the cisternal membranes in GF_3. In the latter it was restricted to the dilated cisternal rims and was absent from the cisternal centers. The AMPase activity found in the fractions by biochemical assay is therefore indigenous to Golgi components and is *not* due to contamination by plasma membrane. Acid phosphatase (AcPase) reaction product was detected within lysosomal contaminants in GF_1 and within many VLDL-filled vacuoles in GF_1 and GF_2, indicating that AcPase activity is due not only to contaminating lysosomes, but also to enzyme indigenous to Golgi secretory vacuoles. G-6-Pase reaction product was present in GF_3 and within contaminating endoplasmic reticulum fragments, but not in other fractions. Thiamine pyrophosphatase (TPPase) was localized to some of the VLDL-filled vacuoles and cisternae in GF_1 and GF_2, and was not found in the cisternae in GF_3. The results demonstrate the usefulness of cytochemical methods in monitoring the fractionation procedure: They have (*a*) allowed a reliable identification of contaminants, (*b*) made possible a distinction between indigenous and contaminating activities, and (*c*) shown, primarily by the results of the TPPase test, that the procedure achieves a meaningful subfractionation of Golgi elements, with GF_1 and GF_2, representing primarily trans-Golgi elements from the secretory Golgi face, and GF_3 consisting largely of cis-Golgi components from the opposite face.

INTRODUCTION

The Golgi apparatus of the rat hepatocyte is less orderly arranged than that of many other cells but nevertheless manifests evidence of polarity since only one side is associated with the formation of recognizable secretory products. The cisternae along one face—referred to as the secretory or trans (cf 1) side of the Golgi apparatus—frequently show dilated ends containing very low-

density lipoprotein particles (VLDLs),[1] and secretory droplets filled with VLDLs (which presumably arise from the same cisternae) typically occur in close proximity. The preferential packaging of secretory products along one Golgi face indicates that in the hepatocyte Golgi, as in other secretory cells, functional specialization exists among Golgi components. Moreover, in addition to morphological heterogeneity there is considerable heterogeneity in cytochemical staining among the Golgi components of the hepatocyte: thiamine pyrophosphatase (TPPase) activity is found primarily in trans-Golgi cisternae along the secretory face and in some closely associated VLDL-filled secretory droplets but is absent from the rest of the stacked cisternae (2, 3), whereas osmium impregnation is limited to cis-Golgi cisternae (4).

Recently, Ehrenreich et al. (1) have developed a new procedure for isolating Golgi fractions from rat liver homogenates which relies on the overloading of Golgi elements with VLDLs by pretreatment of the animals with ethanol. In such ethanol-treated rats, accumulation of VLDLs in the Golgi is sharply increased and involves more cisternae than in the controls. For the fractionation procedure the cumulated VLDLs serve two purposes: (*a*) They act as reliable morphologic markers of Golgi elements upon dispersion of the Golgi apparatus during tissue homogenization, and (*b*) they modify the density of these elements, thereby facilitating their isolation. Using this procedure three progressively heavier Golgi fractions have been obtained which together account for more than 90% of the Golgi elements of the starting material (a total microsomal fraction), as determined by recovery of galactosyltransferase activity, the most useful Golgi marker enzyme available at present. Morphologic analyses of the three fractions by electron microscopy (1) indicated differences in their composition: All three are mixtures of vesicles, vacuoles, and cisternal elements, but the lightest fraction (GF_1) and that of intermediate density (GF_2) contain relatively greater numbers of large and small vacuoles filled with clustered VLDLs, whereas the heaviest fraction (GF_3) is quite different and consists predominantly of flattened "empty" cisternae. The three Golgi fractions are distinguished biochemically (5) from other cell fractions by the presence of galactosyltransferase activity and the absence, or low concentration, of characteristic marker enzymes for endoplasmic reticulum (ER) (G-6-Pase, NADPH-cytochrome *c* reductase, cytochrome P 450), but they also have a number of enzymatic activities (AMPase, G-6-Pase, AcPase) which are generally regarded as markers for other subcellular components, i.e., plasma membrane, ER, and lysosomes, respectively. The enzymologic data could not rule out contamination by any of these elements, while electron microscopical observations indicated limited contamination by lysosomes in GF_1 and GF_2, but could not reliably solve the problem of possible contamination by ER and plasmalemmal fragments in any of the fractions, especially GF_3.

In the present work cytochemical tests for several marker enzymes have been performed on GFs in order to determine: (*a*) in what structures enzyme activities are located (i.e., true Golgi elements vs. contaminants) and (*b*) whether or not the Golgi elements in the three fractions are heterogeneous with respect to these enzyme activities.[2]

MATERIALS AND METHODS

Materials

Liver tissue was obtained from young (120–150 g) male Sprague-Dawley rats fasted overnight and given ethanol (0.6 g/100 g body weight) 90 min before sacrifice.

Adenosine 5′-monophosphate (5′-AMP) (type II, sodium salt), DL-β-glycerophosphate (grade I), D-G-6-P (dipotassium salt), and TPP (cocarboxylase) were obtained from Sigma Chemical Co., St. Louis, Mo. Adenosine 2′-phosphate (2′-AMP) was obtained from P-L Biochemicals, Inc., Milwaukee, Wis. Glutaraldehyde was obtained as an 8% solution from Electron Microscopy Sciences, Fort Washington, Pa.

Methods for Processing Liver Slices

In order to study the distribution of marker enzymes in the Golgi elements *in situ*, cytochemical tests were carried out on liver slices. Liver tissue was

[1] *Abbreviations used in this paper:* AcPase, acid phosphatase; 2′-AMP, adenosine 2′-phosphate; 5′-AMP, adenosine 5′-monophosphoric acid; AMPase, 5′-nucleotidase; ER, endoplasmic reticulum; galactosyltransferase, uridine diphospho galactose: *N*-acetyl glucosamine galactosyltransferase; TPPase, thiamine pyrophosphatase; VLDLs, very low density lipoprotein particles.

[2] These findings were previously published in abstract form (6, 7).

fixed by perfusion through the portal vein as described by Leskes et al. (8). The liver was first flushed with 20 ml 0.1 M cacodylate buffer (pH 7.4), followed by perfusion with 2% glutaraldehyde in the same buffer for 5 min, followed again by flushing with buffer (20 ml). The areas which appeared discolored and hardened were selected, cut into strips of $1 \times 1 \times 3$ mm, and stored overnight in cold buffer containing 0.2 M sucrose. Nonfrozen sections (~40 μm) were prepared with a Smith-Farquhar TC-2 tissue sectioner (Ivan Sorvall, Inc., Norwalk, Conn.), transferred to 20-ml beakers containing 5 ml incubation media, and incubated for 45–90 min at 25°C in a shaker bath (80–100 strokes/min). At the end of the incubation period the sections were washed three times in 0.1 M acetate-Veronal buffer (pH 7.2) containing 0.2 M sucrose, postfixed for 45 min in 1% OSO_4 in 0.05 M acetate-Veronal buffer (pH 7.2), treated with acetate-Veronal-buffered 0.5% uranyl acetate for 45 min (9), dehydrated in graded ethanols, and finally embedded in Epon in BEEM capsules. Detailed techniques for the handling of nonfrozen sections have been published previously (10).

Methods for Processing Fractions

FRACTIONATION: Golgi fractions were prepared from the livers of ethanol-treated rats according to the method of Ehrenreich et al. (1) which involves loading of a total microsomal fraction at the bottom of a discontinuous sucrose gradient, followed by centrifugation at 63,500 *g* for 3 h. The Golgi elements float by virtue of their VLDL load. Three Golgi fractions of increasing density (GF_1 to GF_3) are thereby obtained: the lightest fraction (GF_1) accumulates at the 0.25–0.6 M sucrose interface, the intermediate fraction (GF_2) at the 0.6–0.86 M interface, and the heaviest fraction (GF_3) at the 0.86–1.15 M interface. These fractions were collected and further diluted with 0.25 M sucrose to give concentrations of approximately 0.03 mg protein/ml in the case of GF_1, and 0.3 mg protein/ml in the case of GF_2 and GF_3. The fractions, thus diluted, represented the starting material for the cytochemical tests. Two main procedures were followed: Fractions were either (*a*) fixed, centrifuged, and incubated as pellets, or (*b*) incubated unfixed in suspension.

INCUBATION OF FIXED GOLGI FRACTIONS IN PELLET: Procedures for the processing of fixed Golgi fractions were similar to those described previously for PMN granule fractions (11). Samples (7 cm^3) of each fraction were placed in Spinco #30 cellulose nitrate centrifuge tubes (capacity = 38.5 ml), containing 7 ml ice-cold 1% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4). After 10 min fixation at 0°C, the tubes were filled with 0.1 M cacodylate buffer (containing 0.2 M sucrose) to further dilute the fixative, and centrifuged at 30,000 rpm for 40 min in a #30 rotor on a Spinco Model L ultracentrifuge. The supernatant was decanted, the surface of the resulting pellets was rinsed three times in the same buffer, and 15 ml incubation medium was added to each tube. The tubes were then placed on a mechanical shaker and incubated for 60–90 min at 25° or 37°C. After incubation the pellets were rinsed three times in 0.1 M acetate-Veronal buffer (containing 0.2 M sucrose), and then postfixed in OsO_4 (60 min), treated with uranyl acetate (60 min), rinsed briefly in 70% and 95% alcohol, and placed in absolute alcohol. All preceding steps were accomplished directly in the centrifuge tubes, since the pellets usually remained adherent to the tube walls. During dehydration in absolute alcohol, the pellets and tubes were cut into strips of 1 mm × 3 mm which were placed in vials for subsequent processing. The cellulose nitrate tube backing dissolved in propylene oxide. Specimens were embedded in Epon in flat embedding molds to facilitate orientation during sectioning. The pellet thickness had to be limited to <0.5 mm to assure adequate penetration by all components of the incubation media.

INCUBATION OF UNFIXED GOLGI FRACTIONS IN SUSPENSION: Aliquots (7 ml) of each fraction were placed in #30 centrifuge tubes and 14 ml of a concentrated ($1\frac{1}{2}$ ×) solution of incubation medium, prepared as described below, were added to each tube. The tubes were then incubated for 30 min at 25°C on a mechanical shaker. The reaction was stopped by filling the tubes with cold 1% glutaraldehyde fixative and allowing them to set on ice for 10 min. The tubes were then centrifuged at 30,000 rpm for 40 min, the supernatant was decanted, and the resultant pellets were postfixed in OsO_4 and processed as described above for fixed fractions.

Incubation Media

For incubation of tissue slices and fixed pellets, standard incubation media were prepared according to the original procedures cited below. For incubation of unfixed fractions the concentrations in the media were increased so as to give upon dilution with the fraction samples, final concentrations equal to those used for pellets or tissues.

AMPase: Fractions were incubated at pH 7.5 in Widnell's medium (12) which contains 1 mM 5'-AMP, 0.1 M Tris-acetate buffer, 1 mM Pb $(NO_3)_2$, and 1 mM $Mg(NO_3)_2$. Controls were incubated in media in which 2'-AMP, a competitive inhibitor of 5'-AMP (12), was used as substrate or 10 mM NaF was added to the incubation mixture. In some instances the lead concentration was reduced from 1.0 to 0.5 mM.

TPPase: Incubations were carried out at pH 7.2 in Novikoff and Goldfischer's medium (13) contain-

ing 1 mM TPP, 0.1 M Tris-maleate buffer, 2 mM $Pb(NO_3)_2$, and 5 mM $MnCl_2$.

ACPase: Incubations were carried out at pH 5.0 in modified Gomori media (14) which contain 1 mM β-glycerophosphate, 0.1 M Tris-maleate buffer, and 2.4 mM $Pb(NO_3)_2$. For controls, 10 mM NaF was added to the incubation medium.

G-6-Pase: Fractions were incubated at pH 6.7 either in the regular Wachstein-Meisel medium (15) containing 1 mM G-6-P, 0.1 M Tris-maleate buffer, and 2 mM $Pb(NO_3)_2$ or in the modified medium of Leskes et al. (8) prepared in 0.1 M cacodylate buffer.

Microscopy

Sections were examined either unstained or after brief (10 s) staining in lead citrate (16) in a Siemens Elmiskop 101 operating at 80 kV with a double condenser and a 50 μm objective operture.

RESULTS

Organization of the Rat Hepatocyte

As is well known, the hepatocyte is a multipolar cell with surface differentiations connected with the bile capillaries on the one hand and with the (blood) sinusoid on the other (Fig. 1). The Golgi apparatus and lysosomes are generally concentrated in the vicinity of the bile canaliculi (Fig. 4). One of the established functions of the Golgi apparatus in this cell is the packaging of VLDLs into secretory droplets (1, 17–20). Since these VLDL-filled droplets eventually fuse with the cell membrane along the sinusoidal cell front, they are comparable to secretion granules in other cell types. Normally the packaging operation is for the most part confined to the last trans-Golgi cisternae along the secretory face (i.e., the side from which the secretory droplets emerge). In the ethanol-treated rat, however, there is a dramatic increase in the number of VLDLs found in the Golgi apparatus involving several, and occasionally all, of the cisternae. In addition, increased numbers of secretory droplets are found near the sinusoidal cell front.

Distribution of Enzyme Activity in Situ

TPPase: In liver tissue from ethanol-treated rats incubated for TPPase reaction product was seen: (*a*) along the outer surface of the membrane of bile canaliculus, (*b*) within both rough and smooth ER, including the perinuclear cisterna, and (*c*) within some of the Golgi cisternae (Fig. 2). Within the Golgi, reaction product was restricted primarily to the last trans-Golgi cisternae and to some of the VLDL-filled secretory droplets in the vicinity. Thus the localization was identical with that demonstrated previously for TPPase in normal liver (2, 3).

ACPase: In similar tissue incubated with β-glycerophosphate to demonstrate AcPase activity, reaction product was restricted to lysosomes and to some of the VLDL-filled secretory droplets (Figs. 3–4). It was not seen in elements of the Golgi apparatus or ER.

G-6-Pase: Reaction product was restricted to the ER and was found throughout both the smooth and rough elements, including the perinuclear cisterna (Fig. 5). It was absent from the secretory droplets and stacked cisternae of the Golgi apparatus.

AMPase: The amount of reaction product found in tissue incubated for AMPase varied considerably from one specimen to another and even from area to area within the same specimen, presumably reflecting variations in the degree of enzyme inactivation by fixation. In specimens which appeared well fixed (by virtue of their gross hardness and yellow color and the overall satisfactory preservation of fine structure), reaction product was found on the outer surface of the plasma membrane, but it was restricted to those segments surrounding bile capillaries. It was also seen in some of the VLDL-filled secretory droplets and in lysosomes. In specimens which were less well fixed (i.e., grossly softer and paler yellow, and in which fine structure was less well preserved but enzyme activity was presumably less inhibited), reaction product was seen on the outer surface of the plasma membrane along the entire cell perimeter, i.e., along the space of Disse as well as along the bile capillaries (Fig. 1). It was also detected in some VLDL-filled secretory droplets (Fig. 1, inset) and lysosomes (Fig. 6). In addition, dense deposits, less clearly crystalline in nature, could frequently be seen in association with the outer compartment of mitochondria. No deposits could be recognized in association with Golgi or ER cisternae.

Fixed Fractions

MORPHOLOGY: A morphologic analysis of the fractions briefly fixed in glutaraldehyde confirmed previous findings by Ehrenreich et al. (1) that all three Golgi fractions consist of a mixture of cister-

nal elements and vesicles or vacuoles of varying sizes, but the two lightest fractions (GF_1 and GF_2) are very similar and contain predominantly large and small saccules filled with clustered VLDLs, whereas the heaviest fraction (GF_3) contains predominantly empty, flattened cisternae.

AMPase AND G-6-Pase: In specimens fixed under the conditions described, reaction product was rarely seen in components of any of the fractions. This is in keeping with the known sensitivity to fixation of these two enzymes (8, 12).

TPPase: Reaction product was found within some of the VLDL-filled vacuoles in GF_1 and GF_2, and in occasional cisternae in the same fractions (Figs. 7–8). Relatively greater numbers of reactive vacuoles were seen in GF_1, but the majority of VLDL-loaded vacuoles were unreactive in both fractions. Within GF_3 (Fig. 9) reaction product was rarely seen except for occasional VLDL-filled vacuoles. The fact that TPPase-positive elements are restricted largely to GF_1 and GF_2 suggests that trans-Golgi cisternae (i.e., those along the secretory face) separate preferentially in these two fractions.

AcPase: In GF_1 (Fig. 10) reaction product was seen within morphologically recognizable lysosomal contaminants and within many VLDL-filled vacuoles which showed no morphological indication of involvement with lysosomes. In GF_2 (Fig. 11), the localization was similar except that there were fewer lysosomal contaminants, and the number of reactive secretory droplets was less. In GF_3 the situation was quite different; reactive elements were rarely seen and were restricted to VLDL-filled vacuoles (Fig. 12). In the presence of 10 mM NaF the reaction was completely inhibited in all locations. It can be concluded that the AcPase activity found in the two lightest Golgi fractions is due not only to contaminating lysosomes, but also to enzyme indigenous to Golgi vacuoles.

Unfixed Fractions

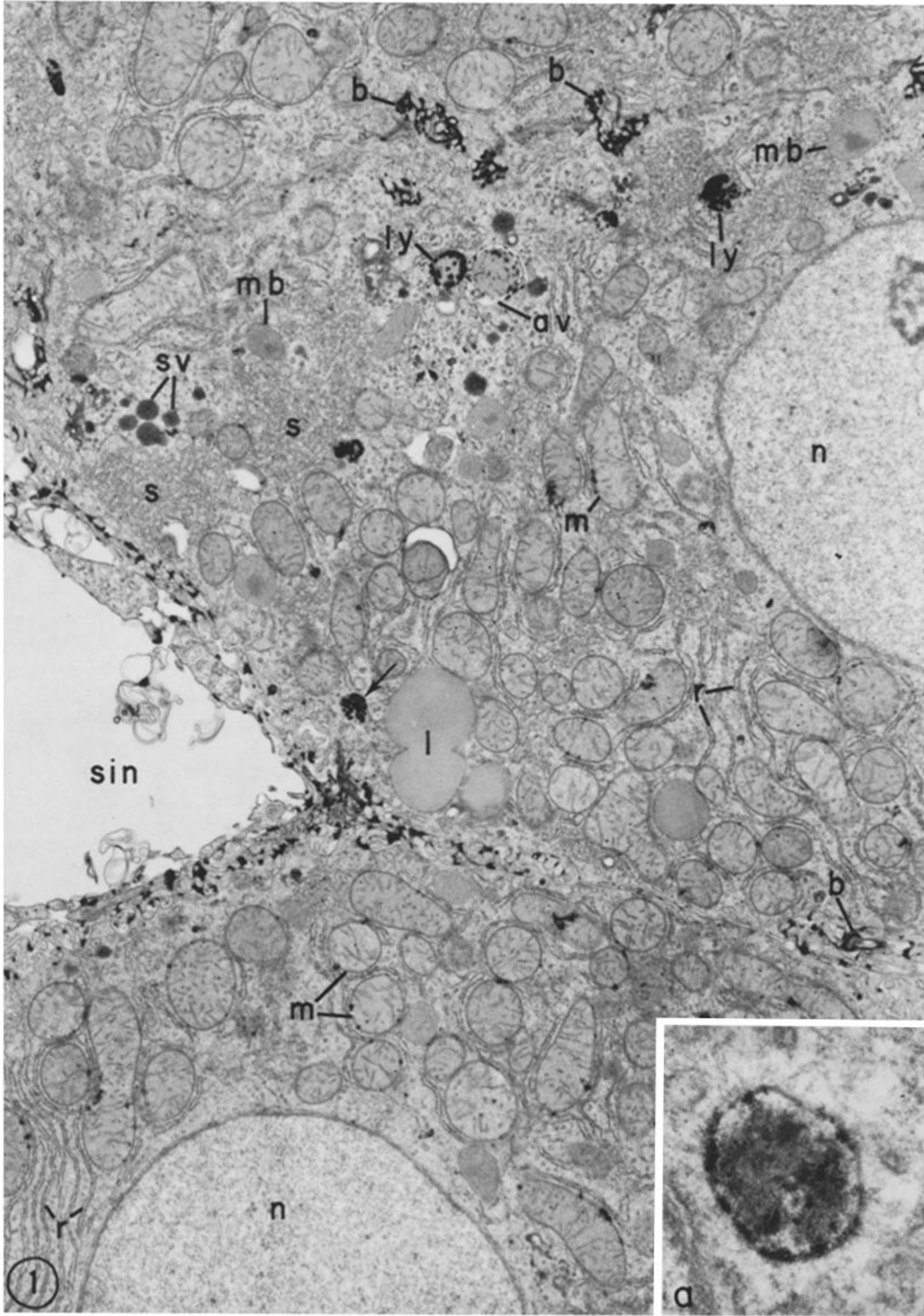
The main difference in morphology between unfixed and briefly fixed fractions is that in the former, more vesicles and vacuoles are seen and cisternal elements are less common than in the latter. Presumably with brief fixation the structure of the cisternae is stabilized, but without fixation there is a pinching off of the secretory droplets from the cisternae during incubation (shaking) similar to the vesiculation of the rough ER which occurs during the tissue homogenization involved in the preparation of a microsomal fraction.

TPPase: The localization of reaction product was similar to that in fixed fractions: It was found in some VLDL-filled secretory droplets in GF_1 and GF_2.

AcPase: The localization of reaction product was exactly as described for fixed fractions, i.e., it was restricted to some of the VLDL-filled secretion droplets in GF_1 and GF_2, and to lysosomal contaminants (Figs. 10–12).

AMPase: In unfixed Golgi fractions incubated in suspension, reaction product was found in all three fractions but differed in its localization. In GF_1 (Fig. 13) and GF_2 it was present mainly in plaques along the inner surface of the membrane of many of the VLDL-filled vacuoles. Deposits were also seen mixed with the Golgi vacuole contents and in some of the lysosomes. In GF_3, the situation was quite different: Reaction product was found along the *outer* surface of the membranes of most, if not all, of the cisternal and vesicular elements (Fig. 14). Typically it occurred on the distended ends of the cisternae and was absent from the flat-

FIGURE 1 Low power field showing several hepatocytes in the liver of an ethanol-treated rat, specimen incubated for AMPase. Lead phosphate reaction product is found along the plasma membrane and is especially abundant on the cell front facing the sinusoids (*sin*) and the bile capillaries (*b*). Reaction product is also present in several lysosomes (*ly*), in an autophagic vacuole (*av*) and some of the VLDL-filled secretory droplets (arrow). (See also *inset a*.) Other VLDL-containing vacuoles (*sv*) appear very dense but lack distinct lead deposits. Dense deposits of unknown composition (enlarged in Fig. 6) are also seen at the periphery of many of the mitochondria (*m*). Microbodies are marked *mb*, lipid droplets *l*, smooth and rough ER, *s* and *r*, respectively, and nuclei, *n*. The *inset* depicts an enlarged VLDL-filled secretory vacuole which contains deposits of lead phosphate in its matrix and especially on the membrane, mostly along its inside surface. Specimen fixed by perfusion for 5 min with glutaraldehyde. Nonfrozen section incubated for 60 min at 25°C in Widnell's medium for AMPase (pH 7.5), postfixed in 1% OsO_4, stained in block in uranyl acetate and embedded in Epon. Thin section stained in lead alone. × 12,000; *inset*, × 60,000.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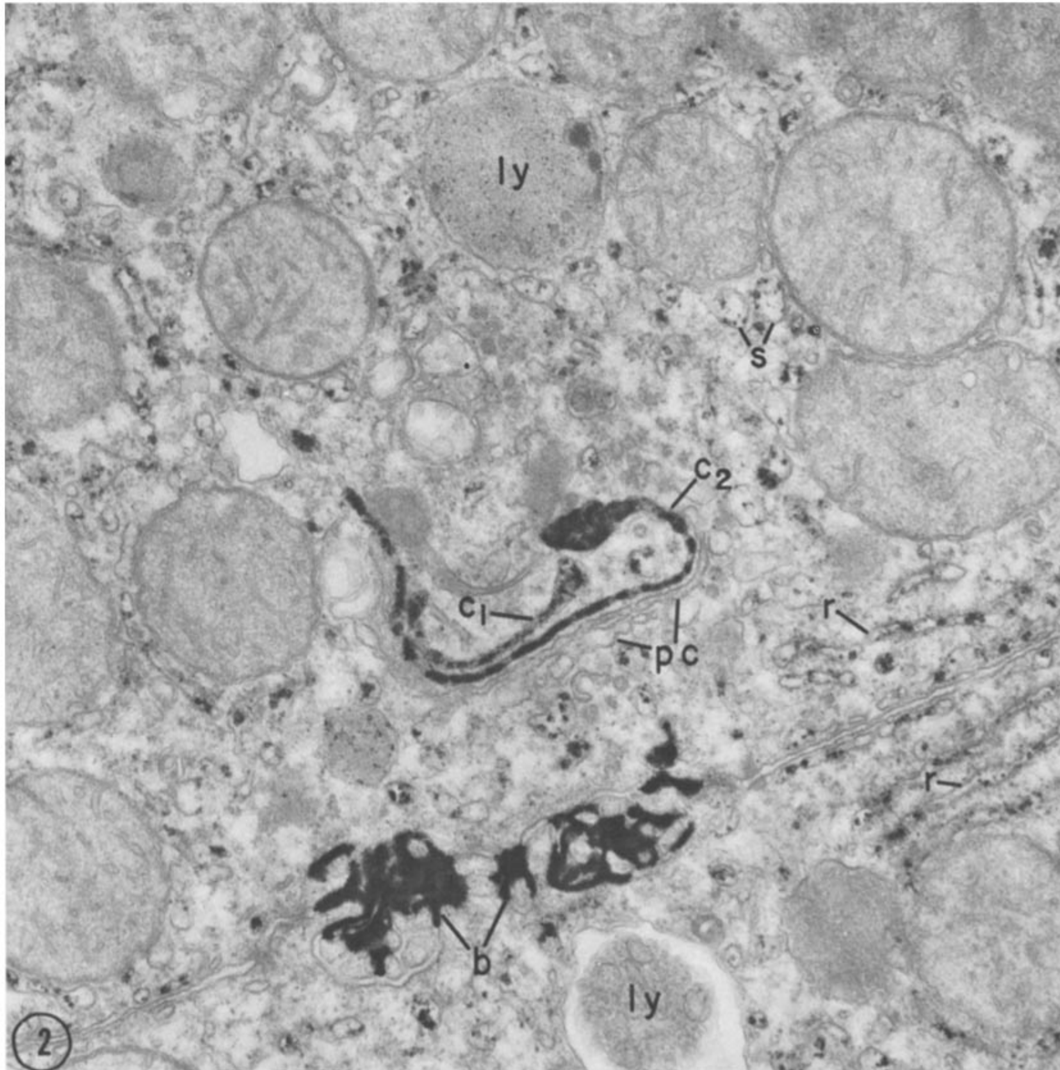
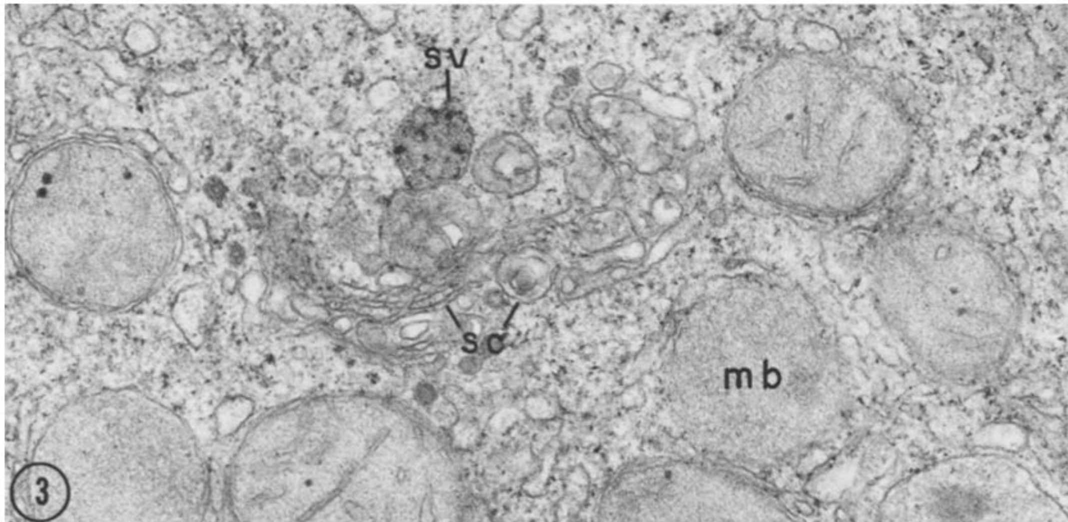
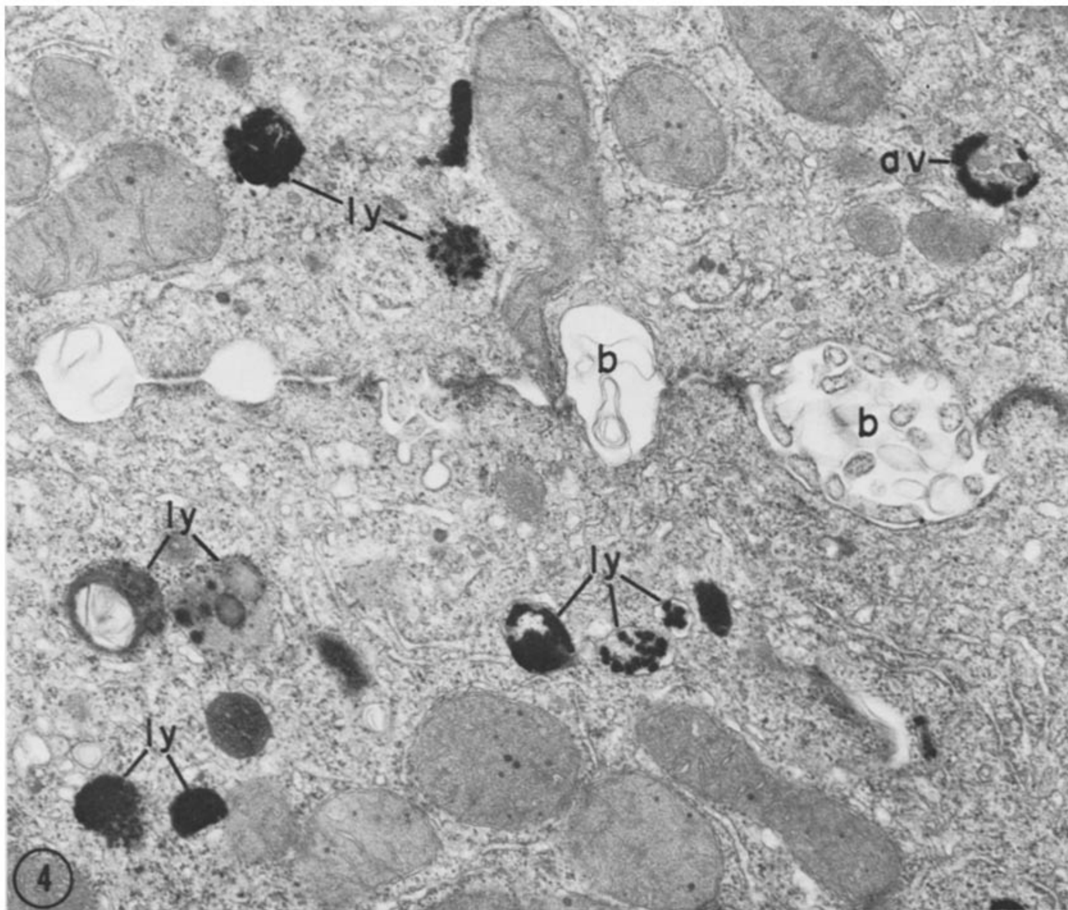


FIGURE 2 TPPase preparation, ethanol-treated rat liver. Heavy deposits of reaction product are seen within two Golgi cisternae (c_1 and c_2) along the concave or trans face of the Golgi apparatus and is absent from several cisternae (*pc*) on the convex or cis side. Heavy deposits are also seen along the plasma membrane where they are restricted to those segments facing bile canaliculi (*b*). Smaller deposits also occur throughout the rough (*r*) and smooth (*s*) ER. *ly*, lysosomes. Preparation as for Fig. 1, except specimen was reacted for 90 min at 25°C in TPPase medium of Novikoff and Goldfischer. × 30,000.

tened centers (Fig. 15). In preparations incubated with 2′-AMP, which inhibits 90% of the microsomal AMPase activity (12), deposits were rarely seen in any of these locations. To rule out the possibility that the observed reaction was due to AcPase or acid nucleotidase activity, fluoride was added to the incubation mixtures. Its presence did not affect the plaquelike deposits present along the inside of the membranes of GF_1 and GF_2 and along the outside of GF_3. However, no reaction product was seen in lysosomes, and deposits were less commonly encountered within the contents of the VLDL-filled vacuoles of GF_1 and GF_2, suggesting that some of the reaction seen in these locations was due to AcPase and/or acid nucleotidase. It is clear, therefore, that the AMPase found by bio-





FIGURES 3 and 4 AcPase preparations; liver of ethanol-treated rat. In Fig. 3, reaction product is present within one of the VLDL-filled secretory droplets (*sv*) along the secretory Golgi face, but is not found in the stacked cisternae (*sc*). Fig. 4 shows several clusters of AcPase-positive lysosomes (*ly* and *av*) located near bile canaliculi (*b*). *mb*, microbody. Specimens incubated for 60 min at 25°C in modified (Barka and Anderson) Gomori medium for AcPase (pH 5.0). Fig. 3, × 35,000; Fig. 4, × 24,000.

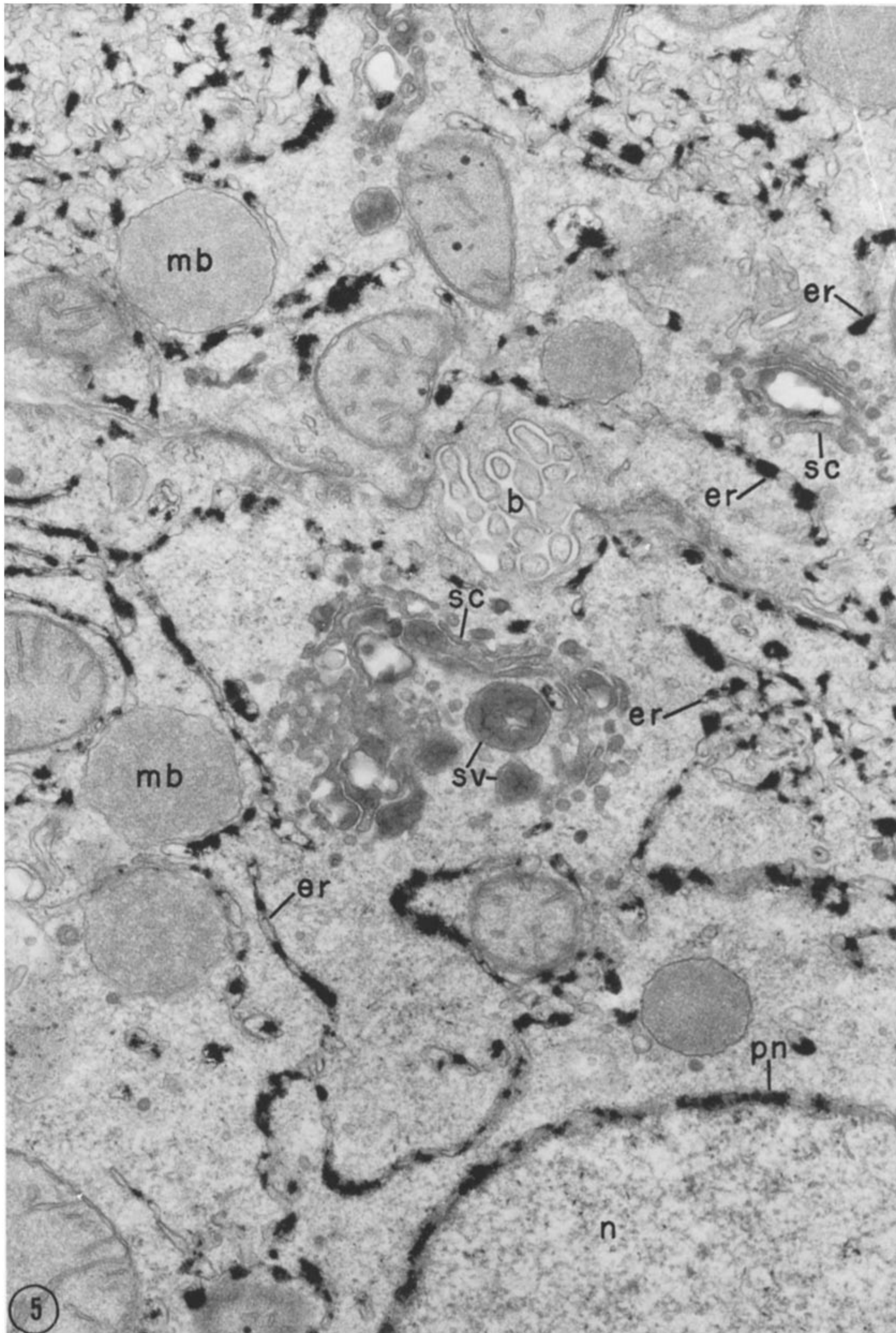


FIGURE 5 G-6-Pase preparations, liver of ethanol-treated rat. Reaction product is seen throughout the ER (*er*), both the rough- and smooth-surfaced parts, including the perinuclear cisterna (*pn*), but it is absent from the secretory droplets (*sv*) and stacked cisternae (*sc*) of the Golgi apparatus. *n*, nucleus, *b*; bile canaliculus; *mb*; microbodies. Specimen incubated for 60 min at 25°C in the G-6-Pase medium of Wachstein and Meisel (pH 6.0). × 31,000.

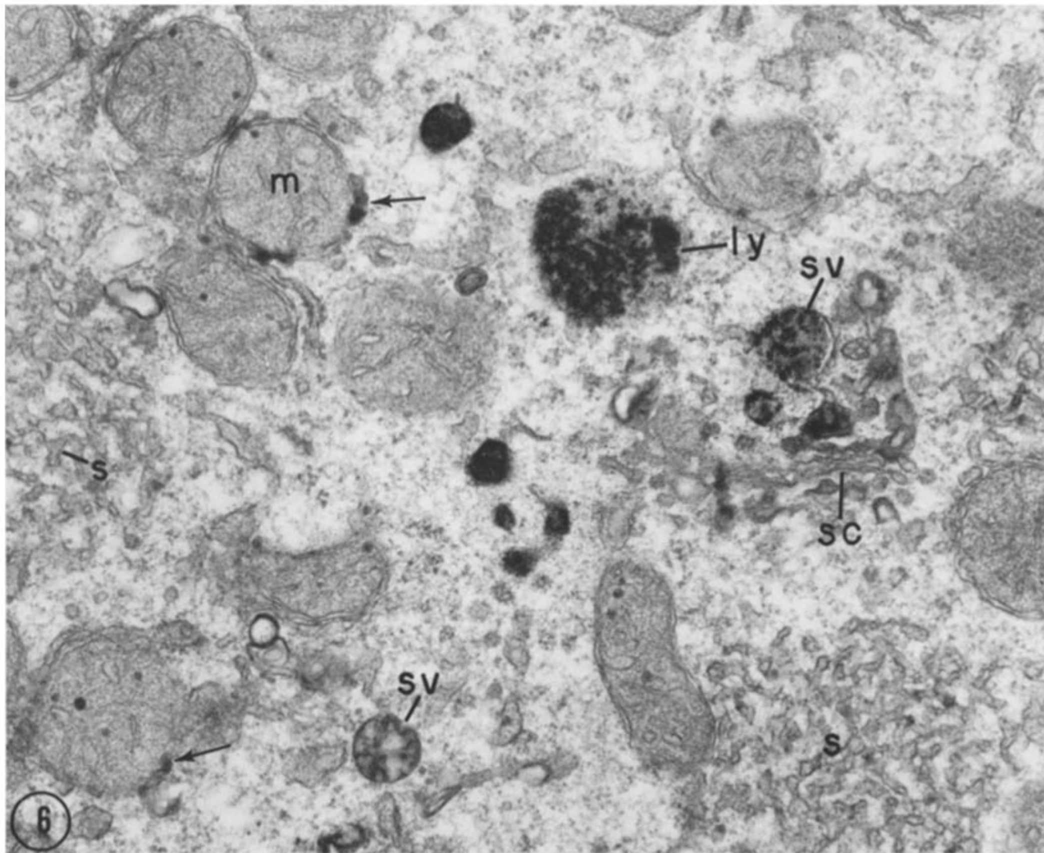


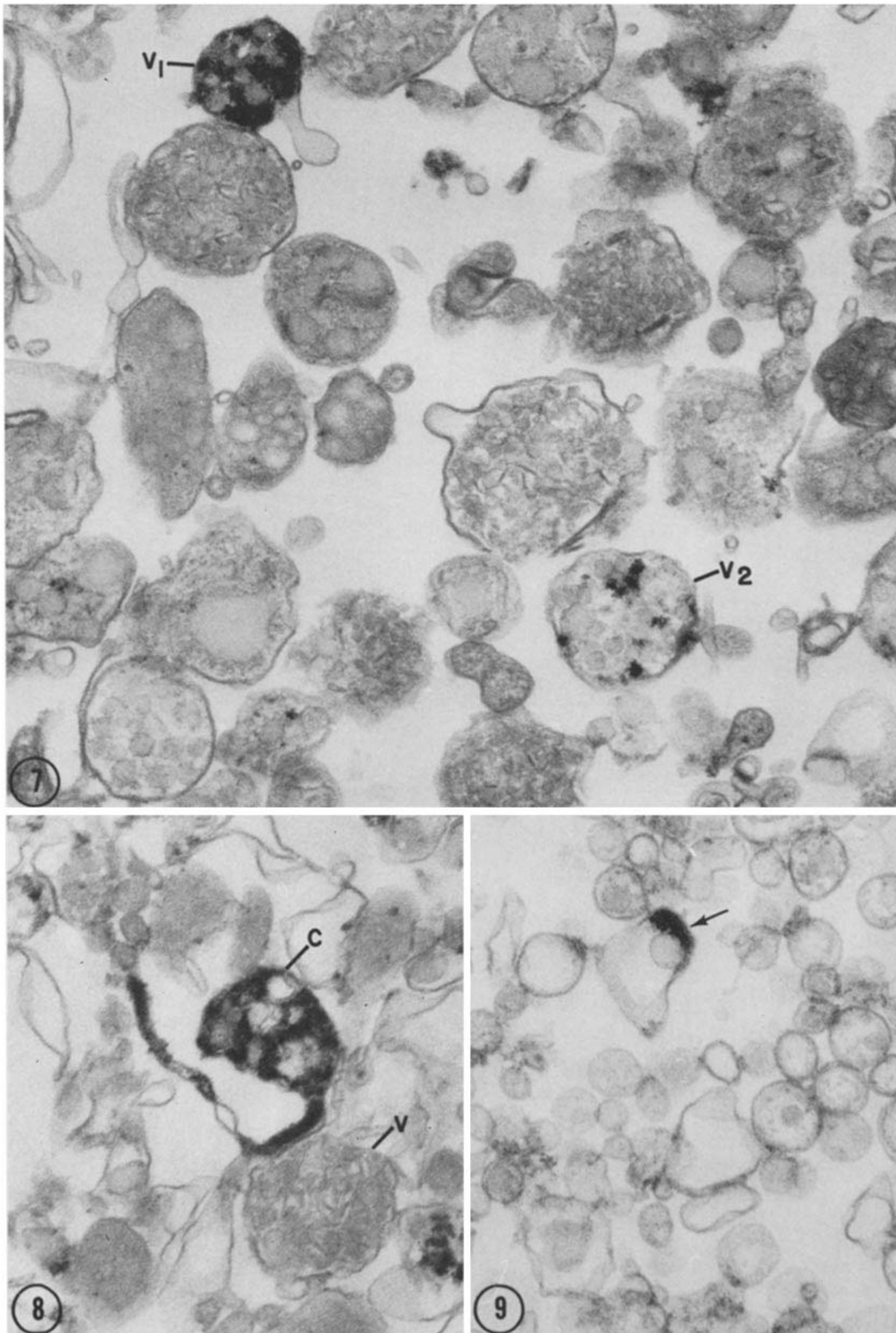
FIGURE 6 AMPase preparation, liver of ethanol-treated rat. Reaction product is present in several VLDL-filled secretory droplets (*sv*) and within a large body, probably a lysosome (*ly*). Dense deposits less crystalline in nature, of unknown origin, are also seen at the periphery of a number of mitochondria (arrows). *s* = smooth ER; *sc* = stacked Golgi cisternae. Specimen prepared as for Fig. 1. × 36,000.

chemical assay in Golgi fractions is not due to contamination by plasma membrane fragments, but is indigenous to the elements of the Golgi complex. The presence of reaction product both on the membranes and in the content of GF_1 and GF_2 is in keeping with the biochemical data (5) which show that upon subfractionation of GF_1, part ($\sim$30%) of the AMPase activity is recovered in the content, and the rest remains associated with the membrane. The reversed localization of reaction product on the outside of the membranes of GF_3 resembles that found by Widnell (12) for ER membranes. The striking restriction of the reaction product to the cisternal rims and its consistent absence from the cisternal centers in GF_3 suggest that in this case the enzyme is localized to specific regions within Golgi membranes and implies that, notwithstanding the general fluidity of membrane components, it is not free to diffuse in the plane of the membrane.

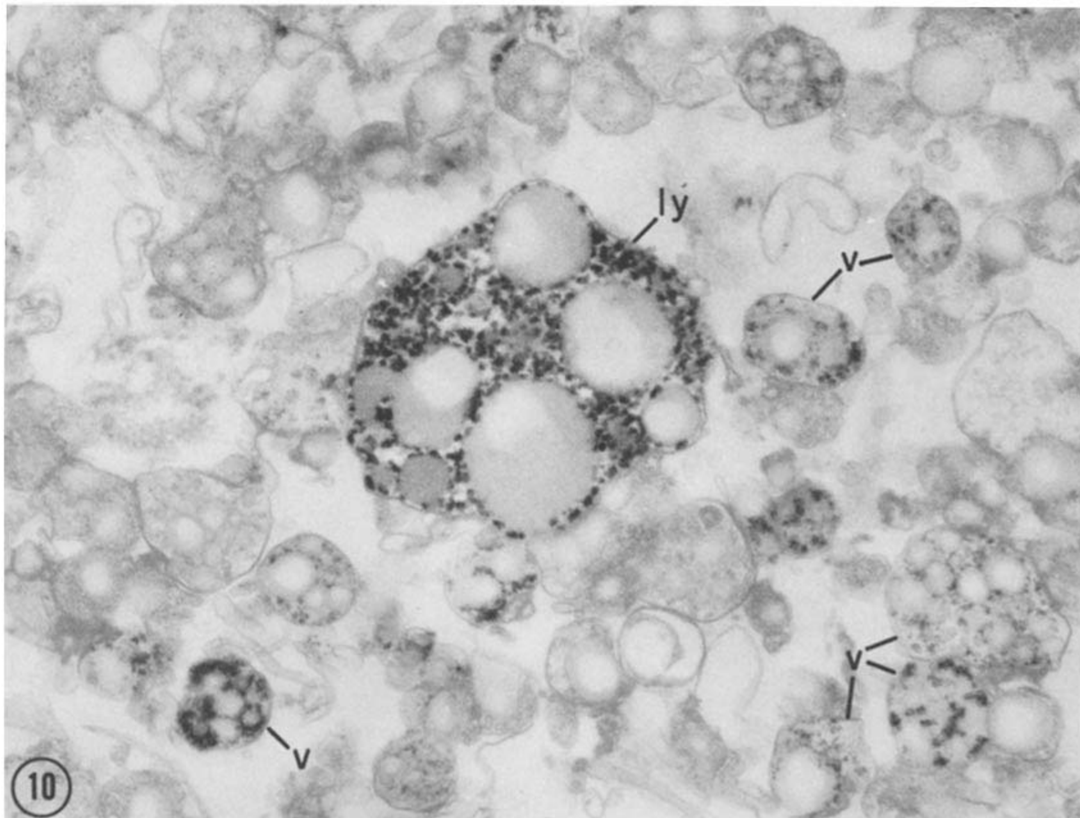
G-6-PASE: No reaction product was seen in elements of GF_1. Lead phosphate deposits were found only occasionally in smooth vesicular profiles in GF_2 and were more numerous in similar profiles in GF_3 (Fig. 16). None were found in any elements which could be identified morphologically as Golgi cisternae, indicating, in accord with the biochemical findings, the presence of some contaminating ER fragments in GF_3 and, less frequently, in GF_2.

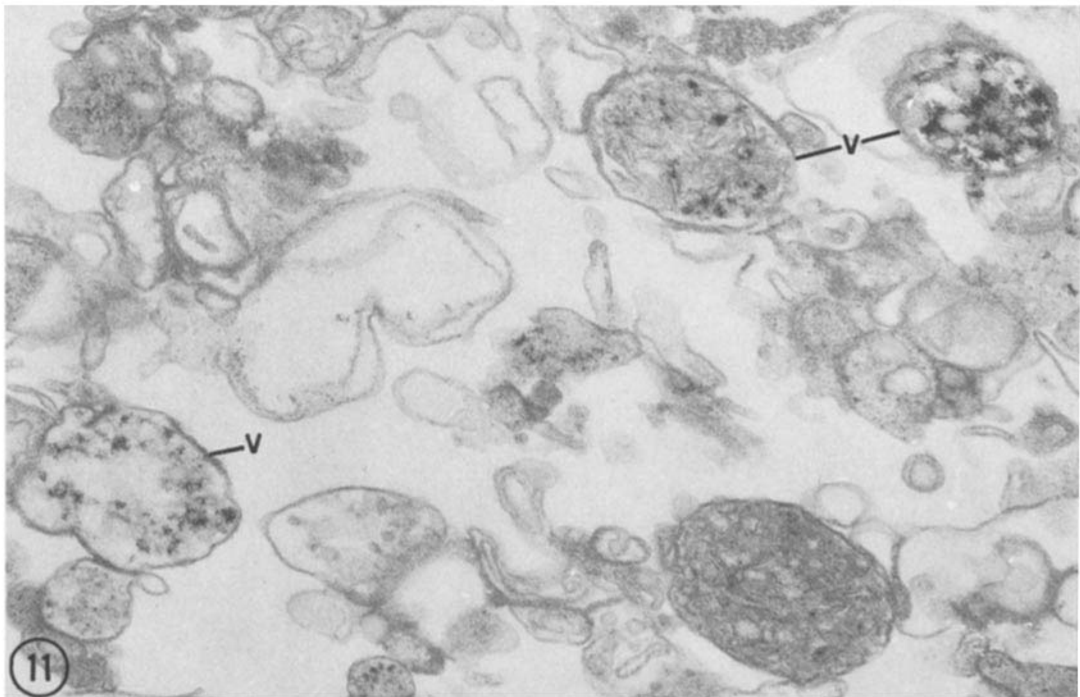
DISCUSSION

The results indicate once again (8, 11, 12) the usefulness of cytochemical methods in monitoring



FIGURES 7, 8, and 9 TPPase preparations of the light (GF_1), intermediate (GF_2), and heavy (GF_3) Golgi fractions. In GF_1 (Fig. 7) reaction product is localized to the matrix of some of the VLDL-filled vacuoles (v_1 and v_2). In GF_2 (Fig. 8) reaction product is found in some profiles (c) representing Golgi cisternae with dilated rims containing VLDLs. In GF_3 (Fig. 9) reaction product is restricted to a few rare VLDL-containing elements (arrow). Fractions were fixed in suspension for 10 min in 0.5% glutaraldehyde, centrifuged, and the resultant pellets incubated for 90 min at 37°C for TPPase. × 60,000.





FIGURES 10 and 11 AcPase preparations of the light (GF_1) and intermediate (GF_2) Golgi fractions. Reaction product is found in lysosomal contaminants (*ly*) in GF_1, and in some of the VLDL-filled vacuoles. (*v*) in both Golgi Fractions. Unfixed fraction incubated in suspension for 30 min at 25°C in AcPase medium, fixed in glutaraldehyde, and then centrifuged and processed for embedding. Fig. 10, × 36,000; Fig. 11, × 48,000.

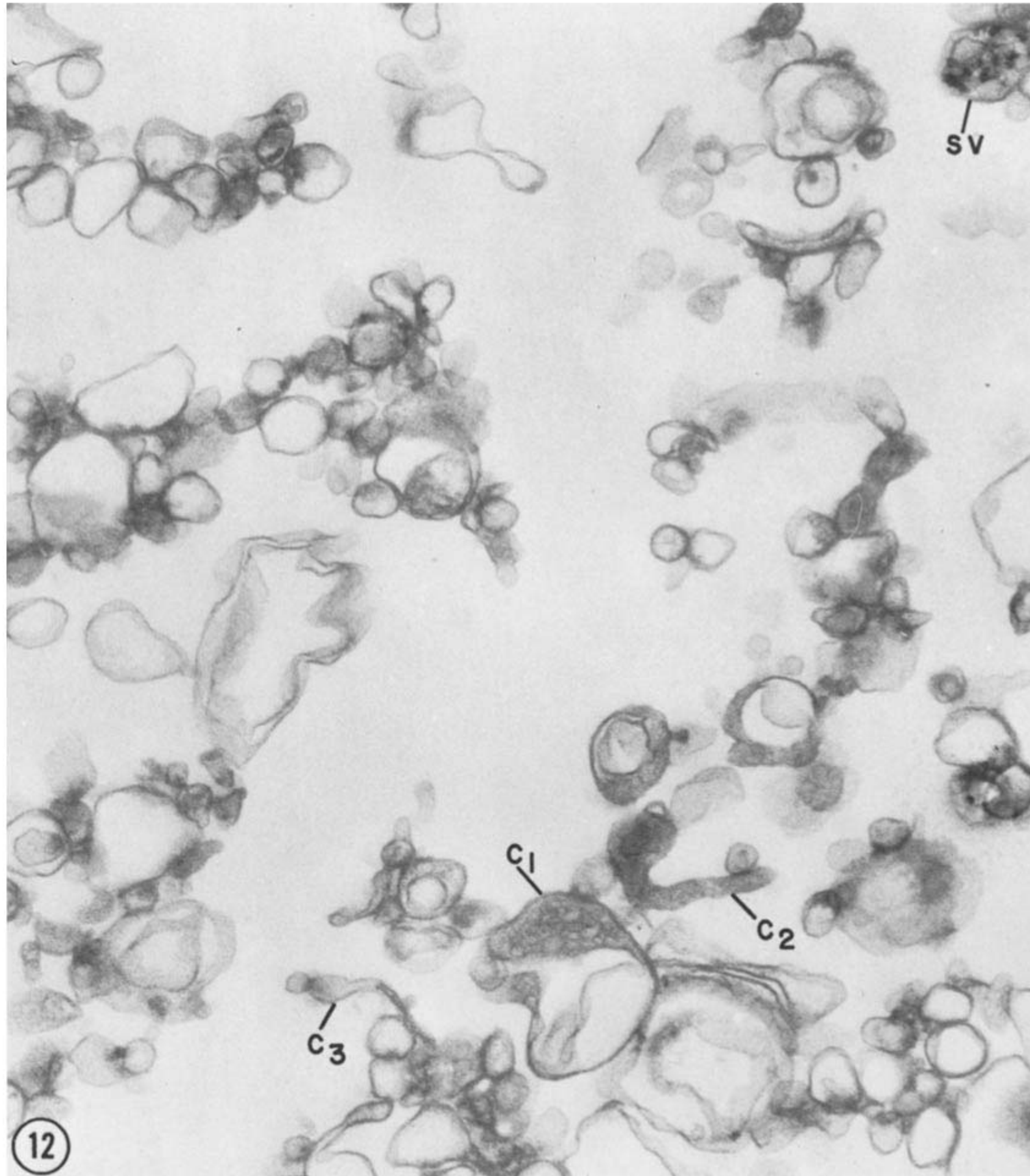


FIGURE 12 AcPase preparation of the heaviest Golgi fraction (GF_3). Very little reaction product is seen in this fraction. Only rare VLDL-containing vacuoles (*sv*) are reactive. Identifiable Golgi cisternae (c_1 – c_3) are free of reaction product. Specimen prepared as for Figs. 10 and 11. × 48,000.

fractionation procedures. In this work they have allowed first, a more reliable identification of contaminants, as in the case of lysosomes in GF_1 demonstrated by the AcPase test, and in the case of the microsomes present in GF_3 and demonstrated by G-6-Pase. Secondly, they have made possible a distinction between indigenous and contaminating activities, allowing the detection of indigenous AcPase and AMPase in Golgi elements. Thirdly, they have shown primarily by the results of the TPPase test that the procedure achieves a meaningful subfractionation of Golgi elements. In all

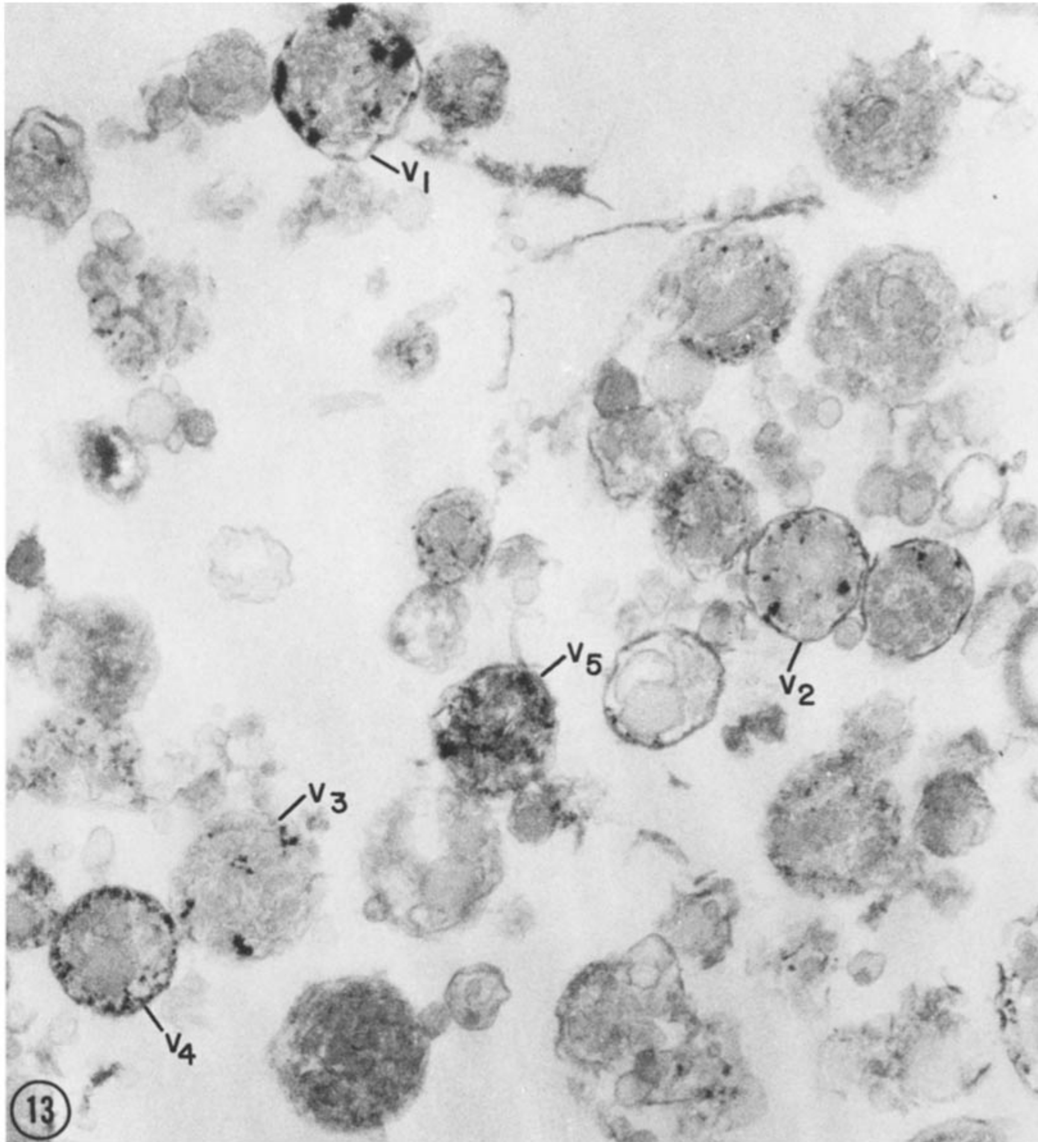


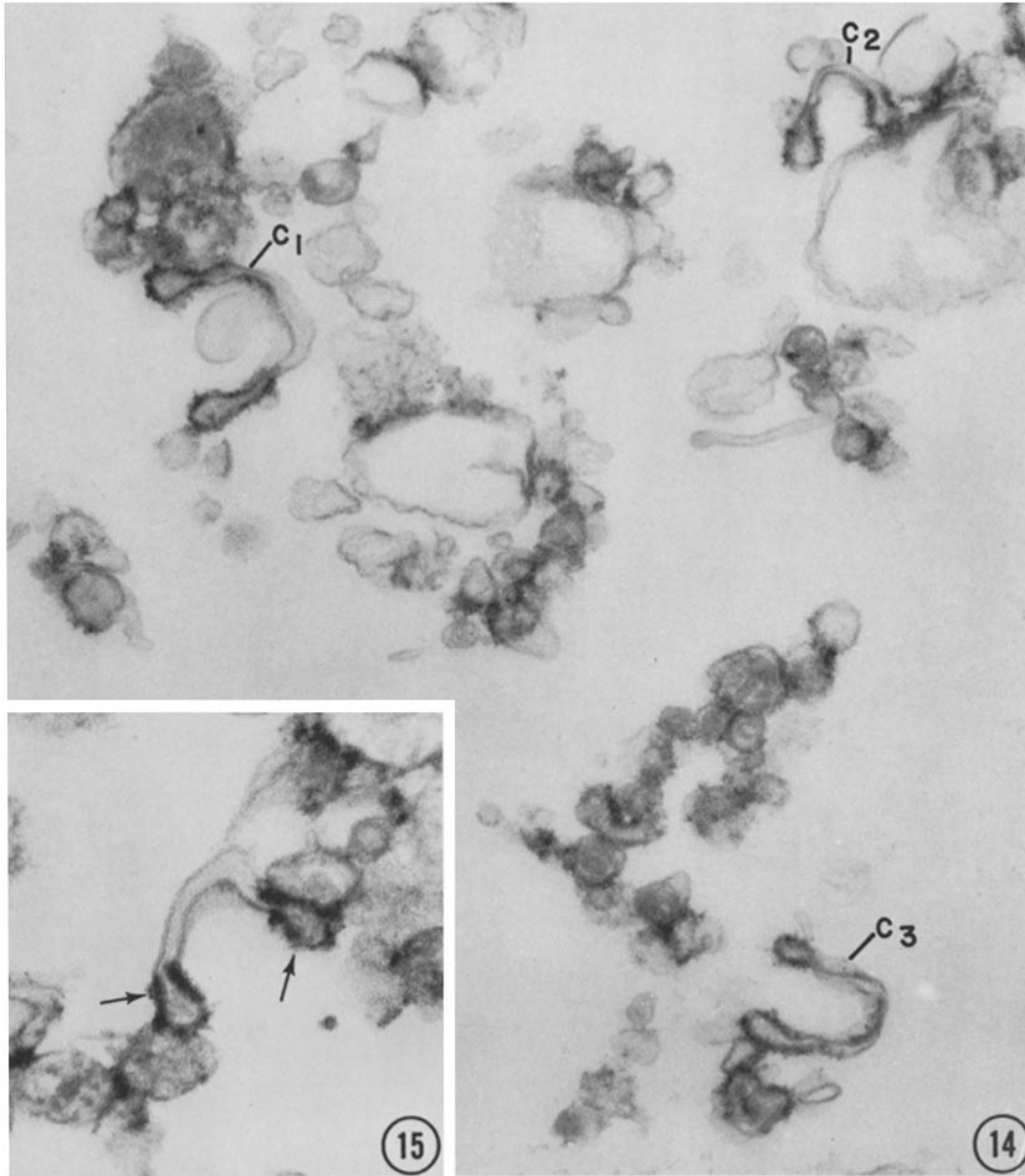
FIGURE 13 AMPase, light Golgi fraction (GF_1). Reaction product is found in some of VLDL-filled vacuoles ($v_1 - v_5$). In many ($v_1 - v_4$) it is located largely in plaques along the inner surface of the membrane of the droplet, whereas in others (v_5) it is also present in the matrix of the body. Specimen preparation as for Figs. 10 and 11, except incubation was carried out for 30 min at 25°C for AMPase. × 42,000.

these instances the cytochemical tests have enabled us to obtain information which could not be obtained by cell fractionation alone.

Reliability of Marker Enzymes

The present results, especially those obtained *in situ*, confirm that G-6-Pase is a reliable marker enzyme for ER in the hepatocyte. As previously reported by Goldfischer et al. (19) and Leskes et al. (8, 21), it is clear that all ER elements—both rough and smooth—are reactive, and, the plasma membrane and Golgi elements are not.

In many tissues TPPase is restricted to Golgi cisternae and therefore is a reliable Golgi marker



FIGURES 14 and 15 AMPase, heaviest Golgi fraction (GF_3). Reaction product is found on many of the cisternal profiles ($c_1 - c_3$). In contrast to the situation in GF_1 and GF_2, it is located on the outside of the membrane and where it typically occurs on the dilated rims (arrows) of the cisternae and is absent from their central regions. Specimen prepared as for Fig. 13. Fig. 14, × 60,000; Fig. 15, × 80,000.

enzyme (13, 22). Several workers (2, 3) have already pointed out that such is not the case for the hepatocyte, since both the ER (2, 3) and portions of the plasma membrane (those facing the bile canaliculi (2, 3, 22) give a positive reaction. However, TPPase has been a very useful enzyme for monitoring Golgi subfractionation, since this enzyme is present only in some Golgi cisternae, i.e., those along the secretory face. From the biochemical and morphologic data it could not be determined whether the fractionation procedure achieved a subfractionation of Golgi elements or

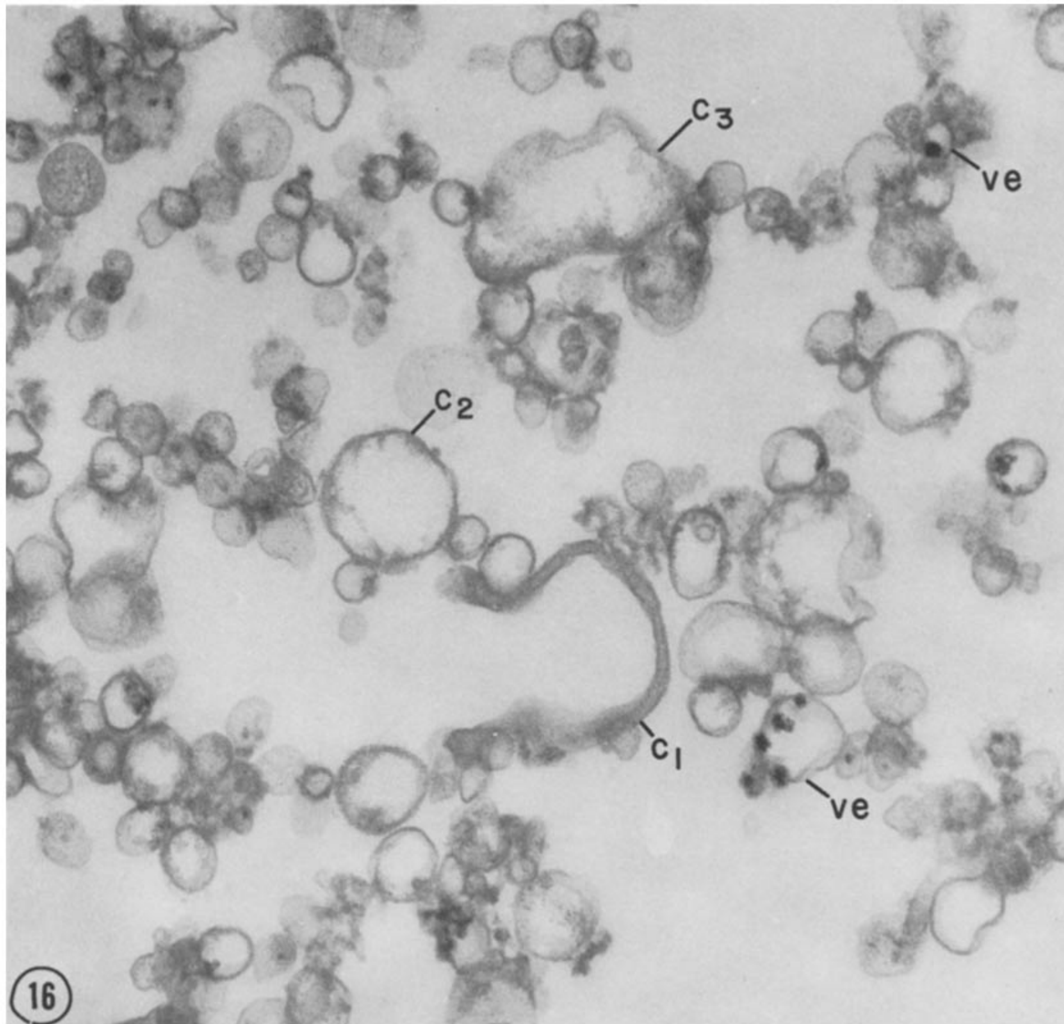


FIGURE 16 G-6-Pase, GF_3. Reaction product is present within several small vesicles (*ve*) which most probably represent contaminating ER fragments. No reaction product is seen in morphologically identifiable Golgi elements of which an elongated cisternae (c_1) and two vacuoles with extracted contents (c_2 and c_3) are seen here. Unfixed fraction incubated 40 min at 25°C. × 60,000.

whether the two lightest fractions represent detached ends of the cisternae indifferent of their location in the Golgi stacks, the heaviest fraction consisting of the cisternal centers that remain after detachment of the VLDL-filled vacuoles. The fact that, except for ER contaminants, TPPase activity was restricted to the two lightest Golgi fractions suggests that at least partial subfractionation of the Golgi apparatus has been achieved, with cis-Golgi elements predominating in the heaviest fraction (GF_3) and the trans-Golgi elements concentrated in the two lightest fractions (GF_1 and GF_2).

Probably the most interesting of the present results are those obtained on AMPase. As far as we are aware, this represents the first clear demonstration of this enzyme in Golgi cisternae. Although AMPase is usually considered to be a plasma membrane marker and is widely used as such (23, 24), this enzyme has recently been shown by Widnell (12) to be indigenous to ER membranes as well as plasma membranes in the hepatocyte. The biochemical data obtained on Golgi fractions indicated that the specific activity of AMPase is intermediate between those of the ER and plasma

membrane fractions (5). The cytochemical results have demonstrated that this enzyme activity is not due to contamination by plasma membrane fragments, but instead is indigenous to Golgi membranes. It is of interest that the localization of the reaction product differed among the three Golgi fractions: It was found on the inside of the VLDL-filled secretory droplets in GF_1 and GF_2 and on the outside of the cisternal elements in GF_3. We are well aware that the localization of lead deposits does not necessarily reflect the true localization of the enzyme. Nevertheless, such a clear-cut differential localization is very intriguing and makes sense in view of the fact that the reaction product of AMPase is localized on the outside of the plasma membrane (see Fig. 1). The inner aspect of the membrane of the secretory droplets becomes the outer aspect of the plasma membrane when such droplets discharge their contents into the sinusoidal capillaries by exocytosis. We have already mentioned that the localization of reaction product on the outside of the heaviest Golgi fraction resembles that of the ER. This finding is also of interest in view of our evidence that these elements are derived from the cis face of the Golgi apparatus which is generally assumed to be in continuity (at least intermittently) with the ER.

Finally, a word about our AcPase results demonstrating this enzyme activity not only in lysosomes, but also in secretory droplets: Historically AcPase has been an extremely useful marker enzyme for lysosomes both from the biochemical (25) and cytochemical (22, 26) standpoints. However, results obtained by cytochemical staining using the Gomori technique have demonstrated that AcPase is not exclusively restricted to lysosomes, but is also found in Golgi cisternae (10, 27–30), specialized regions of the ER known as GERL (31), and around secretory granules, especially immature or forming secretory granules (22, 26, 10). The latter localization was first called attention to by Novikoff and co-workers (22, 26) in a variety of tissues. In some cases, especially in leukocytes (28–30), the ER, Golgi, and granule localization extends to other lysosomal enzymes (e.g., aryl sulfatase) and is clearly associated with the packaging of acid hydrolases into primary lysosomes. In other instances, however, including the VLDL-filled secretory droplets of liver reported here, the AcPase activity demonstrated does not represent a known component of the secretory product, and therefore the significance of this localization remains obscure. It should be added, however, that evidence obtained on the liver (32) as well as on other tissues (33–36) suggests the existence of more than one AcPase in certain cell types.

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