Iron Metabolism and Cell Membranes

II. The Relationship of Ferritin to the Cytocavitary Network in Rat Hepatic Parenchymal Cells

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THE MAMMALIAN LIVER CELL represents an important storage site for non-heme iron. The most important storage form is iron hydroxyphosphate micelles, often combined with protein. One such protein, apoferritin, is well known and combines with iron to form the iron-protein complex, ferritin.[1,2] During periods of iron depletion, iron can be rapidly released from the liver storage sites and returned to the blood stream.[1,3] The subcellular pathways involved in iron uptake, storage, and release are essentially unknown.

It is, however, known that the iron-containing macromolecules are concentrated in the storage phase within the lysosomes of hepatic cells.[4,5] It is not known whether this represents the primary storage site from which iron is again released to the blood or whether this lysosome storage site represents an "end" stage containing iron which is destined only for excretion to the bile duct. Moreover, there are no data regarding the manner in which ferritin gains access to the lysosomes.

The present paper is concerned with the mechanisms of ferritin incorporation into the lysosome system. This has important implications for the understanding of iron metabolism in the liver and of the physiology of the endoplasmic reticulum-Golgi-lysosome pathway. As discussed below, these studies have led us to the formulation of a new concept of the cellular membrane systems involved in the synthesis, transport, storage, and secretion of macromolecules.

Materials and Methods

Iron Loading

Dietary. Young male rats of the Holtzman strain were fed a high-fat, protein-deficient diet supplemented with iron for 3, 7, 14, and 21 days, as described

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previously.[4] The iron-loading diet consisted of corn grits, 77.4%; lard, 19.4%; vitamin mixture, 1.0%; choline, 0.3%; and ferric citrate, 1.9%. Control animals were matched with animals in the experimental group by weight and age and received a standard diet consisting of vitamin mixture, 1.0%; choline, 0.3%; glucose, 66.7%; vitamin-free casein, 18.0%; corn oil, 10.0%, and salt mixture, 4.0%. All animals received water *ad libitum*, and were weighed twice a week. Animals were killed and livers were removed for chemical analysis and electron microscopy after 3, 7, 14, and 21 days.

Parenteral. One group of male rats received five intraperitoneal injections of iron in the form of ferric ammonium citrate (400 μg/100 g of body weight) over a period of 24 hr. Control animals received an equivalent volume of sterile isotonic sodium chloride. Livers were removed for chemical and morphologic study 5 hr after the last injection.

Glucagon and Ethionine Administration

One series of male animals loaded with iron for 3, 7, and 14 days received a single intraperitoneal injection of glucagon (100μg/100 g body weight). One hr following glucagon administration these animals were sacrificed and pieces of liver fixed for electron microscopic study.

Another series of female rats received injections of DL-ethionine in addition to parenteral iron loading as described above. Each animal received a split dose of 4 ml of a 0.25% solution per 100 g body weight (total dose 10 mg DL-ethionine per 100 g body weight) at time 0, and after 15 hr during parenteral iron loading.

Control animals for both glucagon and ethionine groups received equivalent amounts of saline.

Chemical Analysis

Ferritin iron and total iron in the liver were determined by the method of Drysdale and Munro.[6] These methods are modifications of Hill's bipyridyl method [7] for the estimation of iron in the ferrous state and the Laufberger procedure for precipitating tissue ferritin.[8]

Light Microscopy

Sections for light microscopy were fixed in 4% buffered formaldehyde, and embedded in paraffin. Sections were stained with hematoxylin and eosin, or with Perls's stain for iron. Light microscopic observations were also made on 0.5–1 μ thick sections cut from the Epon blocks and stained with alkaline toluidine blue. The light microscopic demonstration of acid phosphatase and iron was carried out with Barka's simultaneous-coupling pararosanilin method [9] using napthol-AS-BI as substrate at pH 6.0 following Perls's stain for iron.

Electron Microscopy

Tissues for routine morphologic analysis were fixed by immersion of approximately 1-mm cubes of liver in cold 1% osmium tetroxide buffered with s-collidine pH 7.4) at 0–4°C. Some animals from each group were fixed by infusing sodium cacodylate-buffered (0.2 M) glutaraldehyde (4%) into portal vein for 15 min followed by immersion fixation in glutaraldehyde for 90 min. After glutaraldehyde fixation the small pieces were washed overnight at 0–4°C in cacodylate buffer containing 7.5% sucrose and postosmicated. Tissue specimens were dehydrated, embedded in Epon, and examined in Hitachi HS-7, HU-11, or HU-

11E electron microscopes after en bloc staining with uranyl acetate.[10] In some instances double staining with uranyl acetate and lead citrate was performed.

Electron microscopic histochemical demonstration of acid phosphatase was accomplished as follows. Slices of liver were fixed for 3 hr in cold (0–4°C), 4% cacodylate-buffered glutaraldehyde and washed overnight at 0–4°C in cacodylate buffer containing 7.5% sucrose. Frozen sections (approximately 50 μ thick) or small cubes of tissue were then incubated according to the Gomori technique[11] for the demonstration of acid phosphatase; the ammonium sulfide rinse was omitted. The incubated tissues were then postfixed in 1% osmium tetroxide, dehydrated, and embedded in Epon. These sections were examined unstained in the electron microscope.

Results

Chemical Analysis

As shown in Table 1, the total liver iron as well as liver ferritin increased sharply with the dietary loading. This increase was almost linear until 21 days of loading. Iron loading by five intraperitoneal injections increased the total liver iron and ferritin iron about threefold as compared with control animals.

Light Microscopy

Paraffin sections of untreated control animals rarely showed blue peribiliary cytoplasmic granules in periportal cells with Perls' method. Sections from animals fed corn grits-lard diet without added iron for 7, 14, and 21 days showed an increased number of blue peribiliary granules.

In iron-loaded animals sacrificed after 3 days of dietary iron loading, the Perls' reaction showed a diffuse blue staining in the cytoplasm of parenchymal cells and a more intense blue staining of sinusoidal cell margins. In acid phosphatase preparations, reaction product appeared as red granules around the bile canaliculi and occasionally dispersed

Table 1. Effect of Dietary and Intraperitoneal Iron-Loading on Amounts of Liver Ferritin and Total Iron.*

	Ferritin iron (μg/g liver)†	Total iron (μg/g liver)†
Control	64 ± 9	124 ± 8
3 days dietary iron loading	207 ± 39	289 ± 8
7 days dietary iron loading	426 ± 59	608 ± 89
7 days dietary iron loading	426 ± 59	608 ± 89
14 days dietary iron loading	631 ± 49	923 ± 105
21 days dietary iron loading	816 ± 121	1142 ± 215
5 intraperitoneal iron injections	247 ± 41	326 ± 62

* In each experiment values are based on 8 animals.

† Mean ± standard deviation.

in other parts of the cytoplasm. This staining pattern for acid phosphatase was identical to that seen in control animals receiving standard diet or five intraperitoneal injections of ferric ammonium citrate.

After 7 days of dietary iron loading, the Perls' stain demonstrated, in addition to diffuse blue cytoplasmic staining, distinct granular staining mostly around the bile canaliculi. The number of blue granules was considerably increased compared to animals which received corn grits-lard diet without added iron for 7 days.

When iron and acid phosphatase were demonstrated simultaneously, the majority of peribiliary granules stained blue-red, suggesting staining by both methods. A few perinuclear granules reacted only for acid phosphatase, while some granules around the bile canaliculi reacted only for iron.

After 14 and 21 days of iron loading the distribution of Perls-positive peribiliary granules was the same as described above but the number and size of the granules appeared to be somewhat increased.

After five intraperitoneal iron injections very little iron stainable with Perls' method was present, though the diffuse bluish staining was similar to that seen after 3 days of dietary loading.

Electron Microscopy

Untreated and Saline Controls. Examination of the hepatic cells in control animals fed with standard diet and injected with saline revealed the usual ultrastructural features.[4] (See Fig 7 in Ref 4).

The peribiliary secondary lysosomes contained a variety of granular and homogeneous materials, but very little ferritin or hemosiderin. In our preparations ferritin particles showed the typical micellar fine structure at high resolution.[4,12,13] They were of medium electron density when compared to the very dense precipitates of lead phosphate in the acid phosphatase preparations. We are using the term hemosiderin to refer to dense irregular particles within digestive vacuoles that do not show the typical ferritin fine structure. Such particles are presumed to represent aggregates of iron hydroxide, possibly combined with protein.[12,13] This type of particle is at the present time poorly defined and the literature concerning hemosiderin is both confusing and controversial.[1,12,14]

Animals Fed Corn Grits-Lard Diet without Added Iron. Examination of hepatic cells after 14 and 21 days revealed an increase in the size and number of peribiliary secondary lysosomes which contained increased amounts of hemosiderin and ferritin when compared with controls.[4] (see Fig 9–11 in Ref 4). Very few autophagic vacuoles were

noted. In addition, the cytoplasm contained numerous lipid droplets and showed dilatation of endoplasmic reticulum and Golgi cisternae. The latter contained numerous homogeneous rounded bodies of moderate density presumed to represent lipoprotein or triglycerides. The mitochondria showed mild swelling of the inner compartment and in many instances paracrystalline densities were noted within the matrix.

Animals Loaded with Iron by Parenteral Injections or Dietary Means. These two groups of animals will be discussed together since the pattern of iron distribution was similar in both cases.

CELL SAP. At all loading periods ferritin particles were seen rather randomly distributed in the cell sap, mostly adjacent to rough endoplasmic reticulum (ER) (Fig 1 and 2), but also near the sinusoidal border of the cell and the bile canaliculus. The only exception to the random distribution of ferritin was a higher concentration of particles around the large ferritin-loaded lysosomes (Fig 13). The amount of cell sap ferritin was difficult to quantitate in thin sections.

ENDOPLASMIC RETICULUM. After dietary or parenteral loading, particles of ferritin were seldom identified within the rough ER at any time interval studied (Fig 2) unless the animals received ethionine. In ethionine-treated animals, however, the numerous lipid droplets within rough ER cisternae often contained ferritin particles (Fig 3). The ferritin particles appeared to be embedded within the lipid droplets from which they could easily be distinguished.

Identification of ferritin within the smooth ER presented considerably more uncertainty. Although smooth-surfaced profiles were sometimes seen to contain ferritin particles, these could not be definitively classified as smooth ER cisternae. They may, for example, have represented portions of Golgi saccules, phagosomes, or other smooth-surfaced structures.

GOLGI APPARATUS. Ferritin particles were seen within the Golgi apparatus by 7, 14, and 21 days of dietary loading and after the parenteral loading experiments. The innermost Golgi cisternae usually contained the most ferritin (Fig 4 and 6). Golgi vacuoles and vesicles along the concave ("maturing") surface of the dictyosomes contained numerous ferritin particles. Acid phosphatase activity was also localized in the innermost cisternae and in surrounding vesicles and vacuoles (Fig 5 and 7). Sometimes it was possible to determine that acid phosphatase and ferritin were present in the same cisternae, vesicles, and vacuoles (Fig 5 and 7). Often, however, large amounts of lead phosphate reaction product precluded any estimate of whether ferritin and acid phosphatase coincided (Fig 11). It was, however, quite evident that some

vesicles filled with ferritin did not contain acid phosphatase reaction product. Ferritin-filled vesicles in the Golgi region were surrounded by a single trilaminar membrane about 80–90 Å thick and often showed a distinct electron-lucent zone just inside the limiting membrane (Fig 11).

Identical-appearing ferritin-filled vesicles were also found in regions of the cell quite distant from the Golgi apparatus (Fig 12). Such regions included the peribiliary cytoplasm and the cytoplasm just inside the sinusoidal plasma membrane. The relationships between ferritin and acid phosphatase in these other vesicles were identical to those described for vesicles adjacent to the Golgi appartus. Occasionally images were observed that suggested fusion between these small ferritin-filled vesicles and the limiting membrane of large digestive vacuoles (secondary lysosomes) (Fig 8).

SECONDARY LYSOSOMES. In 3-day dietary loading experiments and following parenteral iron administration, most secondary lysosomes contained ferritin. The particles were evenly distributed in the lysosomal matrix except for an outer, electron-lucent marginal area, which was often devoid of ferritin. Lysosomes showed considerable variation in size and shape and often had a long, thin "tail" containing ferritin particles. The lysosomes were limited by a trilaminar membrane, about one-third thicker than the outer mitochondrial membrane. After 7, 14, and 21 days of dietary iron loading the distribution of ferritin particles within the lysosomes was similar but the concentration appeared to be increased (Fig 9–11, 17). Approximately one-half of the ferritin-loaded lysosomes also contained lead phosphate reaction product in thin sections (Fig 11). After 14 and 21 days the lysosomes often contained coarse, very dense material interpreted as hemosiderin.

AUTOPHAGIC VACUOLES. It is now generally accepted that the formation of autophagic vacuoles (AV) constitutes an important mechanism for the uptake and degradation of particles as large as mitochondria within digestive vacuoles.[15] Furthermore, the rate of autophagocytosis can be greatly increased by certain experimental treatments such as by the administration of glucagon.[16–18] In a recent study of the latter model, it was shown that in the liver autophagy essentially represents exocytosis or a membrane evagination into the endoplasmic reticulum.[18] That is, early AV consist of a piece of cytoplasm (inner compartment) partially surrounded by a sac. In a later stage, fusion of the cisternal ends occurs, resulting in a double-membrane-limited fragment of cytoplasm. Acid hydrolase appears to be added to this sac (the outer compartment) with subsequent disappearance of the inner mem-

brane, forming a one-compartment digestive vacuole resembling other secondary lysosomes.

Such a mechanism, therefore, could represent a means for enclosure of ferritin particles localized to the cell sap within secondary lysosomes. In control rats only an occasional autophagic vacuole could be recognized. The vacuoles usually contained a mitochondrion or a microbody together with a small portion of cell sap. In the present experiments, animals on the corn grits-lard diet without added iron did not show any apparent increase in the number of autophagic vacuoles. Animals loaded by dietary or parenteral means also showed no structural modification or increase of autophagic vacuoles. When animals loaded by dietary means for 14 days were stimulated with glucagon, an increase in the number of autophagic vacuoles was seen which was similar to that occurring after glucagon administration alone.[18] That is, 5–20 single- or double-membrane-limited autophagic vacuoles were present in the peribiliary area of each hepatic cell sectioned.

It appeared that the highest concentration of ferritin in the newly formed autophagic vacuoles was in the outer compartment (Fig 13), and that the ferritin was distributed in a fashion identical to the acid hydrolase in the newly formed autophagic vacuoles.[18] Occasionally, ferritin particles were observed within the inner compartment of the newly formed AV. However, the concentration in the inner compartment did not differ from that within the cell sap.

MULTIVESICULAR BODIES. In all loading experiments most multivesicular bodies were devoid of ferritin particles. However, after parenteral iron loading or after dietary loading for 7, 14, or 21 days, a few multivesicular bodies contained ferritin particles (Fig 14). Most of the particles were seen in the matrix between the vesicles.

MICROBODIES. No ferritin was seen inside the microbodies in 3- or 7-day experiments or after parenteral iron loading. In 14- and 21-day animals, a number of microbodies contained ferritin particles in their matrix (Fig 14–17). No ferritin was seen in any experiments within the nuclei or mitochondria.

Discussion

The cellular mechanisms involved in the synthesis, transport, and storage of ferritin are poorly understood. It is well known that increased amounts of ferritin are formed following stimulation of the mammalian liver with iron administered parenterally [1,19,20] or via the diet.[4] It also seems well established that a large proportion of the newly formed ferritin is found in the cell sap.[21] Yet, as shown in these experiments, a

substantial proportion of ferritin appears in the cytocavitary network, especially in the portion of the latter that participates in intracellular digestion. Ferritin is also unique in the sense that it is a single molecule that can be directly visualized with the electron microscope.[4,12,13] Therefore, it is potentially possible to follow individual molecules rather than to base conclusions on the essentially statistical type of data that are obtained with labelling experiments. On the other hand, the direct visualization of ferritin in the electron microscope may render it more difficult to distinguish between the major important pathways and peripheral or trivial aspects of protein metabolism.

A New Concept of Intracellular Compartmentation

The present experimental results have made clear the inadequacies of the presently accepted classification of intracellular organelles—especially those systems concerned with protein synthesis, transport, degradation, secretion, and excretion.[15,22–25] For example, ferritin is a protein that is presumably synthesized for intracellular use and yet it is found not only within the cell sap but also in the Golgi apparatus and lysosomes. Furthermore, some ferritin is apparently found in primary lysosomes that are devoid of at least some acid hydrolases—a logical contradiction if previously accepted terminology is used.[15] Moreover, ferritin is found in numerous smooth-surfaced vesicles that cannot be classified with present criteria. Although much ferritin is retained in the cell after iron loading, some ferritin is apparently "secreted" by exocytosis [4] or by other means into the bile canaliculi. The evident complexity in the transport of at least one protein, ferritin, emphasized the necessity to formulate new concepts concerning intracellular compartmentation.

These ideas especially apply to the so-called lysosome system which in the past has been given a rather strict and somewhat artificial definition based on the content of acid hydrolases.[15] Ferritin, a non-enzymatic, newly synthesized protein is found in several compartments including secondary lysosomes and apparently in "lysosomes" devoid of hydrolases. Hence, it is obvious that the entities previously described as lysosomes can be regarded as only part of a larger system of membrane-bound cavities, which includes the endoplasmic reticulum, the microbodies, the Golgi apparatus, the nuclear envelope, the multivesicular bodies, secretory granules, phagosomes, and other vesicles originating from pinocytosis. Only the mitochondria among the membrane-bound organelles are excluded from this system and recent studies regarding the formation of at least the mitochondrial outer membrane

raise some questions about this assertion.[26] We visualize the membrane-bound system as a dynamic network, the continuities, morphologic appearance and contents of which are subject to rapid modulation during both the normal and pathologic activities of the cell. We propose to term this system the cytocavitary network, thus emphasizing the essentially two-phase nature of the cell interior—the cell sap and the contents of the cytocavitary network, which are separated by the membranes of the network. The substructure of the cytocavitary membrane reflects the differences in function in various parts of the system. So far, two types of membranes have been defined: [18,27,28] a thicker membrane found in the maturing face of the Golgi apparatus, the digestive vacuoles, and the secretory granules; and a thinner membrane found in other parts of the system. The transition points between the two are poorly defined but at least one such point is in the Golgi apparatus. In the Golgi, and possibly also in other regions, transitions in morphology and apparently in chemical composition of the membrane occur. There is much evidence that the membranes are essential to the function of many enzymes, for instance those involved in protein synthesis.[29] This suggests that the organization of the cytocavitary network is meaningful in addition to its role as a transport network.

Ferritin Biosynthesis and Transport

Stimulation of the hepatic cell with iron administered parenterally or through the diet has been reported to greatly augment the rate of ferritin formation.[30] It is presently uncertain whether this results entirely from increased apoferritin synthesis followed by addition of iron or whether, at least in part, it reflects stabilization of preexisting apoferritin subunits by iron.[30,31]

It can be assumed that the synthesis of apoferritin subunits occurs in association with cytoplasmic polysomes. We did not find ferritin particles in the nuclei. Moreover, Hicks, Drysdale, and Munro [32] have recently described the occurrence of ferritin synthesis in vitro from cytoplasmic ribosome fractions. It is estimated that in the liver cell approximately 75% of the polysomes are membrane-bound.[29] Cytoplasmic polysomes are generally assumed to exist in two classes—free and membrane-bound.

Hendler [29] has recently emphasized the importance of membranes to protein synthesis. It is difficult with present biochemical techniques to exclude the possibility that at least some "free" polysomes may in fact have been detached from the membrane surfaces during the processes of homogenization and density gradient centrifugation. Certainly, it

seems clear that membrane-bound polysomes are often much more efficient in protein synthesis *in vitro*.[29] On the other hand, Hicks, Drysdale, and Munro[32] reported that the free polysomes were more efficient than a mixed fraction of membrane-bound and free polysomes in synthesizing ferritin *in vitro*. Klavins[33] reached a similar conclusion, but on the basis of electron microscopic and biochemical data presented, it is not certain whether the free polysomes were completely devoid of membranes.

In the present study we found much ferritin in the cell sap. While this may correspond to the true localization (see the discussion of possible artifacts of the EM procedure below), it does not necessarily insure that the ferritin was synthesized in this locus. For example, synthesis may have occurred on membrane-bound polysomes with subsequent detachment to the cell sap. Furthermore, the messenger involved with ferritin synthesis could be easily detached from the membrane and provide another alternative interpretation to that proposed by Hicks, Drysdale, and Munro.[32] The argument that proteins retained by the cell are synthesized by free polysomes and those that are destined for excretion are made on membrane-bound polysomes seems specious in view of the synthesis of acid hydrolases within the rough ER.[34] Also, it appears that protocollagen is almost exclusively synthesized by microsomes,[35] but in autoradiographic studies most labelled protocollagen is found in the cell sap.[36,37] As in the case of ferritin, some protocollagen may be transferred via the ER-Golgi apparatus pathway as evidenced by previous autoradiographic findings.[37,38] Furthermore, Hicks, Drysdale, and Munro[32] reported a significant amount of albumin synthesis by the "free" polysomes in vitro in contrast to the several in vivo studies by Peters[39,40] who found that this protein was predominantly assembled on the rough cisternae. Therefore, in light of our observations and in the context of other reports, it is impossible to decide with certainty at the present time the site of ferritin synthesis. Our own assumption is that the synthesis is most likely to be closely associated with the membranes of rough ER. It is certain that at least some ferritin finds its way into the Golgi saccules and cisternae and hence into Golgi vesicles and digestive vacuoles. In the following section the reasons for assuming that some ferritin follows a pathway similar to known secretory proteins will be given.

Ferritin in the Cytocavitary Network

As described above, ferritin particles were observed within various portions of the cytocavitary network. Low concentrations were found

in rough-surfaced endoplasmic cisternae, in microbodies, and in various smooth-surfaced, membrane-bound profiles. Slightly higher concentrations were found in Golgi cisternae, vacuoles and vesicles, and in multivesicular bodies. High concentrations were found in some vesicles near the Golgi apparatus, and in similar-appearing vesicles in other regions of the cell as well as in large digestive vacuoles, some of which were clearly autophagic. In some instances, acid phosphatase activity and ferritin coincided in Golgi apparatus and in lysosomes. The concentration of ferritin in digestive vacuoles was higher than in any other region of the cell. This raises the question of how ferritin gains access to the cytocavitary network. The following possibilities must be considered.

1. Direct passage through the membrane from the cell sap. As mentioned above, large amounts of ferritin are present within the cell sap. Although ferritin is a very large molecule and present information regarding membrane permeability seems to preclude any possibility of direct transmembrane movement of such a molecule, there are reports regarding effects of macromolecules on membrane systems.[41–43] In the case of the digestive vacuoles such a movement would be against the concentration gradient. Moreover, Ginn, Hochstein, and Trump,[42] in a study of primaquine-induced hemolysis of erythrocytes, did not observe transmembrane passage of ferritin even when the membrane permeability was increased. It is of interest that both red cell membranes and membranes of digestive vacuoles have several features in common including high cholesterol content. In this study the only way in which ferritin could enter the red cell was by the induced endocytosis. Therefore, although the mechanism of membrane penetration cannot be completely excluded, it appears to be highly unlikely in the case of ferritin in the liver cell.

2. Autophagocytosis. As mentioned above, it is well established that macromolecules and supramolecular structures such as mitochondria can get into digestive vacuoles via the process of autophagocytosis. However, increased numbers of autophagic vacuoles were not observed during iron loading and most of those present had ferritin only in the outer compartment, which in fact arises from the cytocavitary network. While some autophagic vacuoles had particles in the outer compartment, this could be explained on the basis of fusion and mixing with other ferritin-containing digestive vacuoles as previously suggested.[18] Furthermore, exocytosis into preexisting structures such as Golgi components or digestive vacuoles was not observed and the multivesicular bodies which we regard as a type of autophagic vacuole had little

ferritin, and most of that was in the matrix. Although it appears that the vesicles within the multivesicular bodies are formed by invagination of the wall [44,45]—ie, microautophagy—very few such vesicles contained ferritin.

While it is impossible to exclude the possibility that some ferritin finds its way into digestive vacuoles by autophagy, the rapid rate of accumulation in the digestive vacuoles in the absence of demonstrable uptake by autophagocytosis makes this seem very unlikely as a primary mechanism. Even when autophagocytosis was stimulated with glucagon, insignificant uptake of ferritin occurred.

3. Transport from rough ER to digestive vacuoles via Golgi apparatus. This is a well-known pathway for proteins that are secreted. In the case of the liver cell, much evidence favors this possibility for albumin.[39,40] The present experiments indicate that this is the most likely pathway, at least for that part of the ferritin that finds its way into the digestive vacuoles. As discussed above, ferritin was observed in rough endoplasmic cisternae, microbodies, Golgi cisternae, Golgi vacuoles and vesicles, and the digestive vacuoles. Such a localization is compatible with the hypothesis that at least part of the ferritin is injected into the cisternae across the cytocavitary membrane, and then is transported to the Golgi apparatus, where it is concentrated in Golgi vesicles, which ultimately fuse with digestive vacuoles. In fact, images suggestive of the latter process were observed. The concentration of ferritin within various parts of the cytocavitary network is quite compatible with this assertion. Ferritin was in very low concentration within the endoplasmic cisternae, in higher concentration in Golgi components, and in highest concentration in the digestive vacuoles. This could mean that transport is most rapid in the endoplasmic cisternae and that a limitation of movement occurs more distally. This is compatible with our previous report [4] of lysosomal excretion of ferritin, which appears to be rather slow in the liver cell. Other interpretations could be made. For example, it is conceivable that apoferritin, which is not visible in the electron microscope, is present in much higher concentration within the endoplasmic cisternae, and that iron is added to form ferritin within Golgi components and/or digestive vacuoles.

Retention of ferritin within digestive vacuoles is probably explained by the recent studies of Coffey and de Duve,[46] who observed that ferritin is stable for long periods of time *in vitro* in lysosomal extracts which rapidly degrade many other proteins. Maunsbach [47] recently studied the fate of hemoglobin and ferritin in the digestive vacuoles of the proximal kidney tubules. Ferritin was apparently degraded much

slower than hemoglobin. In addition, our data indicate that other types of iron-containing aggregates occur in the digestive vacuoles. Such aggregates might represent the effects of denaturation of ferritin to form insoluble iron-containing proteins or might merely represent depositions of inorganic iron compounds.

Our data do not exclude the possibility that ferritin concentrated in the digestive vacuoles by an as yet undisclosed mechanism might appear in Golgi and ER by retrograde movement. There is, however, no precedent for such a mechanism. There remains the possibility that the Golgi apparatus is bypassed in the sense of Novikoff's GERL.[48] This possibility cannot be excluded but does not basically differ from our own interpretation. Finally, there is the probability that ferritin delivered to the bile canaliculus by exocytosis as previously described will be phagocytized by more distal hepatic cells, at least in part, and thus contribute to the complement of ferritin within digestive vacuoles. The experiments of Hampton [49] using retrograde perfusion of colloidal particles into the bile duct system show clearly that such a mechanism can occur.

Intracellular Compartmentation, Protein Synthesis, and Degradation

Our results demonstrate that ferritin can exist within the two principal compartments of the cell—the cell sap and the cytocavitary network. It is extremely unlikely that these localizations represent complete artifacts since two other compartments—namely, the inner and outer mitochondrial compartments and the nucleoplasm—were always completely free of ferritin. The functional significance of this biphasic distribution, though presently unknown, may be of great physiologic significance. For example, this could be the basis for the turnover of predominantly cell sap proteins—a long sought mechanism. They also seem to indicate the importance of the cytocavitary network for the segregation and transport of proteins, which may be either degraded or secreted, depending on functional demand. In the case of ferritin, we could assume that the cell sap pool is more readily depleted upon demand since iron can be easily removed from ferritin along the cell membrane by purely chemical means—ie, reduction. The cytocavitary pool, on the other hand, might store excessive amounts of ferritin which could otherwise be toxic to the cell. Some of the cytocavitary pool is evidently excreted to the bile by exocytosis, whereas direct release to the plasma seems quite unlikely since it is separated from that compartment by two membranes. This could in effect represent a ballast type of reservoir, providing a steadier rate of release during

periods of iron depletion. Finally, the possible importance of ferritin incorporation to the cytocavitary network in the control of protein turnover should be mentioned. It is probable that many cell sap proteins are continually being incorporated into and degraded within the digestive vacuoles. On the other hand, diversion of protein synthesis toward ferritin which is trapped within this system in effect retards such polypeptide breakdown because of the resistance of ferritin to lysosomal digestion.[46]

Technical Aspects and Problems

Ferritin is an ideal molecule for electron microscopic studies of protein synthesis because of its large size and the characteristic structure of its iron-hydroxide core.[13,50] Previous and present studies have demonstrated that a single ferritin molecule can be distinguished *in vitro* and in thin sections of intact cells.[4,12,50]

Little attention has been paid in most studies to the possible solubility of ferritin during fixation and subsequent tissue processing. In contrast to hemosiderin, ferritin is water soluble but probably becomes insoluble through denaturation during the process of fixation. Specificity of localization of ferritin molecules within the various compartments of the liver cell is supported by our finding that no ferritin was present inside the mitochondria or nuclei. On the other hand, the presence of ferritin particles in the cell sap requires explanation. This localization could be a true one or represent, at least in part, leakage from cellular organelles during fixation, and subsequent tissue processing.

In the present experiments some leakage of ferritin from lysosomes during fixation is suggested by an often prominent perilysosomal distribution of ferritin in cell sap. In fact, artifactual leakage of ferritin particles from extracellular space into cytoplasmic matrix during osmium fixation has been previously reported.[52]

Richter described aggregates of ferritin and hemosiderin in the cell sap of rats which were given iron and ethionine for 2 months.[5] He concluded that these aggregates represented contents of siderosomes liberated into the cell sap by dissolution of their surrounding membrane. Against this concept of lysosomal leakage *in vivo* as an explanation of cell sap ferritin is the shortness of the iron-loading period in the present experiments. Moreover, *in vivo* disruption of lysosomal membranes should cause rapid necrosis and lysis of the hepatic cell. Evidence for at least some artifactual ferritin leakage from lysosomes comes from studies that show that lysosomes loaded with Triton WR 1339[15] or

sucrose [53] are mechanically more fragile and often share discontinuities in their limiting membranes under the electron microscope.[53]

Technically, the simultaneous localization of ferritin and acid phosphatase activity in thin sections requires that ferritin particles be distinguished from the lead phosphate reaction product of the acid phosphatase method. The following differences were found to be useful in making this distinction: (1) single ferritin particles had a typical quadruplet-like or rectangular appearance while the smallest lead phosphate precipitates showed wide variations in their shape; (2) the size of the electron-dense core of ferritin measured from 60 to 70 Å; (3) the lead phosphate precipitate was always denser than even the larger clusters of ferritin particles. This was an especially useful criterion in the unstained sections. When both ferritin and lead phosphate were localized within the same organelle, differentiation was difficult and only occasionally possible. Hence, no conclusions could be made regarding ferritin content of organelles heavily stained with lead phosphate. However, from the light microscopy it was evident that most peribiliary lysosomes contained both acid phosphatase and iron. On the basis of electron microscopy the primary lysosomes appeared to have wide variations in their ferritin and acid phosphatase content. Thus, some primary lysosomes seemed to contain only ferritin, while others were probably carriers for both acid phosphatase and ferritin.

Summary

The intracellular localization of ferritin in the liver cell was studied after 3, 7, 14, and 21 days of dietary loading or after parenteral iron loading. In a number of experiments ethionine was given simultaneously with parenteral iron. In others, autophagocytosis was induced after dietary iron loading by a single injection of glucagon. Both after dietary and parenteral iron loading, ferritin particles were seen in different cellular compartments—large numbers of ferritin particles were seen in the cell sap, and ferritin was also found within the *cytocavitary network*. This term is defined as the series of membrane-enclosed cytoplasmic cavities other than the inner compartment of mitochondria. Concentrations of ferritin were as follows: occasional ferritin particles were found within endoplasmic reticulum and microbodies; ferritin was frequently seen within the Golgi apparatus and its associated small vesicles, and within multivesicular bodies; the greatest concentration was found within primary and secondary lysosomes; in none of the experiments was ferritin seen within the nuclei or mitochondria.

Ethionine appeared to trap ferritin within the liposomes of ER. Auto-

phagocytosis was not prominent in the livers after iron loading. When autophagocytosis was induced by glucagon, the newly formed autophagic vacuoles appeared to acquire ferritin by fusion with ferritin-filled lysosomes. The results show that ferritin is localized to two major cellular compartments: (1) the cell sap, and (2) the cytocavitary network. They indicate that the pathway of entry of ferritin into lysosomes is essentially similar to that of acid hydrolases. The functional significance of the ferritin localization in the cytocavitary network and cell sap is discussed from the standpoints of protein synthesis and degradation.

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[*Illustrations follow*]

Legends for Figures

Fig 1. Electron micrograph of liver cell after 21 days dietary iron loading. Ferritin particles *(arrows)* are seen scattered in cell sap between cisternae of rough endoplasmic reticulum. No particles are present within cisternae. Note concentration of particles within smooth-surfaced vacuole probably representing a lysosome *(ly)*. Unstained section. × 217,500.

Fig 2. An area of liver cytoplasm after 21 days of dietary iron loading. Ferritin particles *(arrows)* are present between cisternae of RER; one particle is also seen within a cisterna *(arrow)*. Unstained section. × 347,000.

Fig 3. An area of liver cell after parenteral iron loading and ethionine administration. Note presence of ferritin particles within the liposomes *(arrows)* of endoplasmic reticulum. Ferritin particles are also present in cell sap. Unstained section. × 104,500.

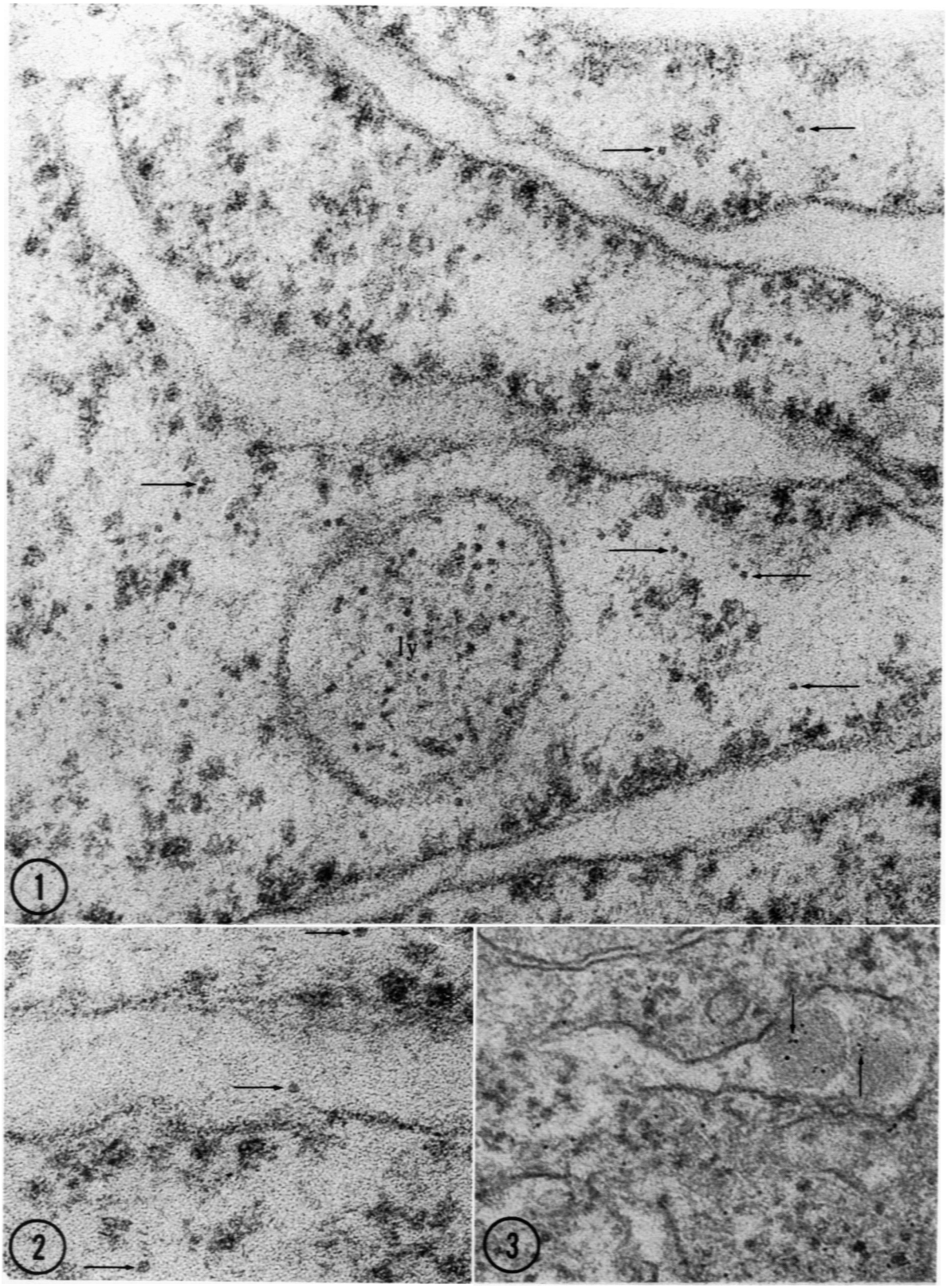
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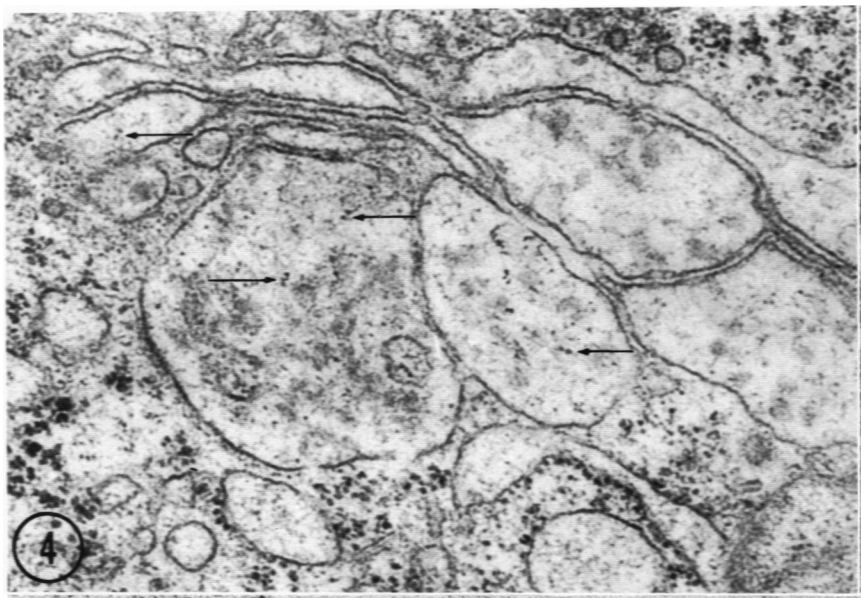
Fig 4. Ferritin particles *(arrows)* in inner cisternae of maturing face of Golgi apparatus. Fourteen days of dietary iron loading. Double stained section. × 96,000.

Fig 5. Ferritin particles *(arrows)* inside Golgi cisternae. Inner cisternae also contain black lead phosphate precipitate as a marker of acid phosphatase activity. Fourteen days of dietary iron loading. Unstained section. × 57,500.

Fig 6. A high magnification of three Golgi cisternae *(g)* showing the presence of numerous ferritin particles *(arrows)* within cisternae. Twenty-one days of dietary iron loading. Unstained section. × 433,500.



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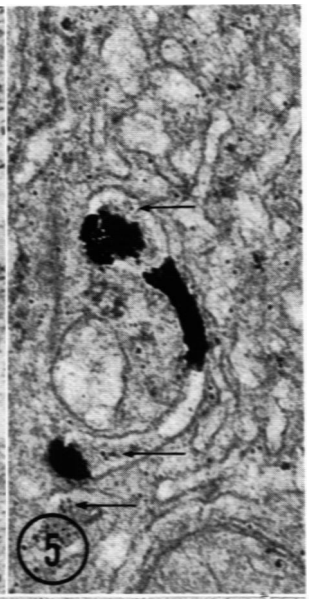

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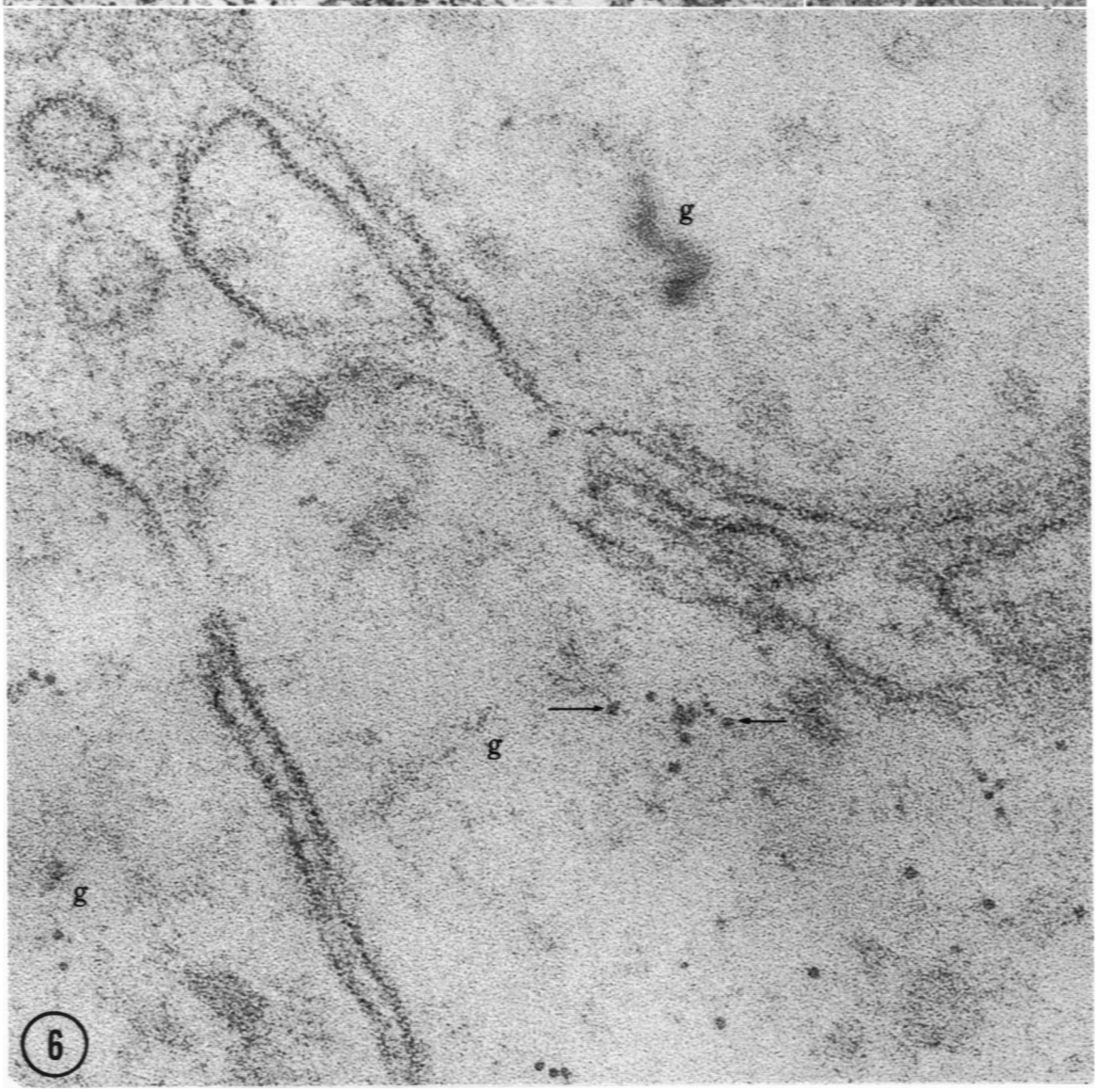

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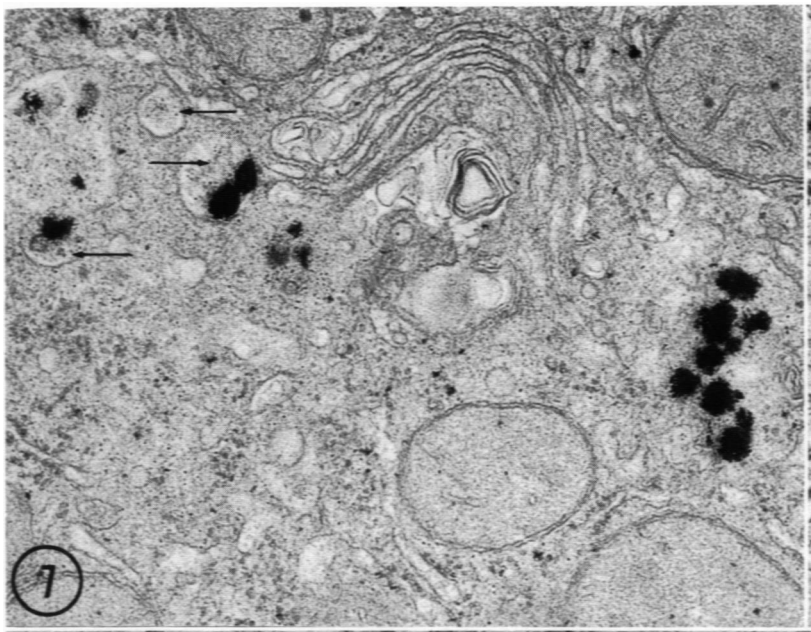
Fig 7. Golgi region from a rat after three days dietary loading. Ferritin particles *(arrows)* are seen inside Golgi vesicles adjacent to Golgi cisternae. Some of vesicles also contain acid phosphatase activity seen as black precipitate of lead phosphate. Unstained section. × 52,500.

Fig 8. Liver cell after 14 days of dietary iron loading and 1 hr after glucagon administration. Preparation was incubated for demonstration of acid phosphatase. Black lead phosphate reaction product is seen within small primary lysosomes. One vesicle *(arrow)* appears to contain only ferritin particles. Note fusion of small ferritin containing vesicle *(double arrow)* with a large ferritin containing secondary lysosome. Unstained section. × 37,500.

Fig 9. Three ferritin-filled lysosomes after 21 days of dietary iron loading. Unstained section. × 115,000.

Fig 10. High magnification of ferritin particles within lysosome after 21 days' dietary iron loading. Note typical micellar structure of particles. Unstained section. × 437,000.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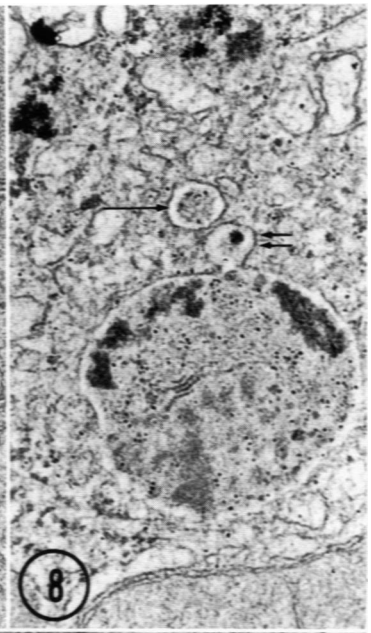
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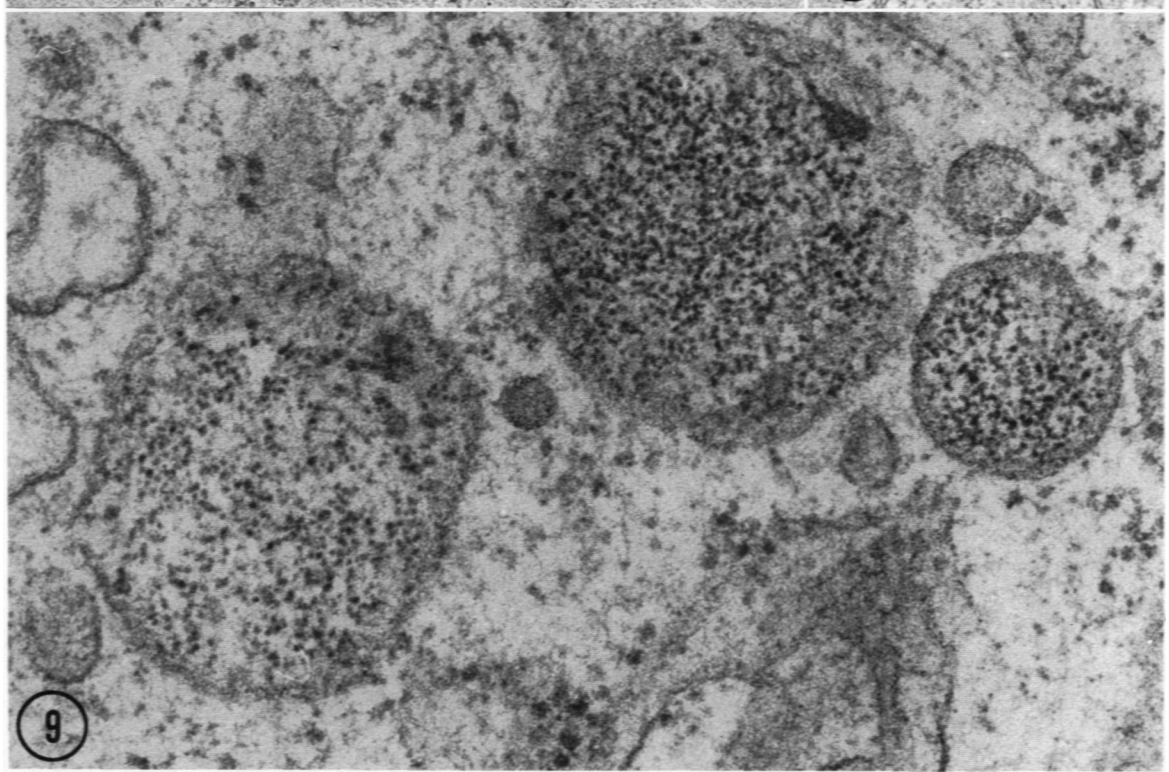
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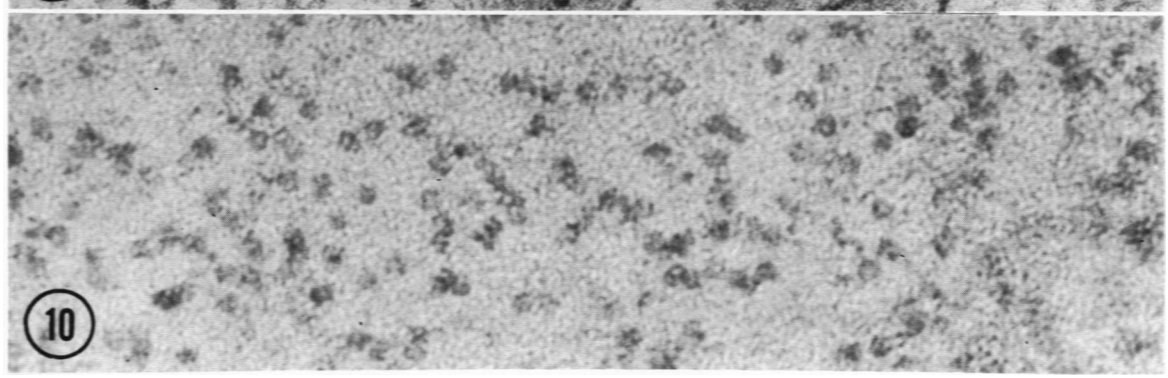
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Fig 11. Ferritin particles inside numerous primary and secondary lysosomes *(arrows)*. Note that acid phosphatase activity is limited to two Golgi vesicles *(ply)*, to a secondary lysosome *(sly)*, and to a circular profile *(cp)*. Seven days of dietary iron loading. Unstained section. × 59,500.

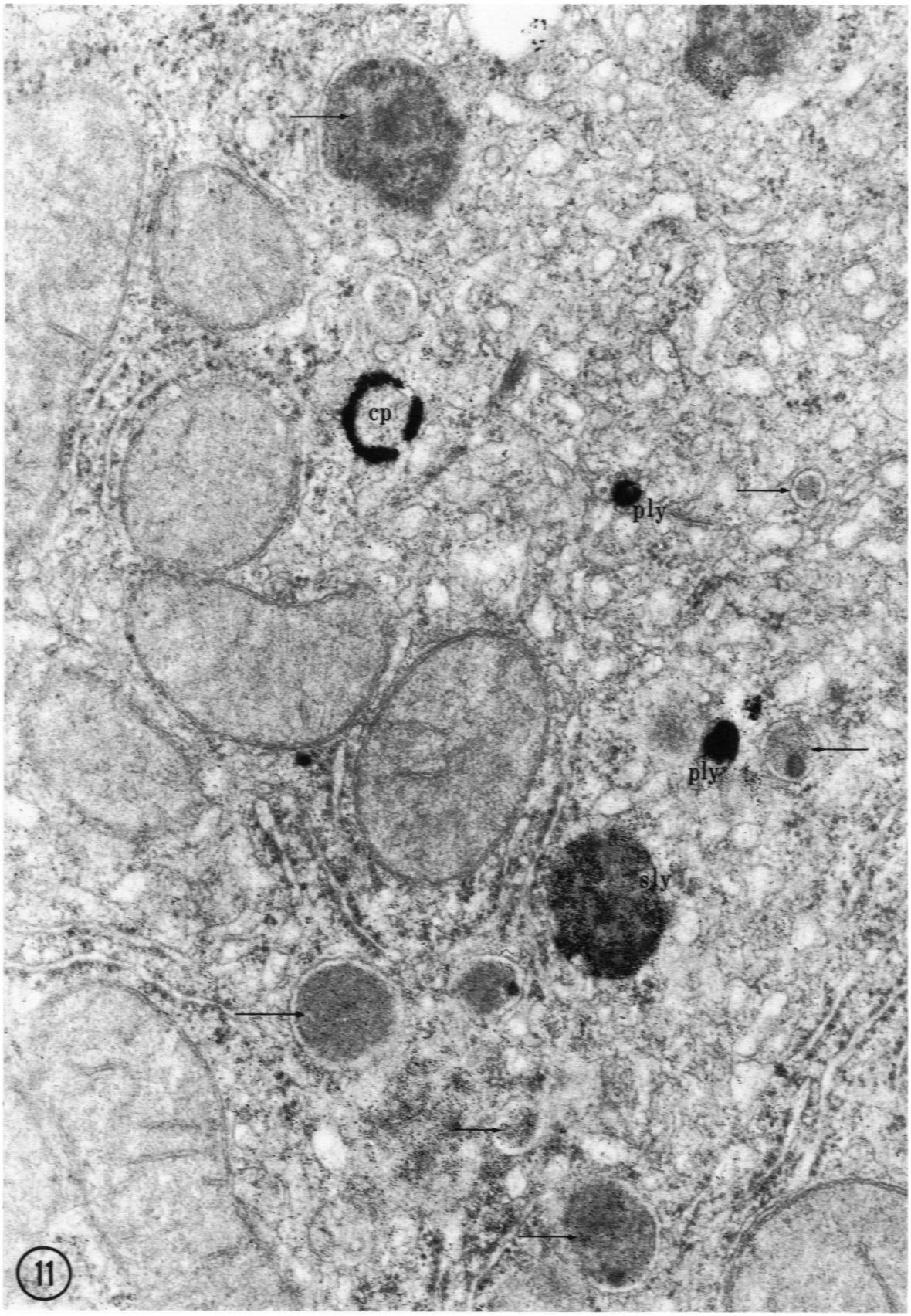

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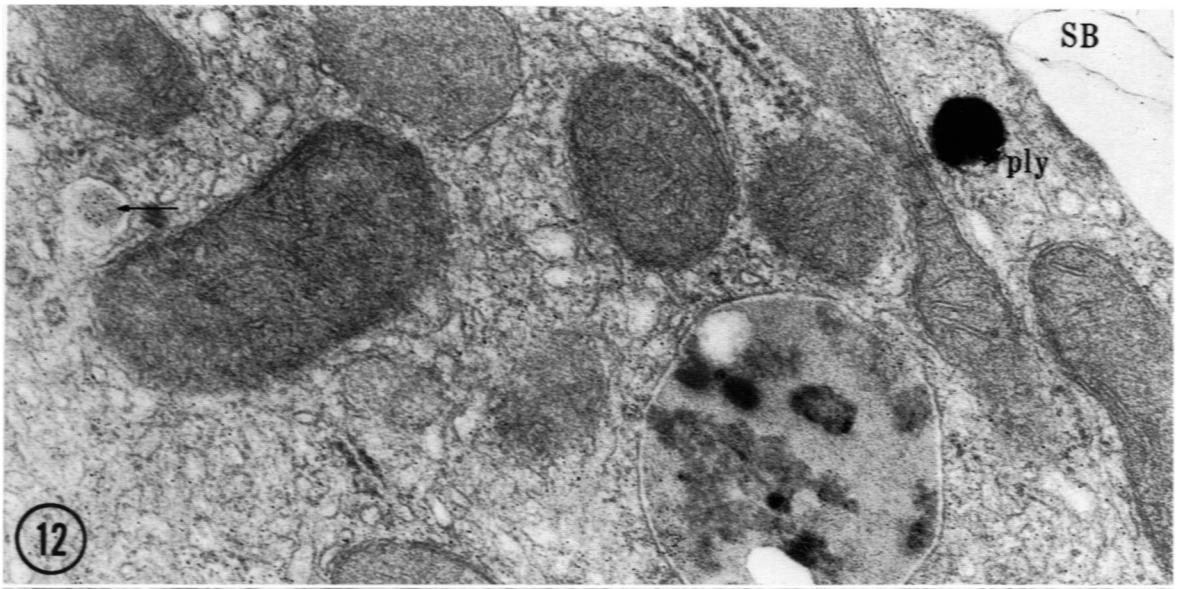
Fig 12. An area of liver cell adjacent to the sinusoidal border *(SB)*. Note presence of probable primary lysosome *(ply)* filled with lead phosphate reaction product and a vesicle containing ferritin particles *(arrow)*. Seven days' iron loading. Unstained section. × 45,000.

Fig 13. Autophagic vacuole limited by double membrane *(arrows)* and containing mitochondrion. Ferritin particles in space between membranes are already present, but there is no lead phosphate reaction product. Note also presence of numerous ferritin particles in cell sap around the AV. Fourteen days' dietary iron loading. Unstained section. × 157,500.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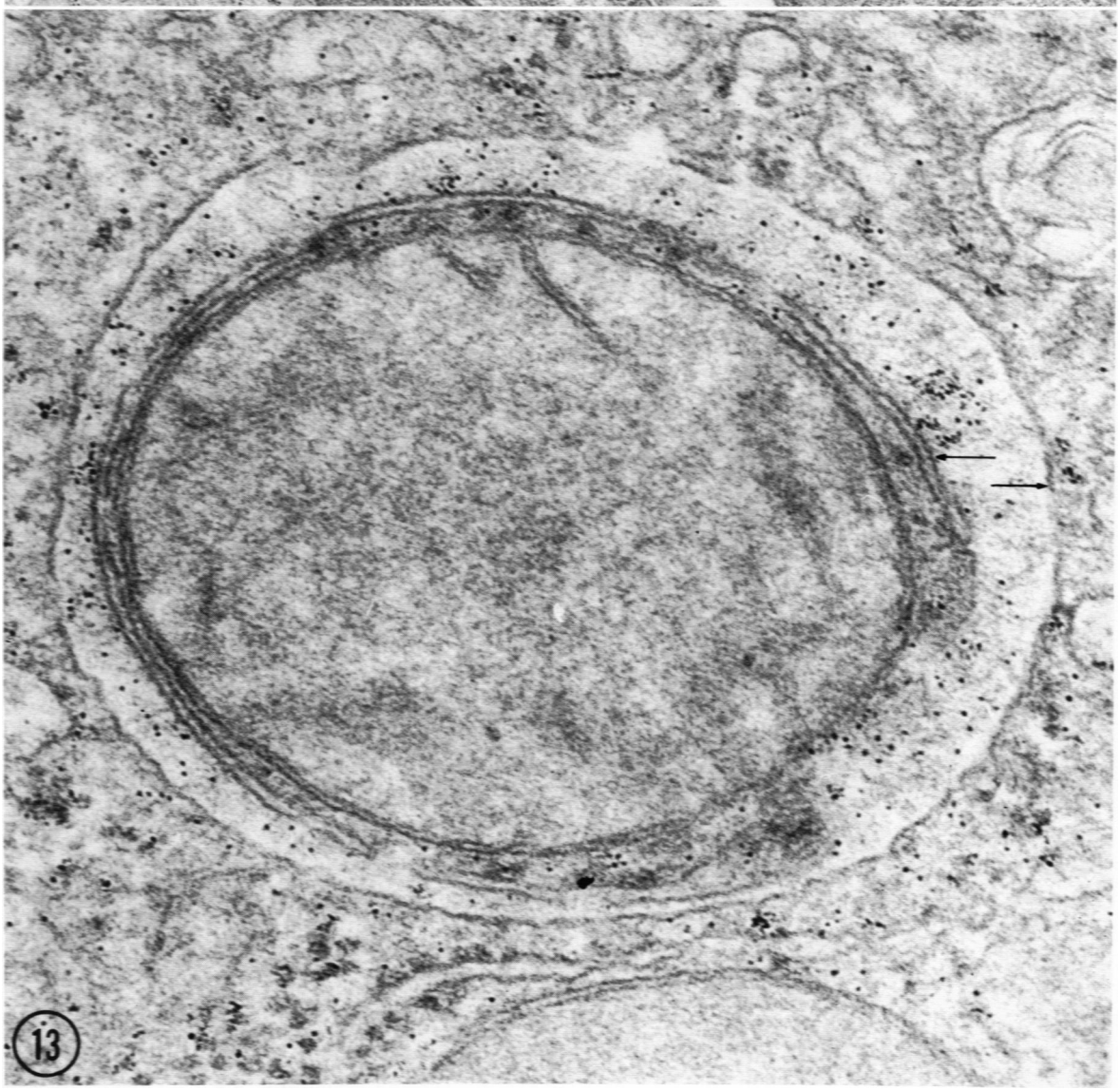
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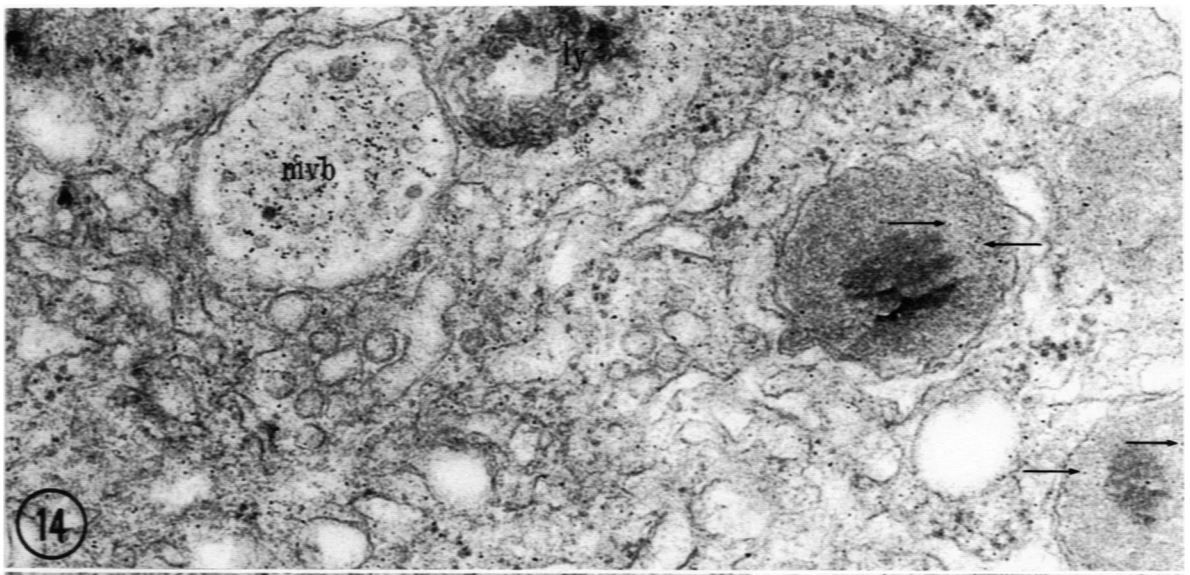
Fig 14. An area of liver cell after parenteral iron loading and ethionine administration. Numerous ferritin particles are seen in the matrix of a multivesicular body *(mvb)* and within lysosome *(ly)*. Some ferritin particles *(arrows)* are also present within two microbodies. Numerous ferritin particles are in cell sap. Unstained section. × 48,000.

Fig 15. Numerous ferritin particles are present inside three microbodies. Dietary iron loading, 14 days. Unstained section. × 54,000.

Fig 16. Microbody after 21 days of iron loading. Ferritin particles are seen in the cell sap and within microbody *(arrows)*. Unstained section. × 89,000.

Fig 17. A lysosome *(ly)* is filled with ferritin particles. Ferritin particles *(arrows)* are also present within typical microbody limited by thin membrane. One ferritin particle *(double arrows)* is seen within microbody-associated, rough-surfaced cisterna. Twenty-one days of iron loading. Unstained section. × 129,500.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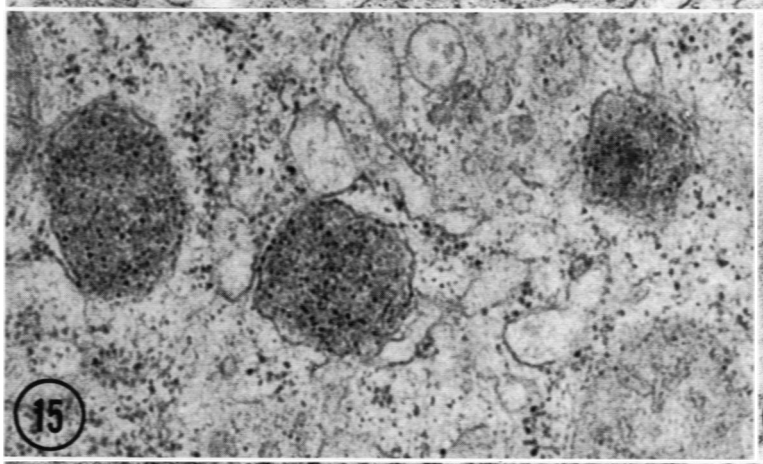
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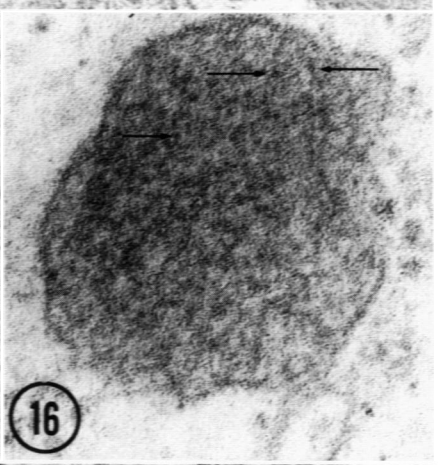
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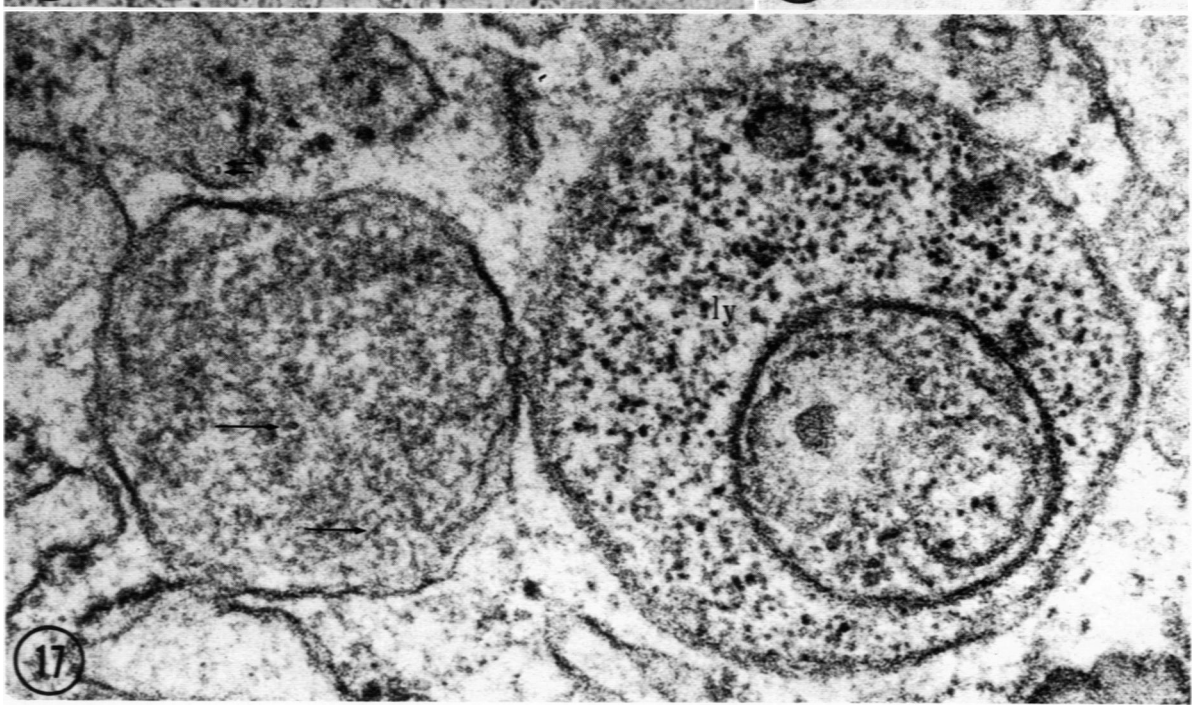
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