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ELECTRON MICROSCOPY OF MEMBRANE-PARTICLE ARRAYS IN LIVER CELLS OF ETHIONINE-INTOXICATED RATS

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Arrays of smooth or rough-surfaced membranes have been observed in liver cells in various experimental conditions. Relatively large, concentric whorls of imbricated paired membranes have been seen in the cytoplasm of parenchymal liver cells of rats fed dimethylnitrosamine (DMNA),[1-3] thiocetamide (TAA),[4-8] alpha-naphthyl isothiocyanate (ANIT),[9] and DL-ethionine (DLE),[10] in guinea pigs given injections of diphtheria toxin,[11] in hepatomas of man,[12] rats,[13] and mice,[14,15] in human [16,17] and murine [18] viral hepatitis and in murine infections with the virus of Rift Valley fever.[19] In the neoplastic lesions [12-15] and in DMNA intoxication [3] the membranes are studded with ribosomes, but in the other lesions ribose-nucleoprotein particles are partially or completely lacking in the whorls. The smooth-surfaced membranous arrays encountered in murine Rift Valley fever are altogether unique since the cisternae between the coiled pairs of membranes contain crossbars which impart to them a banded, ladder-like appearance.[19]

The term "ergastoplasmic *Nebenkern*," coined by Haguenau,[20] seemed applicable to the ribosome-bearing arrays since they are likely to create an image of paranuclear basophilic bodies with light microscopy. A variety of terms have been used to describe the particle-free

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whorls: "fingerprints," [3] *"impreints digitales,"* [5,8] "ellipsoidal bodies," [3] *"figures myéliniques,"* [8] *"masses feuilletées agranulaires,"* [17] *"Nebenkern,"* [1,2,8] *"zytoplasmatische Wirbelbildungen" or "glatte Nebenkerne,"* [7] and *"enroulements membraneux."* [4]

Particles other than ribose-nucleoprotein are sometimes associated with the coiled membrane complexes. Salomon,[5] in a study of the chronic effects of TAA intoxication, described an annular complex, in which a single pair of coiled ribosome-free membranes (*anneaux ergastoplasmiques complets*) were occasionally associated with free glycogen particles randomly scattered in the adjacent hyaloplasm. Herman, Eber and Fitzgerald [10] noted that in the early stages of ethionine intoxication in rats, unidentified dense particles sometimes lay between the adjacent paired membranes of "fingerprints" in liver cells.

Our own investigations of the ultrastructural changes which develop in liver cells of rats during prolonged DL-ethionine intoxication indicated that there were indeed several types of whorled membrane complexes. Some were associated with granules, some were not. It is the purpose of this paper to describe the complexes, to attempt to identify the nature of the particles associated with them, and to deduce from the 2-dimensional images the 3-dimensional form of the membrane-particle arrays.

Material and Methods

White male Wistar (Woodlyn Farm) rats (56), weighing approximately 250 gm. were used. Forty-four rats were maintained on Purina Fox Chow and water which contained 0.5 per cent DL-ethionine (Lot No. 680701, California Corporation for Biochemical Research). Both the food and water were given *ad libitum*. Twelve rats were fed the Chow isocalorically but received water *ad libitum* without ethionine.

The 44 experimental rats were killed at elected times: on the ninth (2), 15th (2), 21st (2), 32nd, 34th, 35th (2), 36th, 38th, 39th, 40th, 42nd (3), 45th (2), 49th (3), 51st (2), 52nd, 56th (2), 63rd (3), 64th, 65th, 71st, 72nd, 77th, 78th, 79th (2), 84th, 93rd, 105th, 106th, 119th and 139th days after commencement of the ethionine-containing diet. The 12 control rats were killed in pairs on the 21st, 42nd, 63rd, 84th, 105th and 126th days. The animals were always killed at 9:30 a.m. after fasting overnight. Material for histologic study was removed from the right lateral lobe of the liver.

Tissues for light microscopy were fixed in 10 per cent aqueous buffered formalin (pH 7.0) or in 10 per cent alcoholic formalin. Sections were stained with hematoxylin and eosin, by the periodic acid-Schiff (PAS) method, or with PAS after saliva digestion for 30 minutes at room temperature.

Small fragments of tissue for electron microscopy were fixed in Palade's buffered osmium tetroxide (pH 7.4) containing 0.25 M sucrose (Caulfield's fixative [21]). Two ml. of fixative were used for each sample. After insertion of the tissue, the vial containing the fixative was maintained at 4° C. for 90 minutes and then kept for 30 minutes at room temperature. The tissues were dehydrated in a graded series of ethanol solutions, and embedded in Epon 812 by the method of Luft.[22] Sections were cut on Porter-Blum ultramicrotomes with glass knives. Some were "stained"

by flotation on uranyl acetate by the method of Watson[23] and picked up on uncoated grids. Others were "stained" with lead hydroxide by the method of Karnovsky.[24] Sections were examined in an RCA EMU-3E electron microscope, using a 100 kv. acceleration potential and initial magnifications of 1,500 to 12,000 times.

Results

LIGHT MICROSCOPY

The administration of an ethionine-supplemented diet led to an extensive disorganization of the architecture of the liver after 4 to 8 weeks. A massive proliferation of "oval" (ductular) cells occurred, and these cells insinuated themselves between the parenchymal cells, dividing lobules into segments, and isolating small groups of liver cells or single hepatocytes. Many liver cells became atrophic or were markedly hypertrophied. The atrophic cells usually had an intensely acidophilic cytoplasm and were, as a rule, totally devoid of glycogen. The hypertrophic hepatocytes had large, hyperchromatic nuclei with multiple nucleoli, and an abundant basophilic cytoplasm. The basophilia was especially prominent in the perinuclear region in the first 4 weeks. Saliva-digestible glycogen could be demonstrated in them only extremely rarely. However, after about 4 weeks on ethionine, glycogen abruptly became abundant in a few of the hypertrophic liver cells. Thereafter the amount of glycogen in the cells, and the proportion of cells affected remained constant until after the 105th day, when the first hyperplastic nodules appeared. The majority of cells in the nodules invariably contained large quantities of glycogen, although the extranodular parenchymal cells remained free of glycogen. The glycogen-rich cells had usually an eosinophilic cytoplasm.

Isocalorically fed control rats had very little glycogen in their liver cells.

ELECTRON MICROSCOPY

The present account will deal only with the changes in liver cells observed between the 32nd and 105th day, that is, after the onset of extensive parenchymal disorganization and prior to the development of hyperplastic nodules. The alterations to be described occurred in hypertrophied hepatocytes, but it was impossible to relate the patterns seen by electron microscopy to changes observed by light microscopy, except by inference.

Perinuclear Glycogen Stores

Glycogen stores were extremely scant even when lead hydroxide "stained" sections were searched for them. The usual plaque-like glycogen areas devoid of other organelles were totally absent. Glycogen

was found in perinuclear locations either as scattered, solitary particles, or as rosettes of particles in rather small clusters (Figs. 1 to 3). Narrow and tortuous or dilated, oval or round profiles or agranular reticulum were always associated with the clusters. The rosettes of glycogen measured on the average 120 mμ with a range from 40 mμ to 200 mμ. However, the majority measured 40 to 80 mμ. The solitary particles and the subunits of the rosettes measured from 15 mμ to 50 mμ in diameter. Individual glycogen particles could not be identified in uranyl acetate "stained" sections, but the clusters were seen as "glycogen holes," because of the "negative staining" imparted to glycogen by the uranyl ion.[9,25]

Identical glycogen deposits were seen in the isocalorically fed animals. These were the only glycogen deposits found in the controls. None of the changes to be described below were encountered in the control animals.

Membrane-Particle Arrays—"Glycogen Bodies"

Complex arrays of profiles of agranular membranes associated with electron-dense particles were found in randomly scattered hypertrophic liver cells. The arrays occurred in two forms:

Polarized Membrane-Particle Arrays. These consisted of concentric arrays of imbricated parallel pairs of agranular membranes arranged in the form of a "fingerprint" (Figs. 4, 5, 8 and 10). The membranes usually enclosed a centrally located quantum of cytoplasmic matrix which almost invariably contained one or several mitochondria, microbodies, vacuoles, lipid droplets or other cytosomes (Figs. 4, 5, 10 and 13). The "fingerprints" were not always complete. On rare occasions we have observed a "fingerprint" shaped like a horseshoe (Fig. 9). The largest of the membranous arrays measured 5.3 μ in diameter and the smallest approximately 2.3 μ.

In the narrow bands of hyaloplasm between the pairs of membranes were particles which measured on the average 400 Å in diameter (Fig. 10). The particles usually comprised a single roughly spherical unit but occasionally consisted of a rosette of aggregated subunits which measured, on the average, 33 mμ each. The single spherical units were far more common than the rosettes. Wide fluctuations were observed in the size of the rosettes and in the number and size of their subunits. The particles were not attached to the membranes and were usually aligned in single file, though sometimes, particularly in the periphery of the whorls, they were more abundant and several layers of the particles lay in the space between more widely separated neighboring pairs of membranes (Fig. 14).

In lead hydroxide "stained" sections, the particle-studded membranous whorls were prominent even at relatively low magnification, because of the apparently uniform, intense electron opacity of the particles. At higher magnifications, it became clear that there were marked variations in the electron opacity of the particles (Fig. 10). We could not be sure that this was not the result of differences in the thickness of sections, though this seemed unlikely since other cytoplasmic structures in their vicinity "stained" evenly. In uranyl acetate "stained" sections the particles could not be seen, but "glycogen holes" [9,25] occurred in the same locations in which the particles were seen in sections "stained" with lead hydroxide (Fig. 11).

Short segments of granular endoplasmic reticulum were found at times near the inner or outer margins of the concentric arrays of agranular membranes (Figs. 5 and 10). The two types of membranes were at times continuous (Fig. 10). The ribosomes, attached to the surface of the former, measured $\sim$ 150 Å and in most instances were readily distinguished from the larger and more markedly electron-opaque glycogen particles between the agranular membranes. "Staining" with uranyl acetate did not diminish the electron opacity of the ribosomes.

Mitochondria lying in close apposition to the particle-studded "fingerprints" were occasionally markedly elongated and aligned with their long axis in parallel with the long axis of the concentric membranous arrays, thus forming a corona on the outer or inner aspect of the "fingerprint." Very rarely such elongated mitochondria were located between the leaves of the "fingerprint."

Nonpolarized Membrane-Particle Arrays. In an over-all view at relatively low magnification, the arrays usually occupied roughly round or oval shaped portions of the cytoplasm. They consisted of a large number of round or oval profiles of cisternae with an average diameter of 250 mμ (Figs. 6 and 7). The cisternae usually did not contain material of appreciable density, though occasional intensely electron-opaque irregular particles were identified in them (Fig. 6).

Near the periphery of these arrays, on occasion the cisternae showed a tendency to align themselves concentrically to form a rosette of vesicles (Fig. 6).

Between the cisternae were particles identical in size, configuration and "staining" properties with the particles found in the "fingerprint" whorls. However, in the nonpolarized arrays, rosettes of particles were somewhat more common than single spherical units (Figs. 6 and 7).

There was no obvious relationship between Golgi complexes and the membrane-particle arrays. The particles sometimes found within Golgi

cisternae consistently showed a lesser degree of electron opacity in lead hydroxide "stained" sections than did the particles in the arrays. Also the "staining" of the particles within the Golgi cisternae was not suppressed by the uranyl ion (Fig. 8a).

The two forms of membrane-particle arrays—the nonpolarized and the polarized "fingerprints"—formed extremes of a range of configurations of these two components. Several arrays were found occasionally in a single cell (Fig. 8). Usually they extended for some distance along the cell membrane, or ran from the cell membrane towards the nucleus. Almost invariably, the nonpolarized arrays were marginal and the "fingerprints" more centrally located.

Three-Dimensional Reconstruction of the Membrane-Particle Arrays. Two reconstructions of the membrane-particle arrays are possible in 3 dimensions:

1. They may be regarded as separate units of more or less polarized profiles. The granule-studded "fingerprints" would correspond to "onion-like corpuscles" described by Palay and Palade in sympathetic ganglion cells of rats.[26] The nonpolarized arrays would consist of bundles of tubules.

2. An alternative reconstruction envisages the various configurations of the arrays as constituting cross-sections of a single body, which resembles a medusa (Text-fig. 2). To this structure we have applied the name "glycogen body." When sectioned at right angles to the long axis of the umbrella and tentacles, variable two-dimensional images would result, as depicted in Text-figure 1. With increasing obliquity of sections in relation to the plane of the pairs of membranes, images would result in which the membranes would be more widely spaced and the particles in the intermembranous spaces more numerous. Such oblique sections would also produce various combinations of the two-dimensional images which are seen in their simplest forms in the cross sections in Text-figure 1 (Figs. 6, 8 and 14).

The two-dimensional Text-figure 1 shows a simplified version of the "glycogen body," since the finding of several closely apposed cross sections in a limited area of a single cell would seem to indicate that the long axis of the "body" can be at times extremely tortuous.

Other Types of Membrane and Membrane-Particle Arrays

In some cells (never in those containing a "glycogen body") are concentric whorls of more or less parallel paired membranes devoid of particles (Fig. 12). These "particle-free fingerprints" were identical with the "ellipsoidal bodies" described by Emmelot and Benedetti[8] and the "fingerprints" of "acidophilic necrosis" in liver cells noted by Albot and Jézéquel.[16,17]

In some cells there were "fingerprints" in which the paired membranes were studded on their outer surfaces with randomly distributed particles with a diameter of ~ 150 Å. The size and electron opacity of these particles was uniform and identical with that of ribosomes (Fig. 15). These membrane-particle arrays correspond to the "ergastoplasmic *Nebenkern*" described by Haguenau,[20] although the RNP particles were less numerous than is usual in these structures. Elongated mitochondria were found in relation to these RNP membrane arrays (Fig. 15), just as they were found in relation to the "glycogen bodies."

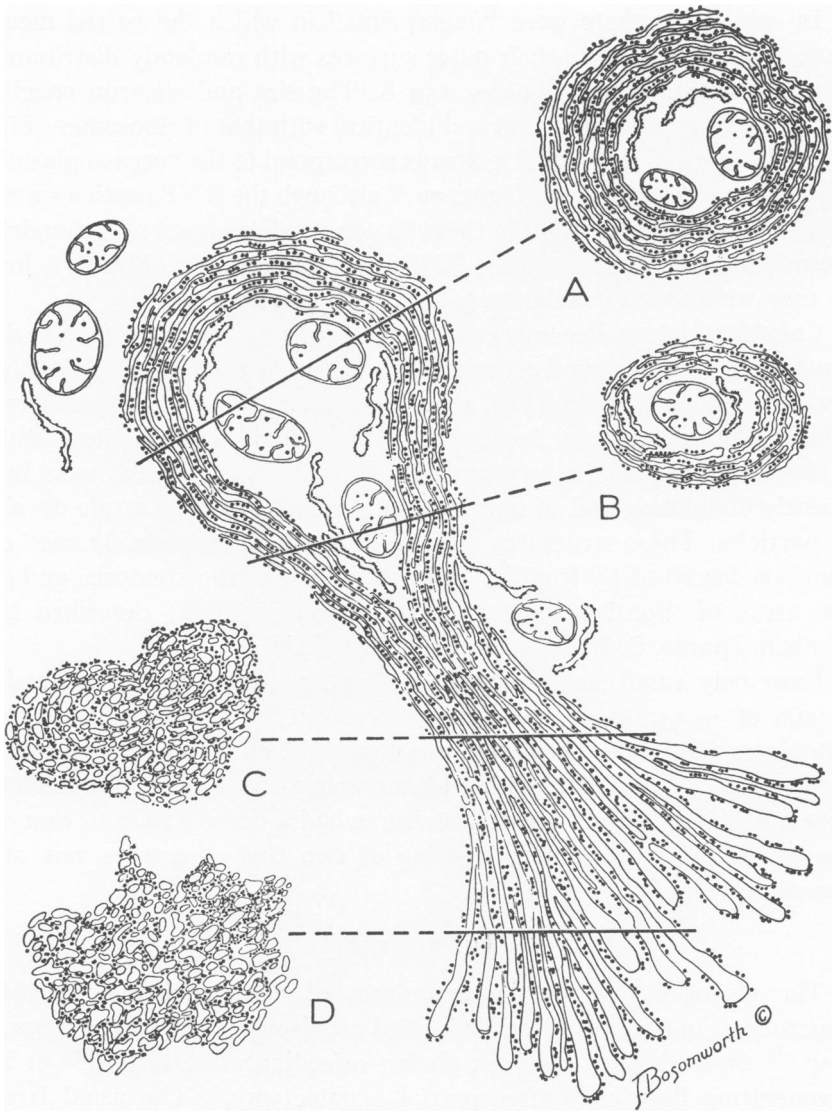
Considerably smaller concentric whorls of membranes (average diameter 1.0 μ) were found occasionally surrounding one or several mitochondria or microbodies (Fig. 16). These membranous arrays differed from the "particle-free fingerprints" and from the "ergastoplasmic *Nebenkern*" in that the membranes were less clearly paired, were frequently undulating and of uneven thickness, and were as a rule devoid of particles. These structures were reminiscent of "myelin figures" of the type described by Rouiller and Simon [8] in hepatic steatosis, and of the areas of "focal cytoplasmic degradation" (FCD) described by Hruban, Spargo, Swift, Wissler and Kleinfeld.[27]

Extremely small smooth-surfaced ring structures with a mean diameter of ~ 250 to 440 mμ were observed in random locations in the cytoplasm (Fig. 17). They were usually single, consisting of two membranes 40 to 50 mμ apart with an intervening dense layer. The central core of these doughnut-shaped structures had a density akin to that of the hyaloplasm. Concentric layering of two ring structures was observed very rarely.

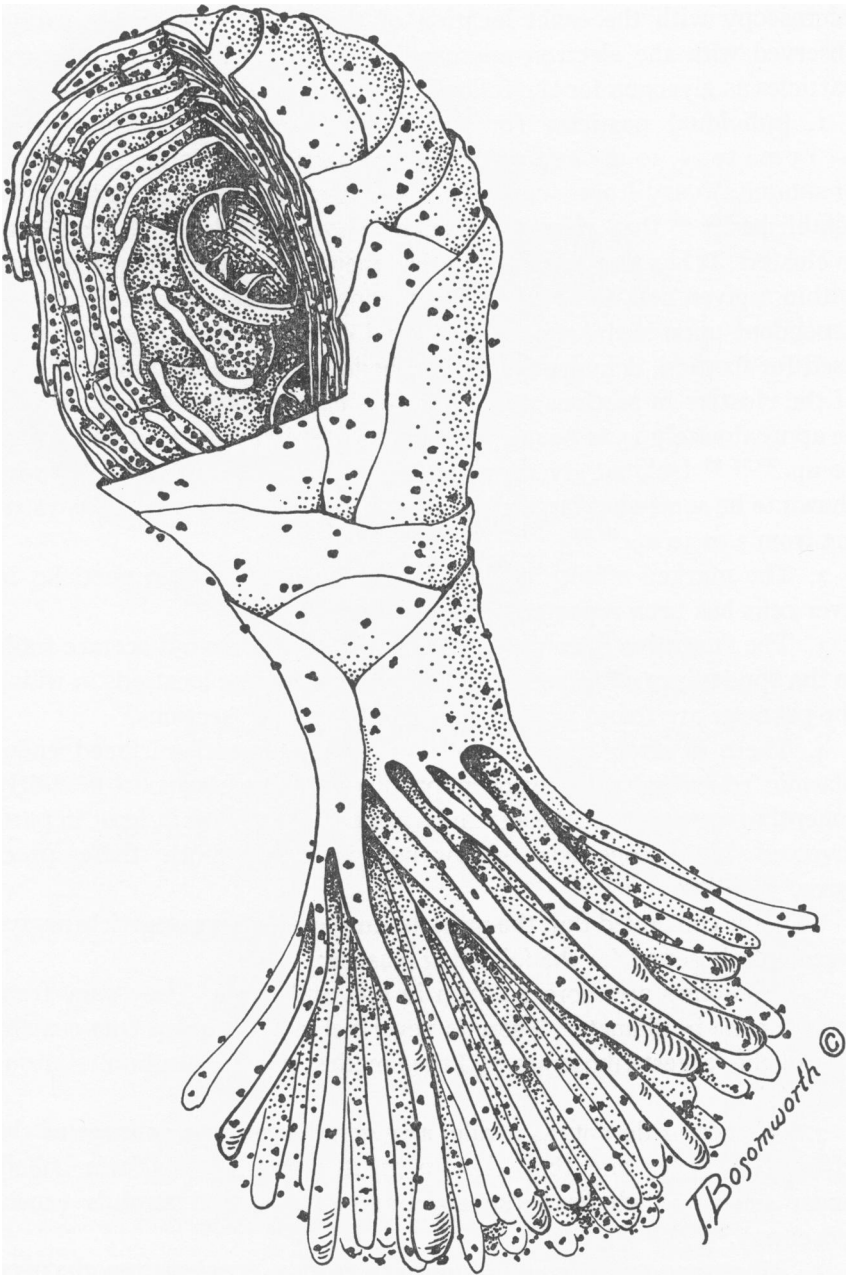
Discussion

The glycogen seen in the cytoplasm of occasional hypertrophic hepatocytes in the liver of ethionine-fed rats is not stored in the normal way,[8,28] or in the manner seen during refeeding after fasting,[29] or in regenerating liver cells after partial hepatectomy.[30] The usual large plaques of glycogen devoid of other organelles are not present. Instead, the newly formed glycogen is always seen in close association with the agranular (smooth) endoplasmic reticulum. Several variants of the particle-membrane pattern can be recognized. In particular, the development of polarized glycogen-bearing "fingerprints" and of nonpolarized arrays of agranular cisternae and particles, which we think constitute a "glycogen body," are unique and have not been described previously.

We have identified the particles related to the membranous arrays as glycogen, although we have been unable to correlate the presence of intracellular saliva-digestible PAS-positive material seen by light



TEXT-FIG. 1. Diagrammatic reconstruction of glycogen-bearing "fingerprints." The membrane-glycogen particle arrays seen in sections are represented by areas A, B, C and D. These are interpreted as being cross sections of a body shown in longitudinal section in the center of the diagram and 3-dimensionally in Text-figure 2. The configuration of the body is shown in its simplest form. It is likely that in reality the long axis of the body is convoluted, since it is common to find several membrane-particle arrays in a single cell (Fig. 8). Area A should be compared with Figure 4, B with Figure 5, C with Figure 6 and D with Figure 7. Note that rough-surfaced endoplasmic reticulum in the central cytoplasmic core (between lines A and B) and outside the body (between lines B and C) is often continuous with the smooth-surfaced membranes of the "fingerprints." Although the diagram shows the particles of glycogen in single files between the cisternae, it is more usual to find the particles in larger numbers in oblique sections of the intermembranous areas (Fig. 14).



TEXT-FIG. 2. Suggested 3-dimensional reconstruction of a "glycogen body." The organelle is reminiscent of a medusa. Particles of glycogen lie around the membranes which constitute the umbrella and tentacles. Cytoplasmic organelles and hyaloplasm occupy the core of the umbrella portion of the body. © University of Toronto, Canada. Reproduced with permission.

microscopy with the exact location of the membrane-particle arrays observed with the electron microscope. We nevertheless consider the particles as glycogen for the following reasons:

1. Individual particles (or subunits of rosettes) measure from ~ 15 mμ to ~ 50 mμ and aggregates of particles (clusters or rosettes of subunits) vary from ~ 40 mμ to ~ 200 mμ in diameter. It has been established[31–33] that glycogen particles show a tendency to aggregate in clusters. It has also been shown that they vary markedly in size even within a given cell, although at least some of these variations may be dependent upon shrinkage artifacts and other effects of the materials used for fixation, dehydration and embedding. The usual range of size of the clusters in sections of mammalian liver cells has been found to be approximately 15 to 80 mμ[29,34–37] and of the individual subunits 15 to 40 mμ.[29,34–36] Isolated glycogen particles of normal rat liver cells were shown to be somewhat larger: clusters 40 to 200 mμ and subunits ranging from 3 to 30 mμ.[31,32]

2. The marked affinity of lead hydroxide for glycogen particles in liver cells has been repeatedly noted.[22,33–36,38–40]

3. The "negative staining" of the particles with uranyl acetate leads to the appearance of "glycogen holes"[9,25] in the same locations in which the particles are found in lead hydroxide "stained" sections.

4. There is close relationship between the smooth-surfaced endoplasmic reticulum and glycogen particles. There seems to be fairly general agreement that the agranular reticulum may be, at least in part, involved in glycogenesis, glycogenolysis or in both these processes.[9,25,33,41–43]

We have concluded that the particles in the membrane-particle arrays were not ribosomes for the following reasons:

1. Ribosomes are more uniform in size and shape. They vary from 10 to 15 mμ in diameter. There is nevertheless an overlap between the size of the largest ribosomes and the smallest glycogen subunits in our material.

2. Ribosomes do not, as a rule, aggregate in clusters as large as the glycogen rosettes and they show a greater tendency to form coiled, linear arrays which have the appearance of beaded strands (cochleosomes).[9]

3. Ribosomes are of a more uniform electron density than glycogen particles when "stained" with lead hydroxide.

4. The electron opacity of ribosomes is not appreciably enhanced by lead hydroxide "staining," whereas even brief exposures of sections to lead hydroxide impart to glycogen particles a fairly intense opacity.

5. "Staining" with uranyl acetate, if anything, tends to increase the

electron opacity of ribosomes, whereas it has a "negative staining" effect upon glycogen in sucrose-containing veronal-buffered osmium tetroxide fixed and Epon-embedded tissue.[9,25]

Some consideration must also be given to the possibility that the particles, which we identify as glycogen, might be viral bodies. Bearcroft has shown virtually identical particles in liver cells of patients with viral hepatitis [44] and in monkeys infected with yellow fever,[45] and considered them to be the mature virus. The rosettes of particles were not surrounded by a membrane, showed no constant relation to the agranular reticulum, and were found usually in perinuclear locations. Somewhat similar, though less distinct structures have been demonstrated in human viral hepatitis by Braunsteiner, Fellinger, Pakesch and Neumayr.[46] Cossel,[47] however, showed that there is at least a suggestion of a membrane around some of the particles in human viral hepatitis and Gueft [48] demonstrated that the viroplasm was probably hexagonal in shape and that it probably possessed two outer membranes. Indeed, various other investigations of viral infections of the liver, such as the spontaneous infections of chick embryos,[49] infections with ectromelia,[50] equine abortion,[51] Rift Valley fever,[19] murine hepatitis [18,52,53] and cytomegalic inclusion disease [54] viruses and observations of the viruses of spontaneous hepatomas of mice [14] all indicate that the viruses are provided with at least one outer limiting membrane at some stage of development and that they have a more or less distinct internal structure. There is, on the basis of this evidence, considerable doubt that the rosettes demonstrated by Bearcroft [44,45] are indeed virus particles. This evidence also seems to indicate that the particles which we have demonstrated are not of viral origin.

The question of the significance of "glycogen bodies" and of the other concentric membranous arrays bear some consideration (Table I). Ribosome-free whorls of membranes have been seen in various normal cells,[26,59,66,67] though they have never been observed in normal liver cells. They have been described in various pathologically altered cells,[64,65] including liver cells.[1-9,11,16-19,50] Ribosome-bearing whorls have been also seen in various normal cells,[15,55-57] but never in normal liver cells. They have been also observed in several pathologically altered cells [15,20,58,60-63] and in altered liver cells.[3,10-15] It thus seems clear that the presence of either type of concentric array of membranes in liver cells is an indication of a pathologic alteration of the cell.

The presence of ribosome-studded whorls might be considered evidence of enhanced protein synthesis by cells, particularly since similar structures in spermatocytes of *Ascaris megalocephala* have been clearly linked to the elaboration of a protein secretory product.[55]

TABLE I

WHORLS OF PAIRED MEMBRANES AND OF PAIRED MEMBRANES WITH PARTICLES DEMONSTRATED IN CELLS BY ELECTRON MICROSCOPY

Species	Cells	Experimental conditions	Designation and description	Particulate component	Ref.
Chironomus larva	Salivary gland cells	Normal	Ergastoplasmic *Nebenkern; Nebenkern*	Presence of granules not clearly demonstrated	20
Ascaris megalocephala	Spermatocyte	Normal	Concentric pairs of ergastoplasmic membranes enclosing lipid and, later, proteinaceous material	Palade granules attached to membranes	55
Cambarus virilis (crayfish)	Spermatocyte	Normal	"Tubulate lamellar system"—ellipsoidal lamellar set intersects a number of straight lamellae	A few RNA-containing granules in the marginal region of the system	56
Spicula solidissima (surf clam)	Oocyte	Normal	Yolk nuclei resemble "fingerprints" with approximately circular cross sections	150 Å particles studded on membranes	57
Fowl	Rous No. 1 fowl sarcoma cells	Transplanted Rous sarcoma in breast muscle	Convoluted maze of ergastoplasm	Ribosomes attached to membranes	58
Mouse	Intermediate layer of transitional epithelium of urinary bladder	Normal	"Membranous whorls"; concentric membrane layers with core of small vesicles	Absent	59
Mouse	Peritoneal cells	Injection of sarcoma 37 cell into peritoneal cavity	Rings of elongated rough-surfaced cisternae of endoplasmic reticulum	Ribosomes on outer surface of limiting membranes not profuse	60
Rat	Pituitary cells	Estrogen-induced adenoma	Ergastoplasmic *Nebenkern*	Granules attached to membranes at periphery of whorls	20
Rat	Sympathetic ganglion cells	Normal	"Onion-like corpuscles"; whorls of shallow cisternae more loosely packed at periphery than center	Absent	26

Mouse	Pancreatic acinar cells	Fasting followed by refeeding	Cytoplasmic "formation centers"; whorl-like, onion-layered "granular membranes"	Not clearly stated	61
Rat	Pancreatic acinar cells	DL-ethionine intoxication	"Osmiophilic whorls"; whorls of ergastoplasmic membranes, denser than normal	Heavily stained ribosomes attached to membranes	62
Rat	Pancreatic acinar cells	DL-ethionine intoxication; recovery phase	"Whorls of regenerating ergastoplasm"	RNP particles attached to membranes	63
Guinea pig	Germinal epithelial cells of testis	Normal and necrosis *in vitro*	"Elaborate concentric systems" resembling structures that have often been interpreted as "intracellular myelin figures"	Small granules presumed to be ribonucleoprotein adhere to membranes	15
Mouse	Argyrophil cells of gastric mucosa	36 hours fasting and 12 hours water deprivation	"Large systems of membranes"	Absent	64
Mouse	Ehrlich ascites tumor cells	Injection, Newcastle disease virus, peritoneal cavity of tumor-bearing mice	"Inclusions" elliptical or circular in profile; concentric lamellar systems	No particles attached to membranes; particles in center of whorls may be subunits of virus or ribosomes	65
Cat Guinea pig Hamster	Adrenal cortical cells	Normal	Lamellar complexes forming myelin figures or onion ("fingerprint") images of microspirals or myelin figures	RNP particles are absent; ferritin molecules found on membranes	66
Macaque monkey	Presumably oxyphil cells of parathyroid	Normal	Whorl-like structures consist of laminated agranular membrane pairs concentrically disposed about a central core	Absent	67
Mouse	Liver cells	Viral hepatitis (MHV 3)	Concentric laminae of smooth endoplasmic reticulum	Absent	18
Mouse	Liver cells	Rift Valley fever	Concentric lamellae of paired membranes with regular banding . . . within cisternae	Absent	19

(*Table I continued on next page*)

(*Table 1 continued*)

Species	Cells	Experimental conditions	Designation and description	Particulate component	Ref.
Man	Liver cells	Viral hepatitis	*Masses feuilletées agranulaires;* masses of smooth membranes replace cytoplasm	Ribosomes rarely on surface of membranes	16, 17
Mouse	Liver cells	Mouse pox virus hepatitis (ectromelia virus)	Moribund or necrotic cells contain "whorls of membranes"	Not stated	50
Rat	Liver cells	Alpha-naphthyl isothiocyanate intoxication	Smooth-surfaced "fingerprint" arrays	Absent	9
Rat	Liver cells	Dimethyl-nitrosamine intoxication	(1) Pairs of membranes form concentric arrays. (2) Elaborate masses of double membranes form "fingerprints," myelin figure or ellipsoidal bodies	(1) RNP granules adhering to external surface of membranes. (2) Absent	3
Rat	Liver cells	Dimethyl-nitrosamine intoxication	*Nebenkern*—sponge-like core, surrounded by a continuous shell of several membranes closely packed together	Absent	1, 2
Rat	Liver cells	Thiocetamide intoxication	*Anneaux ergastoplasmiques complets*—single cell of smooth endoplasmic reticulum. *Impreintes digitales*—concentric whorl of smooth-surfaced membranes	Glycogen particles in hyaloplasm	5
Rat	Liver cells	Thiocetamide intoxication	*Enroulements de membranes lisses*	Absent	4
Rat	Liver cells	Thiocetamide intoxication	*Zytoplasmatische Wirbelbildungen* or *RNP-arme bzw. Freie Glatte Nebenkern;* large concentric membrane complexes	Absent	7
Rat	Liver cells	Thiocetamide intoxication	*Geschichtete oder Gebündelte Membrancomplexe;* concentric membranes like locks of hair. Even spacing of membranes in chronic, uneven in acute intoxication	Absent	6

Rat	Liver cells	Thiocetamide intoxication	"Fingerprints" or *Nebenkern* of concentric membranes	Not stated whether particles present	8
Rat	Liver cells	DL-ethionine intoxication	Inclusion body consisting of whorl of paired, smooth-surfaced membranes	Some dense particles between adjacent membranes	10
Rat	Liver cells	Carcinoma induced by p-amino-azotoluene	*Bulbes d'oignons*—pallisaded ergastroplasmic membrane arrays	RNP particles on membranes	13
Mouse	Liver cells	Spontaneous hepatoma	Spiral whorls of endoplasmic reticulum around viral particles	Ribosomes attached to membranes in whorls	14
Guinea pig	Liver cells	Diphtheria toxin	*Wirbelbildungen*—concentric arrays of membranes	Absent	11
Man	Liver cells	Hepatoma	*Nebenkern*—concentric arrays of lamellae	Ribosomes attached to membranes in whorls	12

The particle-free "fingerprints" may be regarded as evidence of regeneration of the ergastoplasm. This view of Benedetti and Emmelot,[1] based upon observations of Fawcett and Ito,[15] holds that this type of "fingerprint" is "a reaction to injury rather than degeneration." On the other hand Albot and Jézéquel[16,17] suggested that the particle-free "fingerprint" is a form of degeneration of the ergastoplasm, which they designated "acidophilic necrosis." This interpretation can be justified on the basis of findings of others[9,41,68] that the aggregation of agranular reticulum in cells enhances cytoplasmic eosinophilia, although it is not at all clear whether the tinctorial change is not simply a reflection of the loss of ribosomal proteins.

Another interpretation is to regard the agranular "fingerprints" as part of a *de novo* hypertrophy of the smooth-surfaced endoplasmic reticulum.[6,7] If this is the case, then it would seem likely that it might indicate an increased metabolic activity of cells, since this form of the endoplasmic reticulum has been linked to various metabolic processes of cells.[43] There is no morphologic confirmation of this assumption, since no structurally identifiable metabolite has been recognized in relation to these membrane complexes. The finding of glycogen particles in relation to some of the ribosome-free arrays in our experiment would support the view that at least some of the ribosome-free fingerprints might be engaged in active anabolic processes. In the case of ethionine poisoning, the "glycogen bodies" might therefore indicate the development of a specific metabolic function in liver cells.

We based our investigation upon the premise that the metamorphosis of normal to neoplastic cells is more likely to occur in few rather than in many cells. Hence it seemed expedient to search for alterations of liver cells which were rare, which could be interpreted as probably "regenerative" rather than "degenerative," and which were at the same time unique and distinct when compared with the changes induced by noncarcinogenic chemicals. Our own preliminary observations indicate that most of the alterations which occur in hepatocytes of animals maintained for a prolonged period of time on an ethionine-supplemented diet are aptly labeled as "degenerative" in that they are likely to be associated with a profound disturbance of cellular metabolism. These alterations are nonspecific since they have been induced by various other forms of insult, all of which either render the existence of liver cells precarious or actually place their very survival in jeopardy.

Depletion of glycogen is one example of such a nonspecific injury of liver cells. However, the capacity of liver cells to store glycogen seems to play a significant, and perhaps a more specific role in one phase of ethionine carcinogenesis. Initially, as with all other hepatic carcinogens,

there is a marked reduction or a total loss of the ability of parenchymal liver cells to store glycogen. Later, the development of nodular hyperplasia (regenerative hyperplasia) is associated with a return of the glycogen-storing capacity in some cells. In the hyperplastic nodules large vacuolated cells with an eosinophilic cytoplasm are filled with glycogen, although those in the surrounding liver are almost totally devoid of it. Although some of the hyperplastic nodules are reversible, others are irreversible, even if ethionine is removed from the diet, or its effects are counteracted by the addition of methionine to the diet. The irreversible nodule which cannot be distinguished morphologically from the reversible one is apparently the last recognizable distinctive non-neoplastic morphologic lesion in the histogenetic sequence terminating in neoplasm. This does not necessarily mean that neoplasms actually arise from these nodules, although there is strong suggestive evidence that this is the case.[69,70]

In studying the sequential ultrastructural changes which precede the development of the hyperplastic nodule, we have observed the early abrupt glycogen depletion and the subsequent failure of replenishment of glycogen stores which can be readily seen by light microscopy and which persists for about 4 weeks. After this time, and prior to the appearance of recognizable hyperplastic nodules, a very few cells can be found by light and electron microscopy which apparently resume their glycogen storage function. We are, of course, uncertain whether this occurs in cells which will be destined to initiate or participate in the growth of the hyperplastic nodules. It seems intriguing, nevertheless, that the resumption of glycogen storage occurs only in very few cells and that at a later date this aptitude is so prominent a feature of some cells which constitute the hyperplastic nodule, at which time the glycogen-storing activity remains in abeyance in liver cells not involved in the nodular hyperplasia. Whether this finding is meaningful in terms of the normal to neoplastic cell transformation must await further study of this phenomenon.

Porter and Bruni [41] in their excellent study of the earliest phase of 3′-Me-DAB carcinogenesis came to the conclusion that the dye or its derivative destroy liver cells which have differentiated to the level where they store glycogen. "Hence, in any population of new liver cells arising in the process of regeneration and repair, those that mature will in time be destroyed. Only cells which, through mutation, lose the normal tendency to differentiate for glycogenesis will survive and so will be selected out for continued growth and proliferation." [41] It will be clear from the above discussion of our experiments that our suggestions seem to be diametrically opposed to those of Porter and Bruni.[41]

Summary

The liver cells of rats, maintained on a normal diet supplemented with 0.5 per cent DL-ethionine in their water supply, undergo a variety of nonspecific alterations during the first 4 weeks. Glycogen storage ceases almost completely and the lobular architecture becomes severely disorganized as a result of proliferation of ductular cells. Glycogen storage is resumed in a very few cells after the fourth week. The usual intracellular "glycogen areas" are not found. Instead, glycogen particles occur always in intimate relationship to the agranular reticulum. Some cells develop complex glycogen-membrane arrays which we have referred to as "glycogen bodies" on the basis of a 3-dimensional reconstruction. These arrays are unique and have to be distinguished from other forms of membranous or of membrane-ribosome whorls which are devoid of glycogen. Since the latter arrays have been found in conditions other than after the administration of carcinogens, they are regarded as nonspecific evidence of cell injury.

The resumption of glycogen storage by a few cells, observed between the 32nd and 105th day of ethionine feeding, subsequently becomes a prominent feature of some cells which participate in the formation of hyperplastic nodules. The glycogen storing capacity remains in abeyance in cells outside the nodules. There is strong presumptive evidence that neoplasms arise exclusively within the hyperplastic nodules. Hence reactivation of the glycogen-storing ability in spite of continuing exposure to the carcinogen might indicate that some liver cells acquire a "resistance" to its action, and that such cells, by their participation in nodular hyperplasia, might play an important role in the neoplastic transformation.

Addendum

Since submitting this paper for publication we have noted that Clementi [71] described single or multiple "fingerprints" (*impronta digitale, immagini a vortice*) which were devoid of any particles, in 25 per cent of liver cells in ethionine-fed rats. It is not clear at what stage of the process these structures developed.

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LEGENDS FOR FIGURES

Unless otherwise stated, micrographs were prepared from sections stained with lead hydroxide. One μ scale is indicated by the line in the right lower corner of the micrographs.

FIG. 1. A perinuclear store of glycogen found in a parenchymal liver cell on the 32nd day of the experiment. Glycogen (GL) appears mainly in the form of rosettes of particles although occasional solitary particles are also seen. The entire area is permeated by haphazardly distributed vesicular profiles of the agranular (smooth-surfaced) endoplasmic reticulum (AR). GR, granular (rough-surfaced) endoplasmic reticulum; CYT, cytosome; MT, mitochondrion; NE, nuclear envelope. × 18,000.

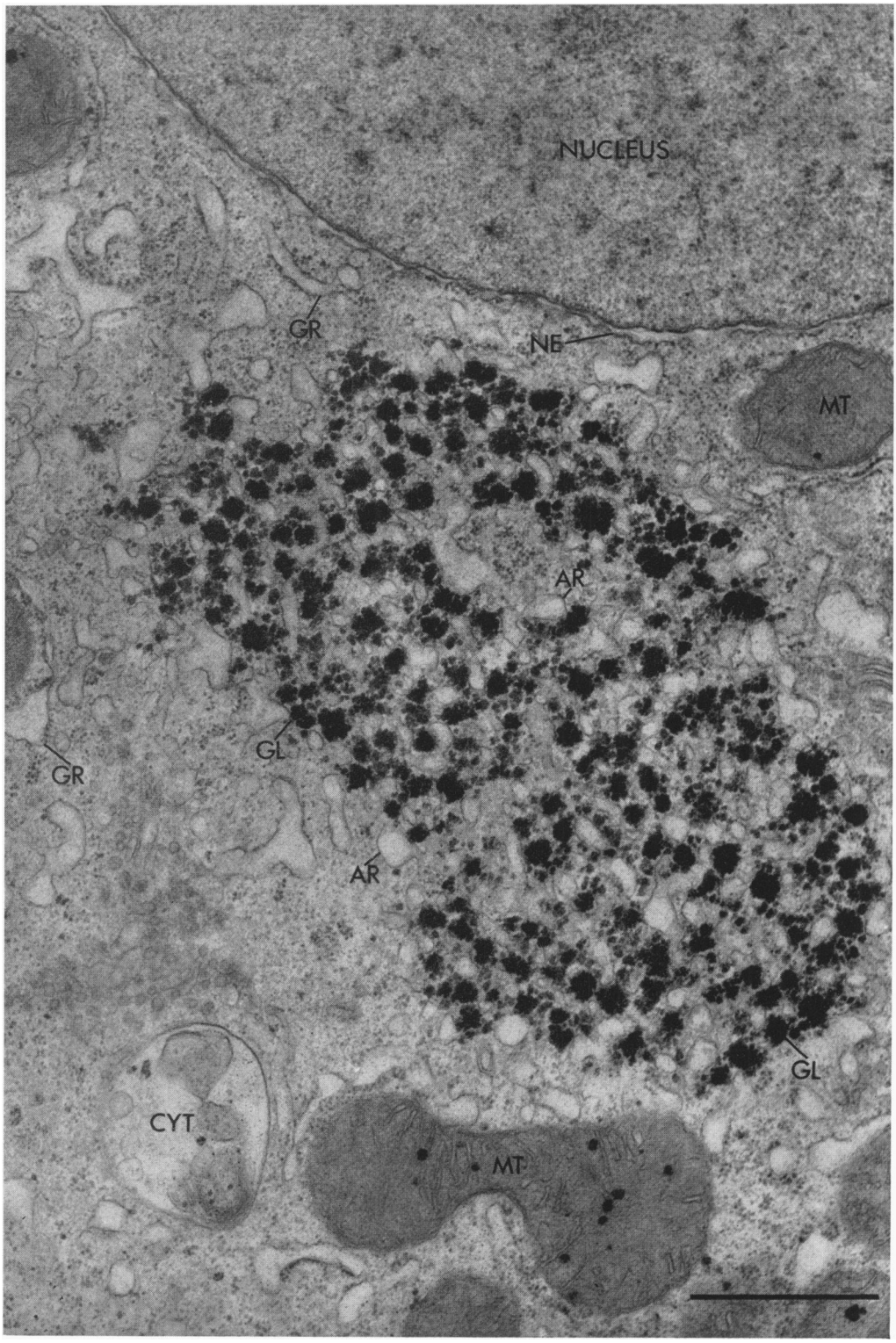
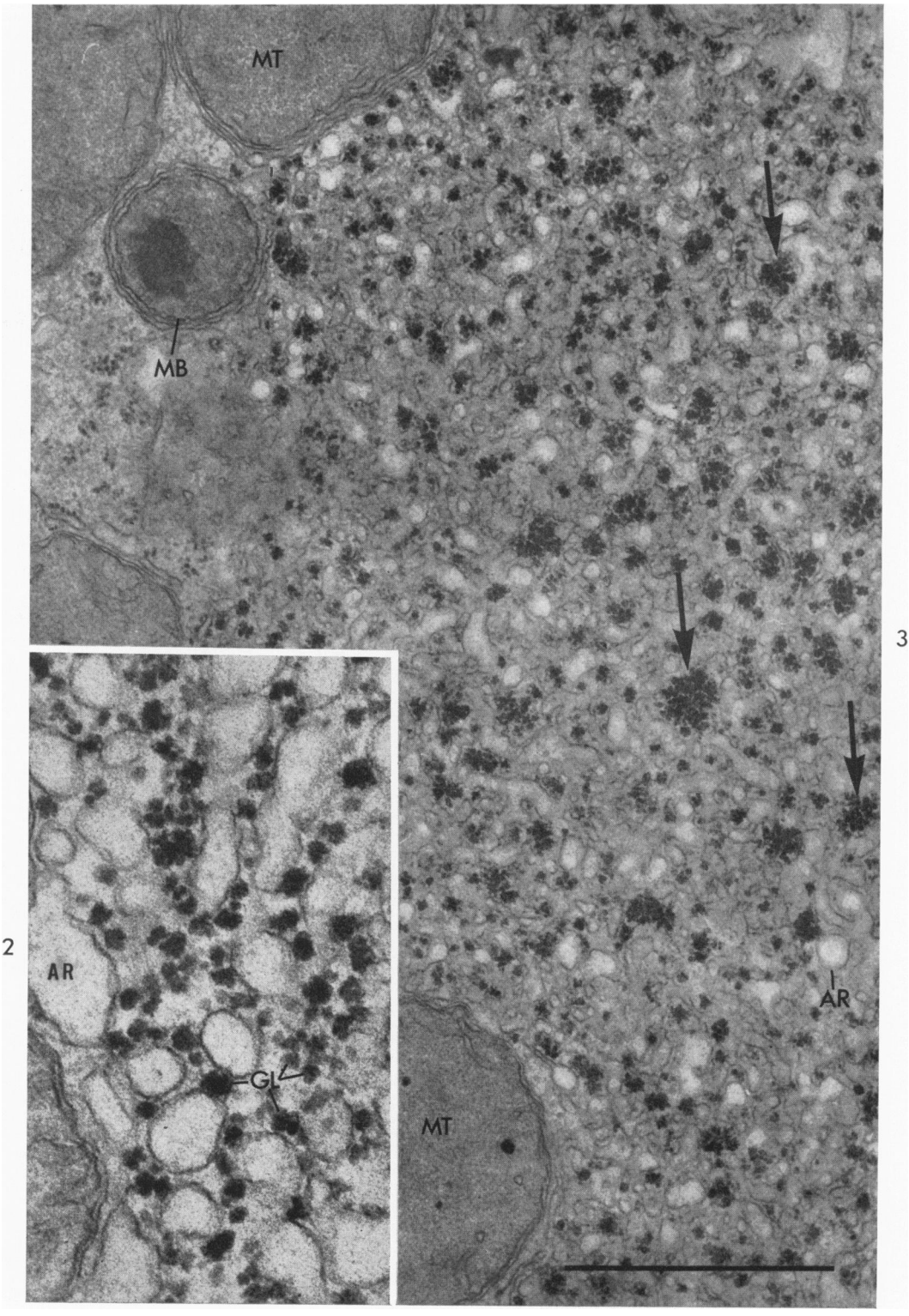

NUCLEUS
GR
NE
MT
AR
GL
GR
AR
GL
CYT
MT

FIG. 2. The particles in one of the glycogen-membrane arrays, similar to one seen in Figure 6 at lower magnification. There is uneven "staining" of the particles of glycogen (GL) and occasional aggregation into rosettes. AR, agranular reticulum. × 71,680.

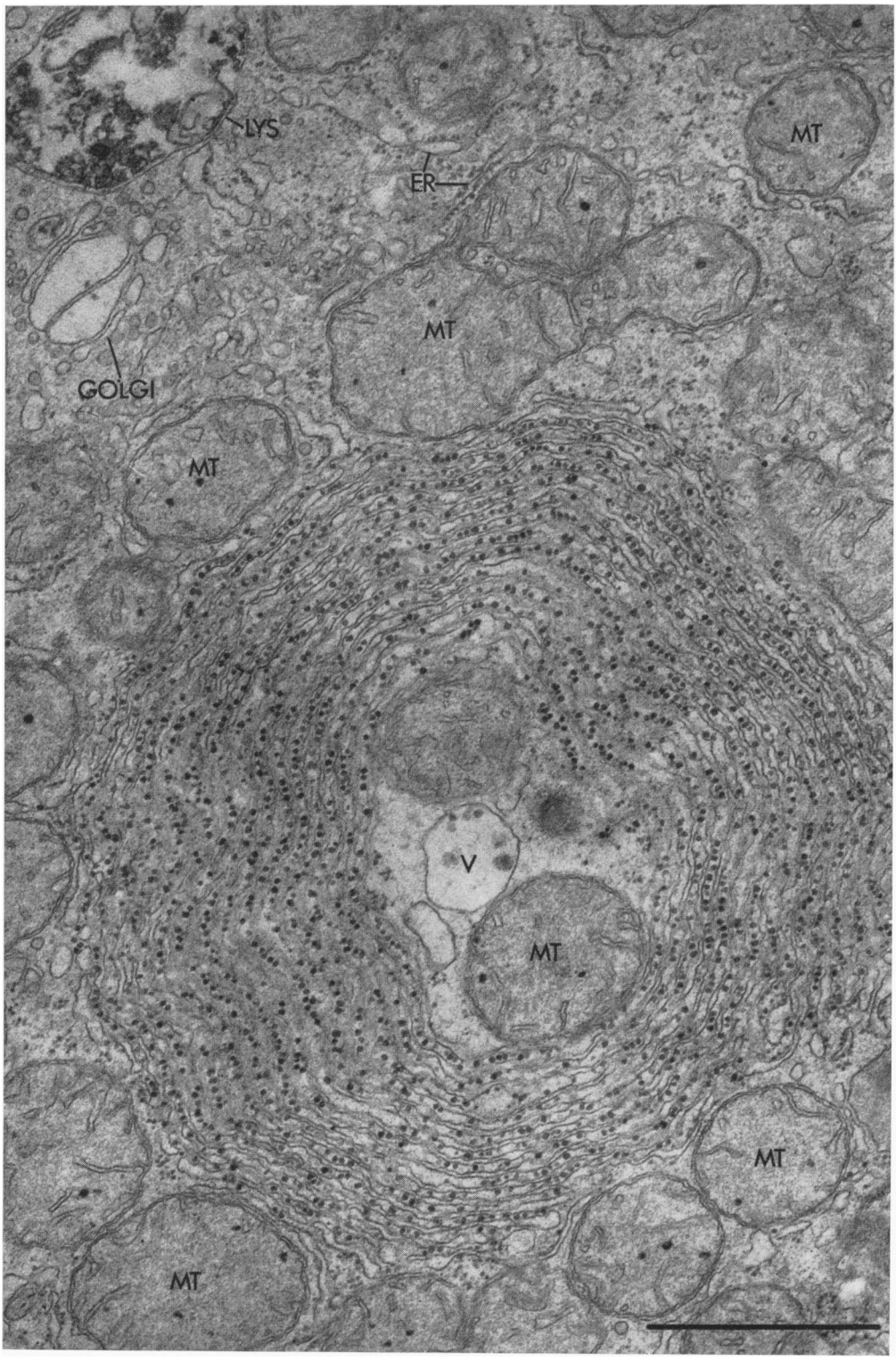
FIG. 3. A perinuclear glycogen store permeated by vesicles of agranular reticulum (AR). Relatively large rosettes of particles (heavy arrows) are shown. Compare the marked electron opacity of the glycogen particles with the relatively less opaque ribosomes seen at the left upper margin of the micrograph immediately adjacent to the microbody (MB). MT, mitochondria. × 39,690.



2

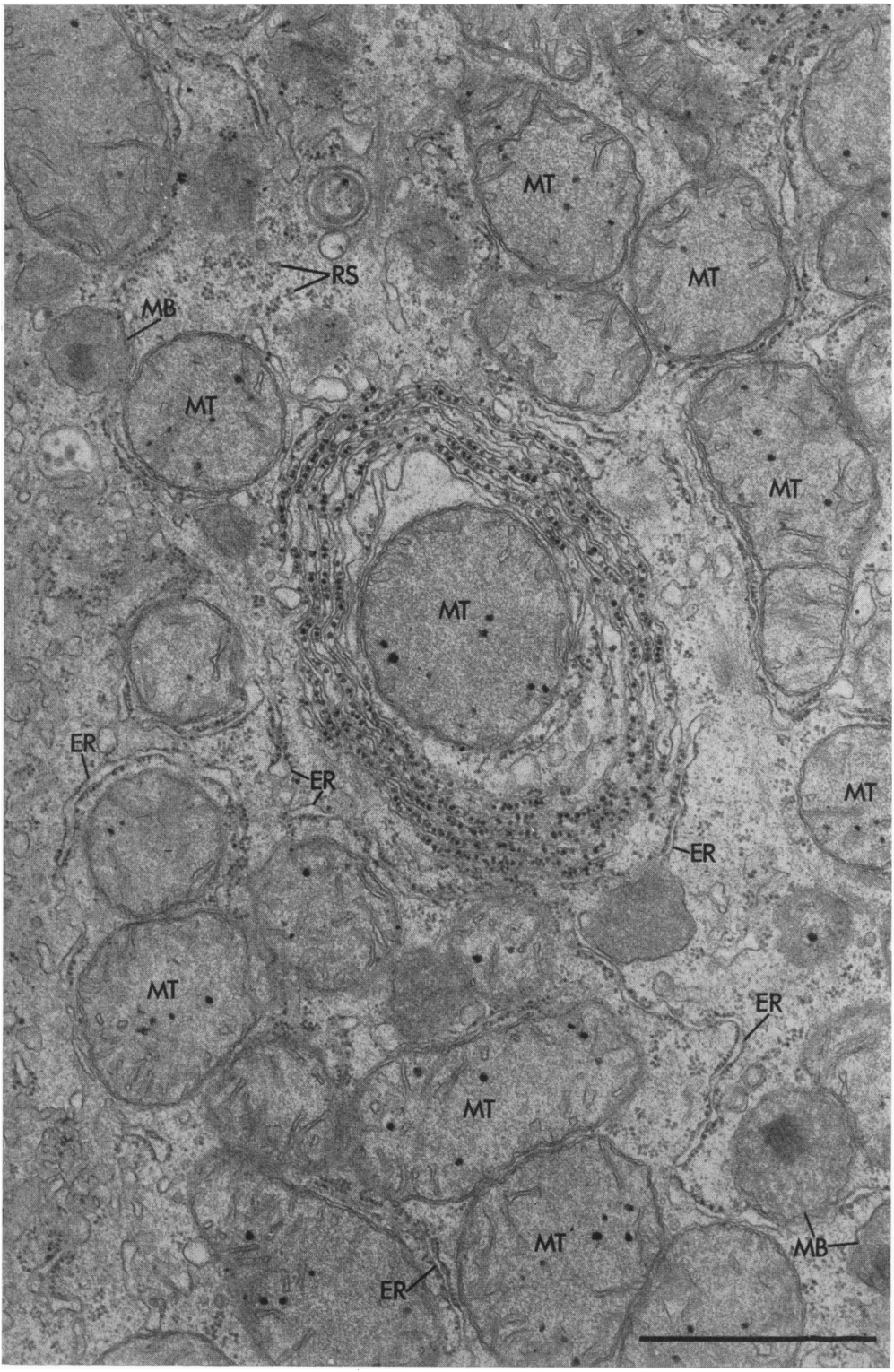
3

FIG. 4. A large glycogen-studded "fingerprint" occupies a part of the cytoplasm. The epicenter contains a core of hyaloplasm, a mitochondrion (MT) and vesicles (V). Most of the glycogen particles are aligned in single rows and show little tendency to aggregate in rosettes. This figure corresponds to cross section "A" in Text-figure 1, and probably corresponds to a cross section of the umbrella of the medusa depicted in Text-figure 2. LYS, lysosome; ER, rough-surfaced endoplasmic reticulum. × 32,480.



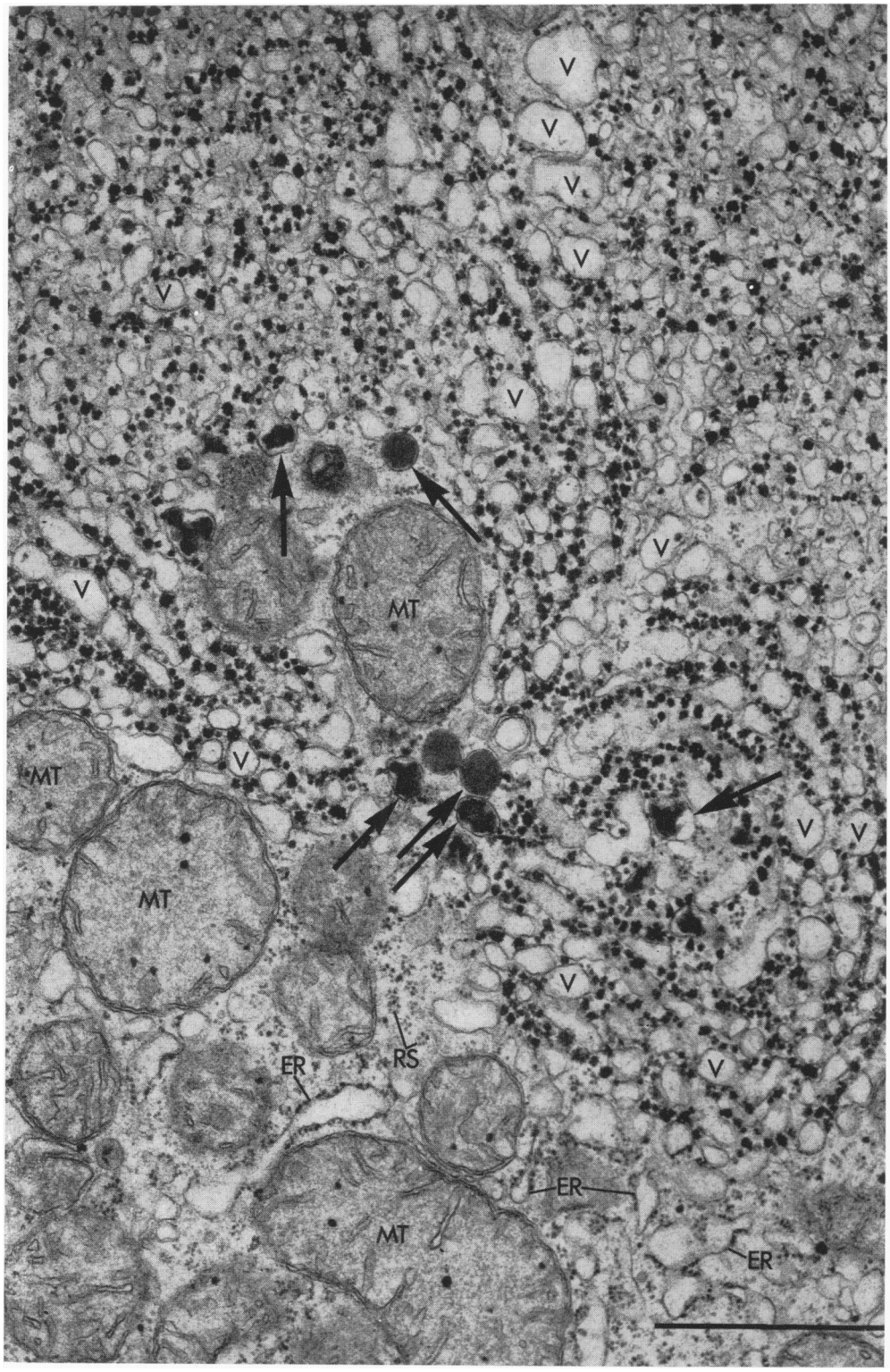
4

FIG. 5. A glycogen-studded "fingerprint" occupies a small portion of the cytoplasm. A mitochondrion (MT) is enclosed in its central part. This "fingerprint" corresponds to cross section "B" in Text-figure 1, and probably corresponds to the area of transition from umbrella to tentacles in Text-figure 2. There is marked electron opacity of the glycogen particles when compared with ribosomes both free (RS) and attached to membranes of the rough-surfaced endoplasmic reticulum (ER). MB, microbody. × 34,160.



5

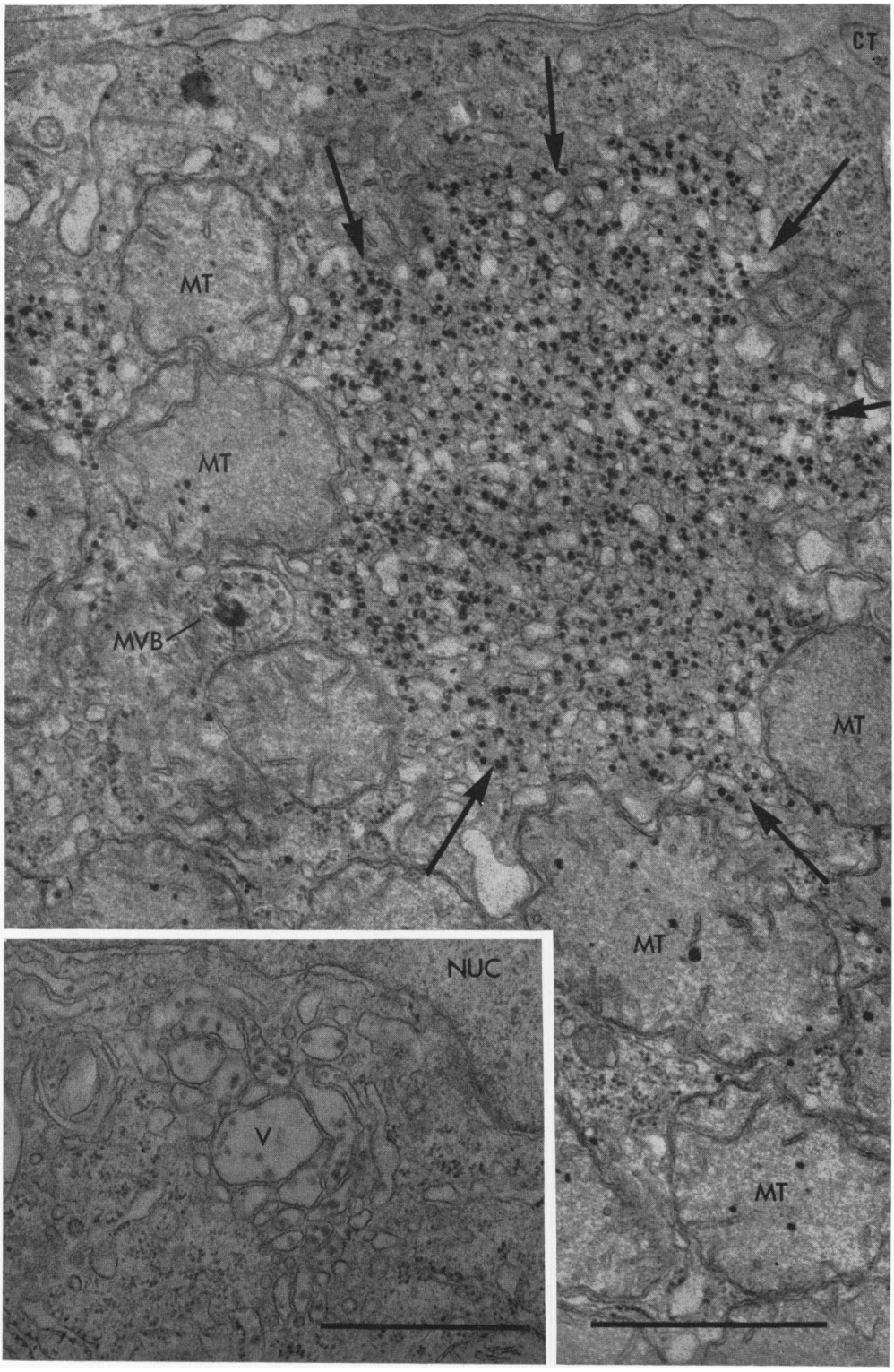
FIG. 6. An "unpolarized membrane-particle array" occupies part of the cytoplasm of a parenchymal liver cell. This corresponds to cross section "C" in Text-figure 1, and probably to a cross section of the upper reaches of the tentacles in Text-figure 2. The vesicular profiles (V) of the agranular reticulum show a tendency to become aligned in a circular pattern, encompassing some mitochondria. The heavy arrows point to vesicles which contain dense particles in their lumens. Free (RS) and membrane-bound ribosomes (ER) are small in comparison with the glycogen particles in the array. MT, mitochondria. × 34,160.



6

FIG. 7. An "unpolarized membrane-particle array," the circumference of which is outlined by heavy arrows, occupies a peripheral location in the cytoplasm. This area corresponds to cross section "D" in Text-figure 1, and probably corresponds to a section through the free ends of the tentacles of the medusa-like "glycogen body" depicted in Text-figure 2. Most of the particles are solitary rather than aggregated into rosettes. CT, connective tissue; MVB, microvesicular body; MT, mitochondria. × 34,160.

FIG. 8a. A perinuclear Golgi zone shows the presence of dense particles in the cisternae (V). The particles can be clearly distinguished from glycogen particles since they retain their electron opacity in this "stain." NC, nucleus. Uranyl acetate. × 30,780.

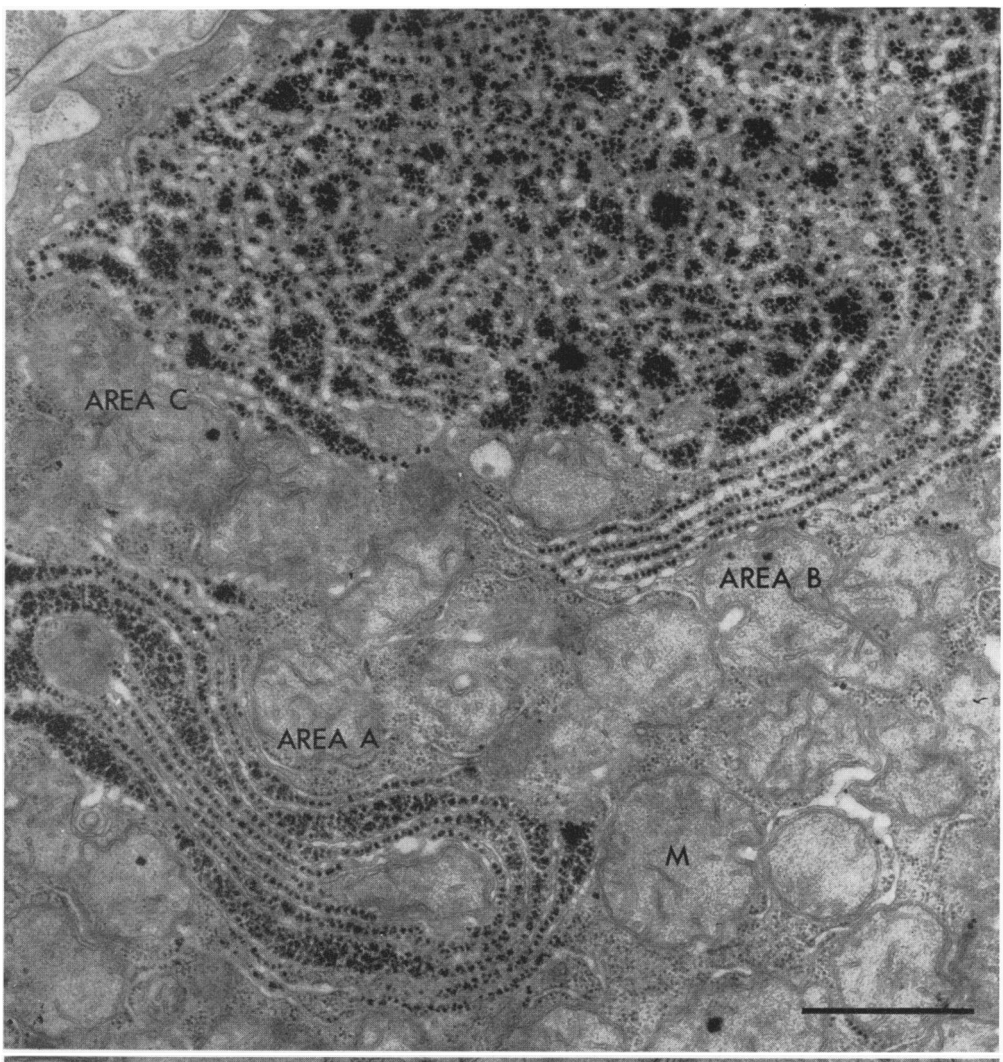


7

8a

FIG. 8. The periphery of a parenchymal liver cell is seen in the left upper corner. Area "A" of the cytoplasm is occupied by a glycogen-studded "fingerprint" which is sectioned partly tangentially and consequently the glycogen particles between the paired membranes appear more numerous in some areas. Area "B" shows part of a "fingerprint" whereas area "C" shows a "nonpolarized membrane-particle array." These multiple cross sections have led us to the supposition that they form part of a single "glycogen body." M, mitochondrion. × 22,400.

FIG. 9. An incomplete horseshoe-shaped "fingerprint" lies between closely packed mitochondria (M). × 25,200.



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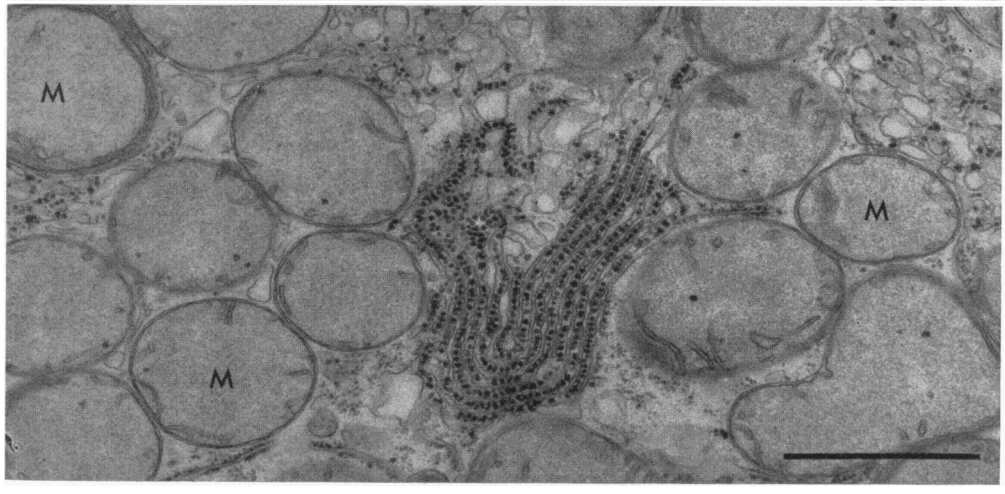

9

FIG. 10. Higher magnification of a "fingerprint" similar to those depicted in Figures 4 and 5. The epicenter of the body is occupied by a mitochondrion (MT). Glycogen particles (GL) exhibit uneven "staining" intensity. The arrows point to ribosomes attached to membranes bounding cisternae of the endoplasmic reticulum. Continuities between the ER and the glycogen-studded agranular reticulum (AR) can be seen in the lower center of the micrograph. The glycogen particles in the "fingerprint" show little tendency to aggregate in rosettes. × 49,410.

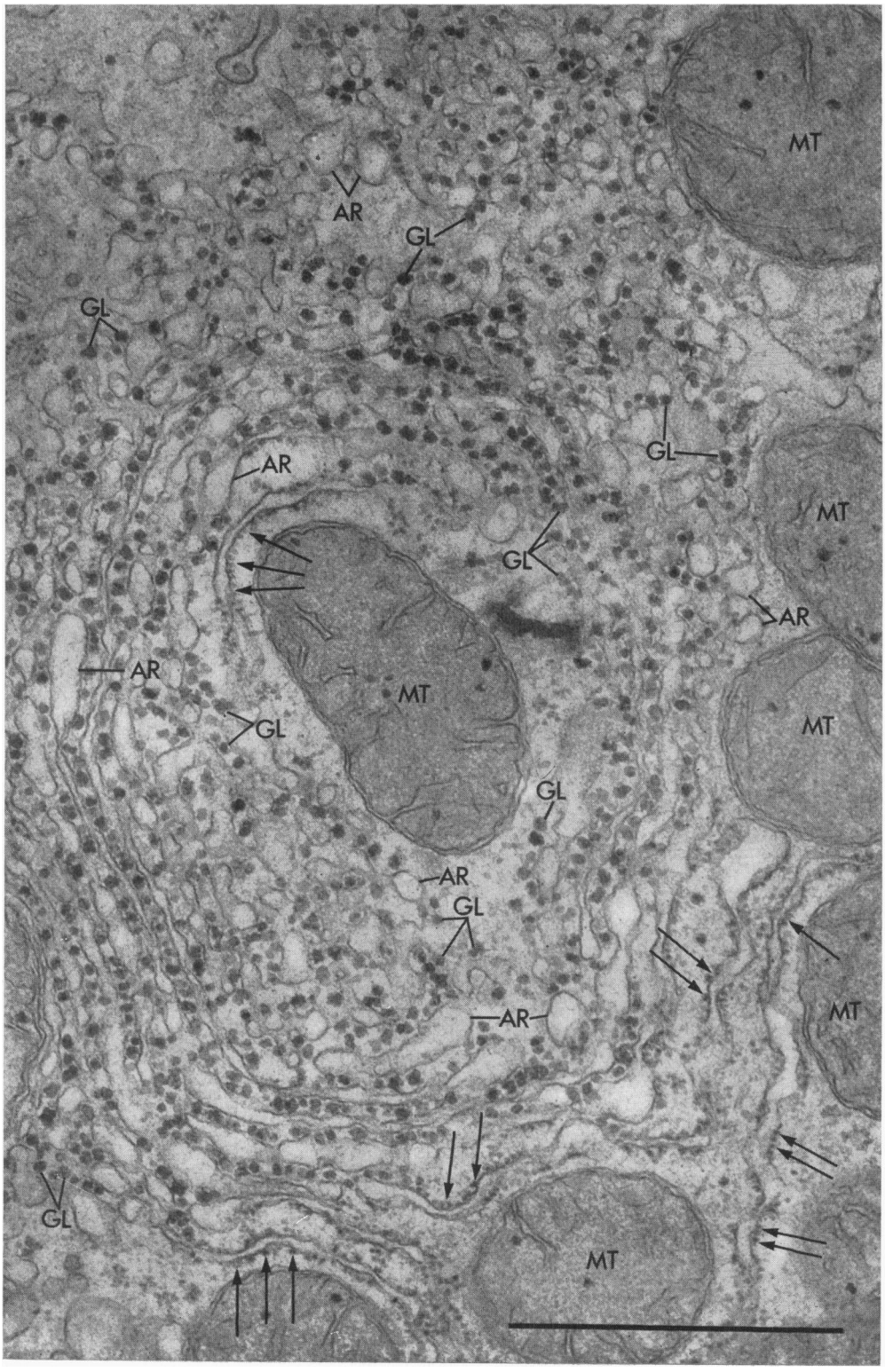

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FIG. 11. A "fingerprint" occupies part of the cytoplasm of a liver cell. In its epicenter lie 3 mitochondria (M) and numerous mitochondria surround the periphery of the "fingerprint." The heavy arrows point to "glycogen holes" which result from the "negative staining" of glycogen particles with this "stain." V, vacuole. Uranyl acetate. × 34,160.

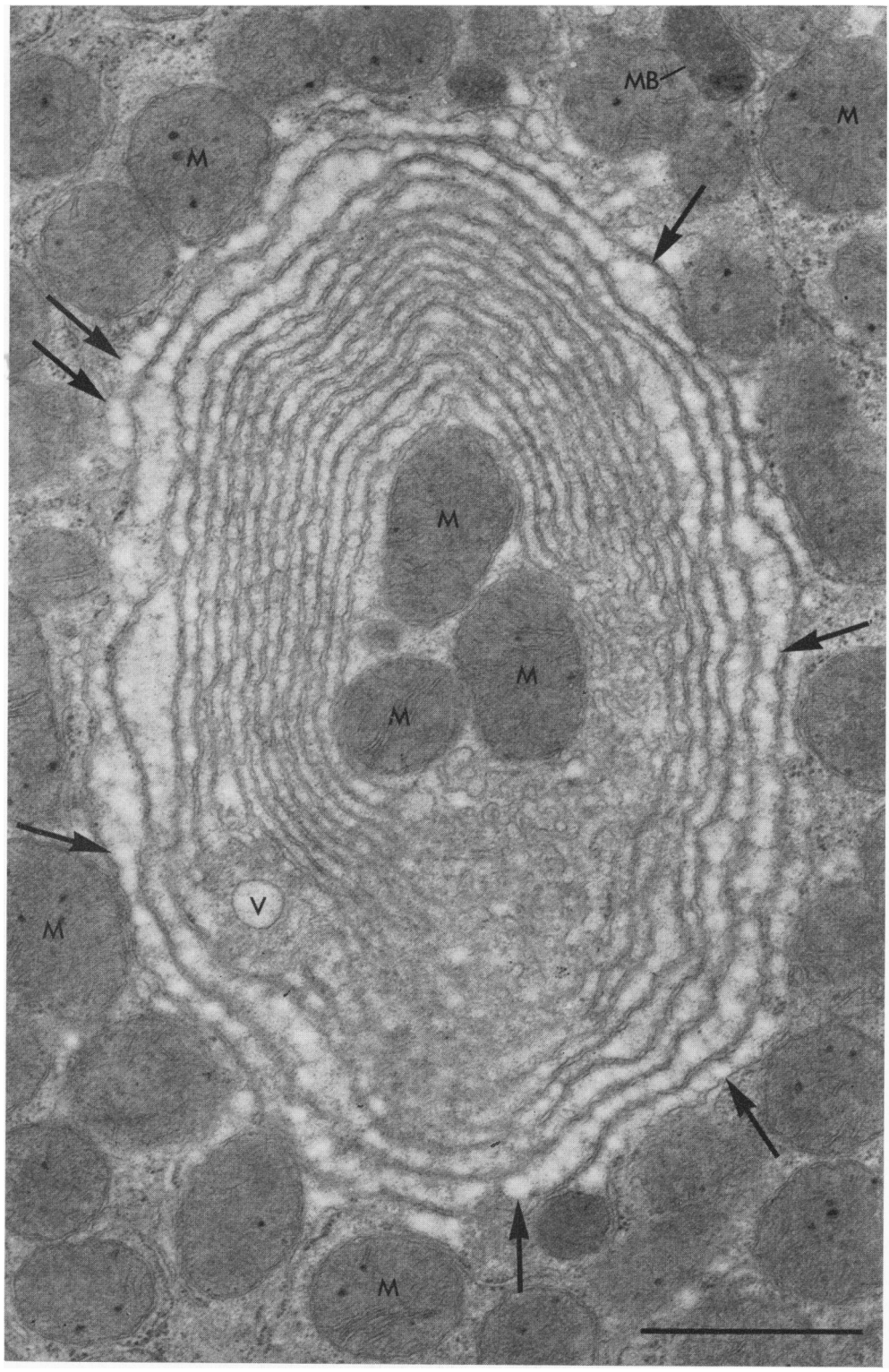

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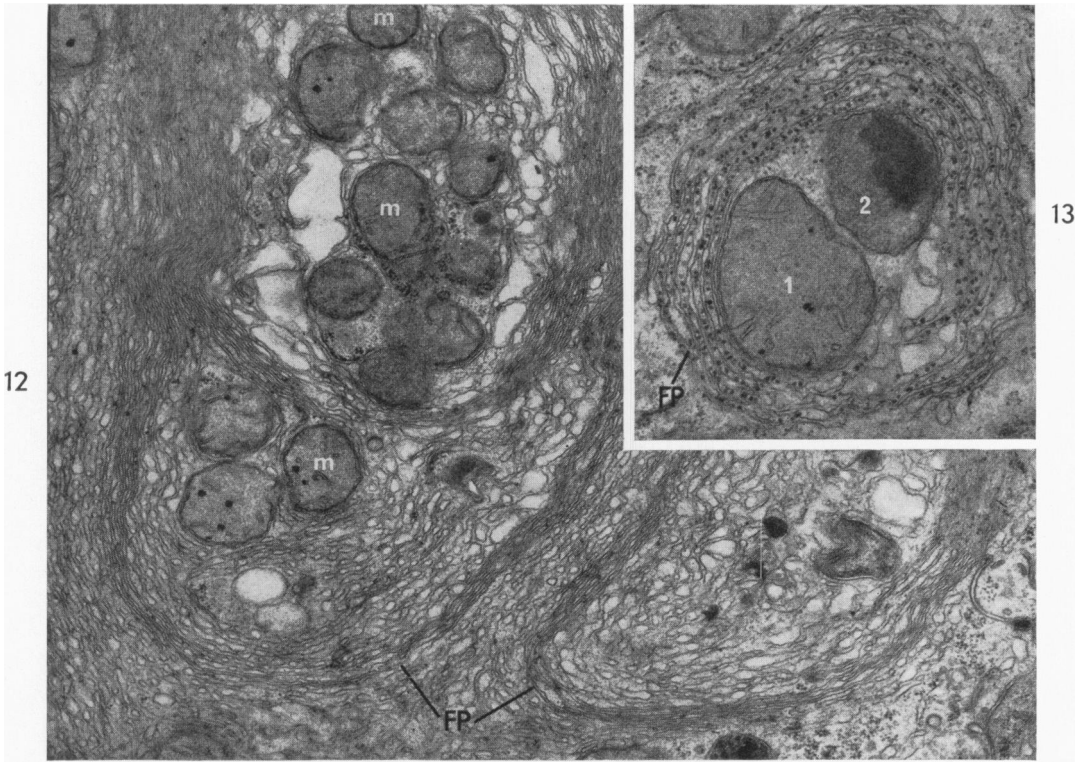
FIG. 12. Two adjacent particle-free "fingerprints" (FP, "ellipsoidal bodies") occupy part of the cytoplasm of a parenchymal liver cell. Numerous mitochondria (m) are enclosed within the epicenter of the "fingerprint" on the left. × 17,920.

FIG. 13. A glycogen-studded "fingerprint" (FP) encloses a mitochondrion (1) and a microbody (2) as well as several vacuoles in its epicenter. × 22,400.

FIG. 14. A tangential section through a "glycogen body" is seen. In this fashion a compound image of a "fingerprint" and of a "nonpolarized membrane-particle array" is produced. cm, cell membrane; m, mitochondrion. × 13,300.



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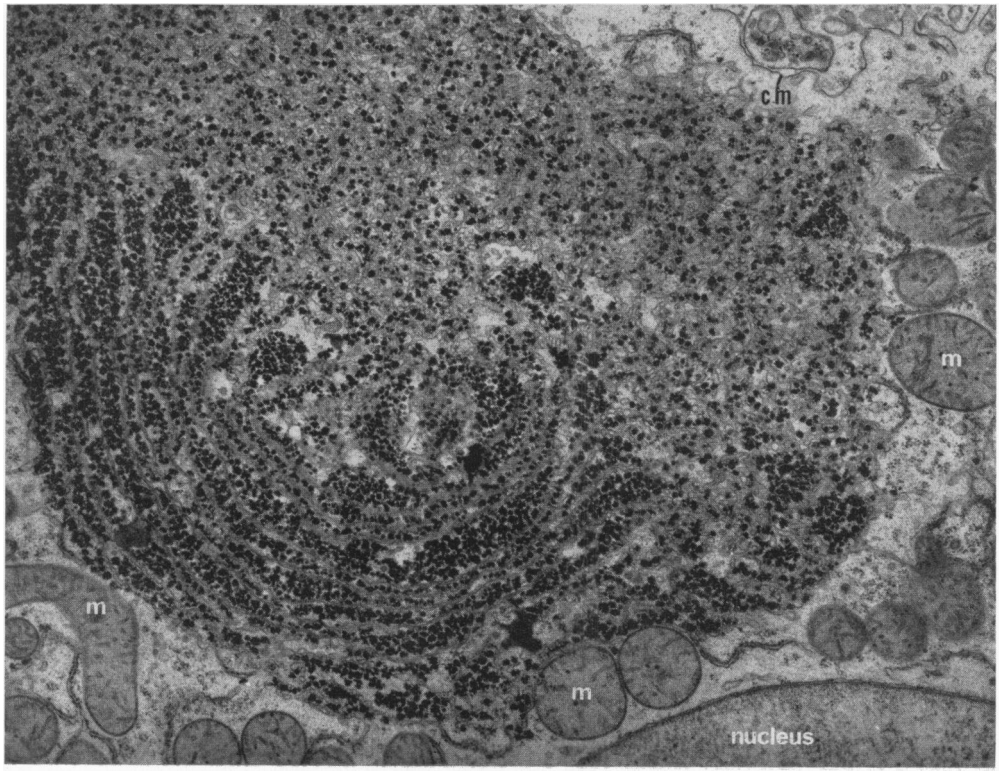

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FIG. 15. The heavy arrows point to two adjacent particle-studded "fingerprints." The particles here are identical in size with ribosomes which are found randomly scattered in the hyaloplasm. Mitochondria (m) are strikingly elongated and curved around the outside or the inside of the "fingerprints." There is extreme prominence of the intramitochondrial opaque granules. × 25,200.

FIG. 16. Within the cytoplasm of a liver cell are seen two particle-free "fingerprints." The membranes which constitute them are not paired and are rather undulating. Arrow "1" points to a "fingerprint" enclosing mitochondria and arrow "2" to one enclosing a single microbody. These structures probably constitute areas of "focal cytoplasmic degradation." m, mitochondria; er, endoplasmic reticulum. × 24,300.

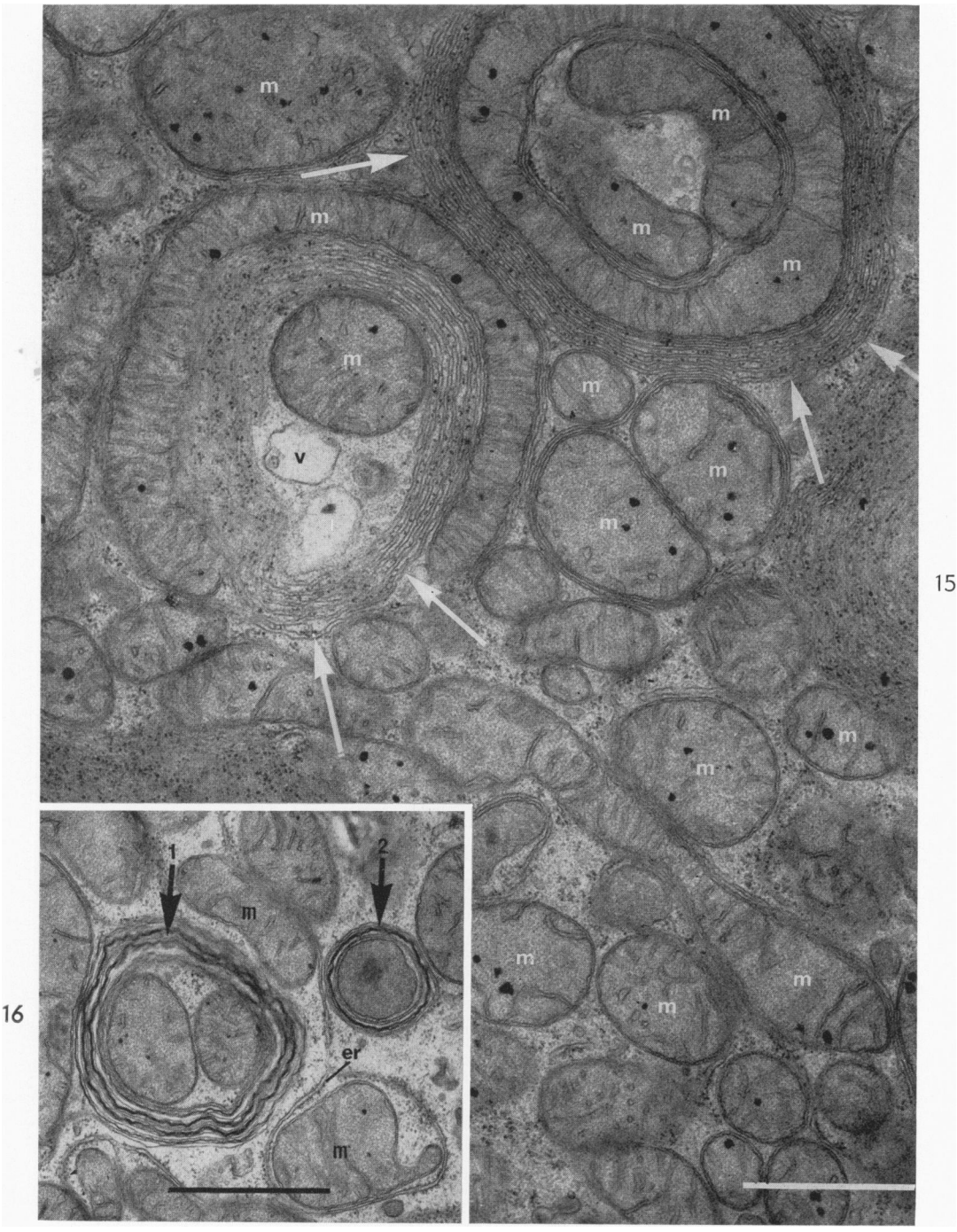

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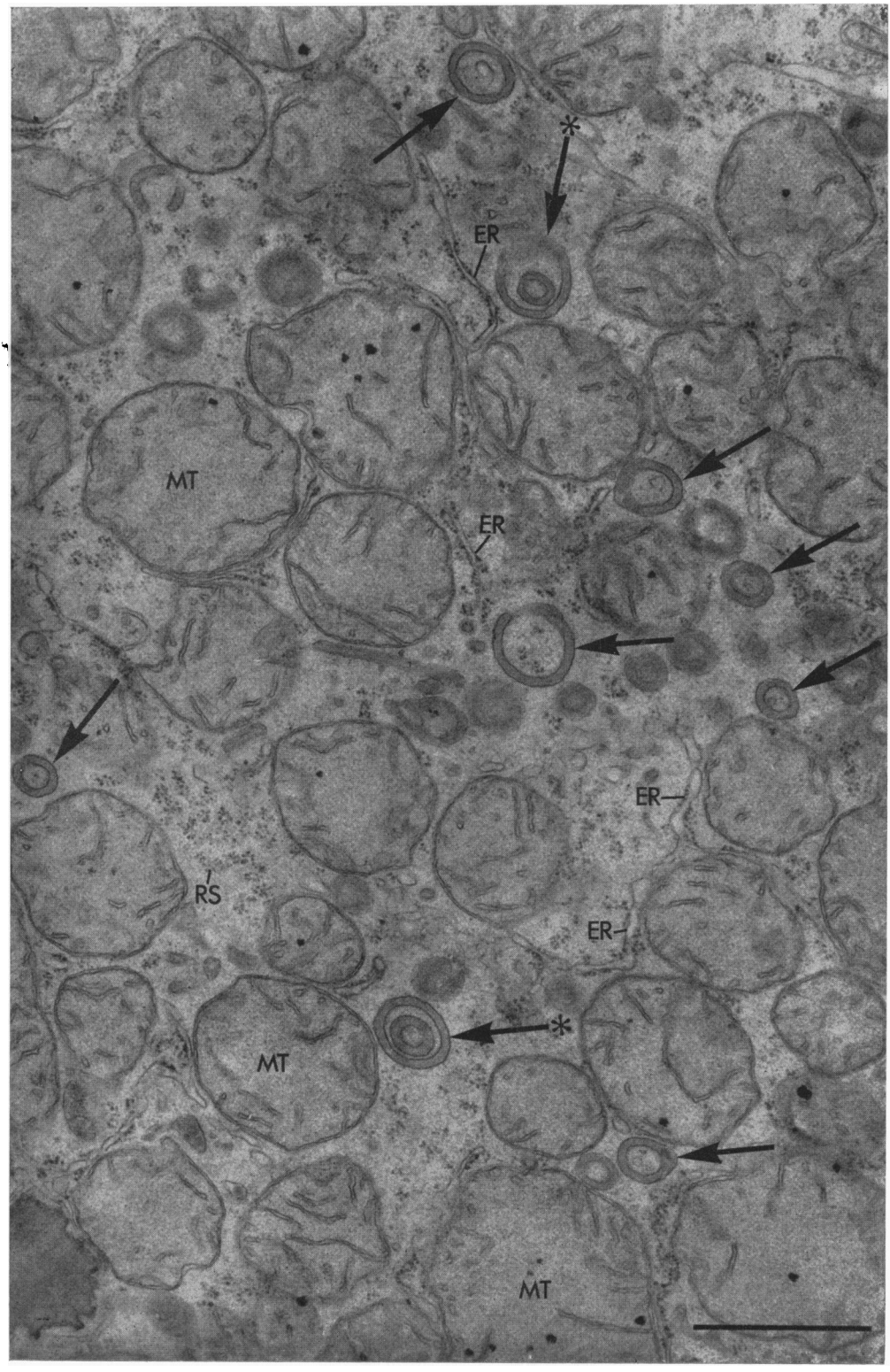
FIG. 17. The cytoplasm of a parenchymal liver cell contains several ring-shaped profiles of parallel membranes separated by an intervening dense zone (heavy arrows). The arrows marked with an asterisk indicate the rarely observed concentric layering of such ring structures. MT, mitochondria; ER, endoplasmic reticulum; RS, ribosomes. × 26,880.



17