A STUDY OF HEMOSIDEROSIS WITH THE AID OF ELECTRON MICROSCOPY

With Observations on the Relationship Between Hemosiderin and Ferritin *

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Although hemosiderosis develops in many circumstances (1) in man and animals, and though hemosiderin has long been recognized as an important product of iron metabolism, the formation and structure of hemosiderin in tissues have not as yet been elucidated at fine structure levels. In recent years numerous studies have indicated a close chemical relationship between the iron storage compound ferritin and hemosiderin. Through the important contributions of Laufberger, Kuhn, Granick, Michaelis, and others (2–9), considerable information regarding the chemical nature of ferritin has accumulated, and Farrant (10) has provided an elegant demonstration of the structure of the ferritin molecule by means of electron microscopy. In general, these studies have proceeded from the isolation of ferritin *in vitro*. Its presence in intact tissues has been inferred from compelling evidence. Novikoff *et al.*, Palade and others (11–13) have indicated that certain electron-dense particles which they have observed in electron micrographs of cells in liver and spleen, and of cell fractions from the liver, might represent ferritin.

In electron micrographs taken for other purposes, the author recently observed large, dense aggregates of similar particles in liver cells. Further study with the light microscope revealed that the aggregates were visible as "hemosiderin" granules. The present report deals with observations on the fine structure and disposition of hemosiderin in different cells in rats and in man, and under varying circumstances. These observations indicate that inside cells, ferritin is one of the constituents of hemosiderin. In this study information obtained through light microscopy and chemical procedures has been correlated with the findings made by means of electron microscopy.

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Materials and Methods

General.—Initial observations employing light and electron microscopy were made on hemosiderin deposits in hepatic parenchymal cells and reticulo-endothelial cells of rats that had been given a diet containing DL-ethionine for 2 months. Crystalline ferritin was obtained from the pooled livers of these animals. In a similar way the livers of normal control rats were also studied. In further work other rats were given repeated intraperitoneal injections of hemoglobin solutions to produce hemosiderosis. The livers, spleens, and kidneys of these animals were then examined with light and electron microscopes. For comparison, tissue from spleen and liver of a case of Cooley's anemia with massive hemosiderosis was obtained at an operation (splenectomy and liver biopsy), and studied by means of light and electron microscopy.

Animals.—For experimental purposes male and female albino rats of Wistar strain, weighing at least 150 gm., were used.

Diets.—All animals were given food and water *ad libitum*. In the experiment with ethionine a group of rats was given a powdered diet containing adequate amounts of protein, carbohydrate, fat, minerals, and vitamins ("normal protein test diet," Nutritional Biochemicals Corporation, Cleveland), to which DL-ethionine (Nutritional Biochemicals Corporation) was added so as to constitute 0.75 per cent by weight. Control rats were given the diet without ethionine. In the other experiments the rats were fed purina chow.

Hemoglobin.—Crystalline rat hemoglobin was prepared from the blood of normal rats, obtained by cardiac puncture. The blood was drawn into heparinized syringes and expelled into oxalate bottles. Following centrifugation the plasma and buffy coat were drawn off, the red cells suspended in an equal volume of 0.67 per cent NaCl solution, and again sedimented by centrifugation. The supernatant solution was drawn off, and the same process of washing and centrifugation was repeated. Following withdrawal of the saline solution, distilled water at 37°C. was added to the sediment so as to produce a fourfold increase in volume. At this point in the procedure crystallization usually started, but most of the hemoglobin remained in solution. To each 5 ml. of the crystal suspension 2 ml. of toluene was then added, and the mixtures vigorously shaken for 10 minutes. Following subsequent centrifugation the creamy top layers were drawn off, the remainder warmed in a water bath for 30 to 60 minutes to effect solution of the crystals. Following this, the hemoglobin solutions were filtered through Whatman No. 1 filter paper, and the filtrates rapidly chilled to effect crystallization. Well formed crystals were obtained in this way, corresponding to the descriptions of rat hemoglobin given in the detailed account of Reichert and Brown (14). These crystal suspensions were kept in a deep freeze at −10°C. until they were used. Prior to use they were again checked with the light microscope.

Injections.—Male and female rats were given intraperitoneal injections of 5 ml. of suspensions of crystalline hemoglobin containing approximately 0.8 gm. of hemoglobin as determined by means of a routine alkaline hematin method (15). Five injections were given at 2 day intervals, and the animals were sacrificed 3 days following the last injection.

Human Tissue.—A splenectomy and liver biopsy were performed on a 10 year old boy with clinically established Cooley's anemia who had received many transfusions of whole blood. The tissues were obtained immediately following removal.

Preparation of Tissues.—As routine, animals were anesthetized with ether, and tissues excised prior to death. Blocks not exceeding 1 mm. in greatest dimension were fixed in 1 per cent OsO_4 solution buffered at a pH of 7.3–7.4 with a potassium barbiturate—barbituric acid buffer to which crystalline sucrose was added just before use so as to constitute 5 per cent (weight per volume). The tissues were generally fixed for 1½ hours though occasionally

the periods of fixation were shorter. The blocks were dehydrated in graded concentrations of ethyl alcohol and embedded in *n*-butyl methacrylate or in a 9:1 mixture of methyl and *n*-butyl methacrylate. Sections for light and electron microscopy were prepared with a Porter-Blum microtome. For routine light microscopy the sections were stained with basic fuchsin or with hematoxylin.

To confirm the presence of hemosiderin, a modification of Perls' Prussian blue test (Berlin blue test) for ferric iron compounds was employed, as described by Lillie (16). It was also found that in order to bring about the reaction in material embedded in methacrylate, it was necessary to dissolve the methacrylate with warm acetone or toluene. Following such treatment the reaction could be brought about with ease. To make more general surveys with the light microscope, larger blocks were fixed in neutral formalin, dehydrated, embedded in paraffin, and sectioned at 3 to 4 microns with a Spencer microtome. The Prussian blue test was then performed in the same manner (16), and sections were also stained with hematoxylin and eosin, eosin and methylene blue, and iron hematoxylin.

Electron Microscopy.—Most of the microscopy was performed with an RCA electron microscope, model EMU 2b at 50 kv.; but parts requiring high resolution were done with a Siemens Elmiskop 1 at 60 and 80 kv., care being taken to reduce factors detracting from high resolution. A number of observations were made with an RCA EMU 3b microscope. Magnifications were calibrated with a diffraction grating replica, and by spraying latex particles, 1386 A in diameter, upon the sections examined. Sections were mounted on copper specimen grids coated with carbon films.

Crystallization of Ferritin.—This was done according to a procedure described by Granick (6).

EXPERIMENTS AND RESULTS

(*a*) *Hemosiderin in Parenchymal Liver Cells of Rats.*—The initial observations concerning hemosiderosis were made on liver cells of rats that had been given the diet containing ethionine for 70 days.

It was found by means of light microscopy that many of these animals had large amounts of typical brown hemosiderin granules in the hepatic parenchymal cells, and in Kupffer cells, whereas no hemosiderin was found in the livers of 24 control rats that had been kept on a normal diet. Application of the Prussian blue test confirmed this observation. When viewed with a polarizing microscope, the granules proved to be isotropic. Of a series of 24 rats, half male, half female, six (2 males and 4 females) were found to have severe hemosiderosis of the liver. In the remaining animals much less hemosiderin was found in the liver though its presence was always easily demonstrable. Fig. 1 shows the degree of hepatic hemosiderosis in one of the severe cases. Figs. 3 and 4 show the presence of iron-positive granules as well as fat within liver cells. Sections of liver tissue from 4 such rats were also examined with the electron microscope. The findings were similar in all, and examples are shown in Figs. 5 to 9. The majority of liver cells contained large aggregates of electron-dense particles, some aggregates measuring up to 3 μ in cross-section. It was then ascertained that under the light microscope these aggregates were represented by the brown, iron-positive "hemosiderin" granules. The massiveness of the hemosiderin deposits demonstrable with the light microscope, and the electron-denseness, size and number of deposits seen with the electron microscope did not admit of any logically consistent alternative interpretation. The identity of the iron-positive granules and of the electron-dense aggregates was confirmed by comparison of serial sections of individual cells.

It should be noted that there were abnormal changes in many of the liver cells: the presence

of abnormal amounts of fat, nuclear and mitochondrial changes, and evidence of necrobiosis (Fig. 5), but these changes, being outside the scope of the present paper, will not be considered here.

Figs. 6 to 9 reveal that the hemosiderin deposits are composed of innumerable dense particles. Most of these particles have diameters between 30 and 80 A and correspond in size and density to the particles seen by others in so called lysozomes of liver cells (11). It is noteworthy that the dense particles in the liver cells of rats treated with ethionine occur not only in large aggregates, but are sometimes also scattered through the cytoplasm of liver cells (Fig. 9). It can also be seen in Figs. 6 to 9 that many aggregates of the particles forming the hemosiderin granules are relatively homogeneous, and are frequently surrounded by membranes. In Fig. 8 a membrane delimits the aggregate indicated by an arrow, and a membrane-like structure (crista?) projects into the aggregate. In addition, rather homogeneous, densely packed aggregates are often seen without any associated membranous structures (Figs. 6 and 9). Sometimes they are present outside cells, and also in sinusoidal endothelial cells and macrophages in the livers of rats treated with ethionine; in these cells too, the light microscope discloses such aggregates as brown granules that give the Prussian blue reaction. When associated with membranous bodies inside parenchymal liver cells, the aggregates differ from the so called lysozomes by virtue of their relative homogeneity, dense packing, and usually by their larger size. But many of the particles in the aggregates are evidently of the same order of magnitude as those found in lysozomes; *i.e.* between 50 and 60 A. It was further found that while the size of particles within the aggregates varied considerably, particles scattered through the cytoplasm displayed much greater uniformity, their mean diameter being 58 A, with a standard deviation of 7 A.

While the Prussian blue test revealed the presence of iron in discrete hemosiderin granules, it also produced a diffuse blue hue in the cytoplasm of many liver cells. This phenomenon has been known for a long time (16), and has generally been ascribed to diffusion of the Prussian blue from hemosiderin granules and/or differences in solubility of constitutents of the hemosiderin granules under varying circumstances. Yet, as in the present instance, of three adjoining liver cells one might show only discrete blue granules, another a diffusely blue cytoplasm, and the third both, discrete granules and a blue cytoplasm. It appears to the author that electron micrographs provide the basis for a better explanation, *viz.*, the presence of diffusely scattered particles in the cytoplasm of many liver cells. Though aggregates of such particles are large enough to be seen with the light microscope as discrete blue granules in the Prussian blue test, scattered particles are too small to be individually visible. Yet, if enough particles be scattered through the cytoplasm, enough Prussian blue would be formed to be visible.

Fig. 6 shows an aggregate that displays a certain pattern. It can be seen that electron-dense particles are separated or surrounded by "clear spaces." To account for this pattern, it can reasonably be assumed that the "clear spaces" represent matter that is much less dense to electrons, and is associated with the particles, holding them together in an aggregate. Such non-electron-dense matter most likely includes the protein moiety of hemosiderin. Both, hemosiderin and ferritin, contain protein moieties as well as iron micelles, and Farrant (10) has shown that the protein moiety of

ferritin surrounds the iron micelles. It is therefore proposed that the aggregate depicted in Fig. 6 is composed of electron-dense particles representing iron micelles (presumably iron hydroxide–phosphate complexes), and of a protein matrix situated around and between the particles.

To determine whether ferritin can be readily obtained from the hemosiderotic livers of rats treated with ethionine, portions of such livers that had been kept frozen were pooled and processed for extraction of ferritin according to well known procedure (6). Typical crystals of rat ferritin were obtained with ease. The identity of these crystals was confirmed by means of simple chemical and crystallographic procedures that have been described by others (6, 8, 17). A study of the ultrastructure of these crystals is now in progress. By contrast, equal amounts of pooled liver tissue from control rats failed to yield crystalline ferritin when the same procedure for its isolation was used.

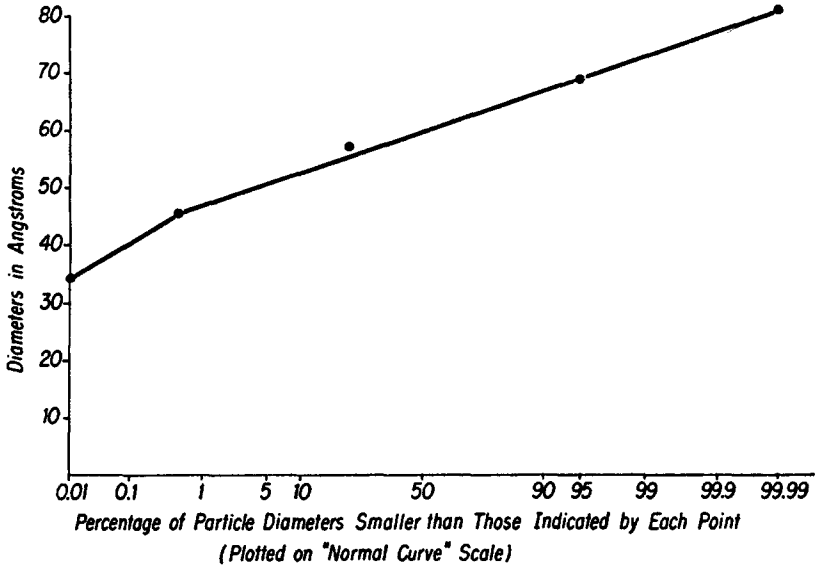
(*b*) *Hemosiderin Deposits Resulting from Injections of Hemoglobin.*—To enlarge the information on the fine structure of rat hemosiderin the following experiment was done:—

Twelve Wistar rats, six male and six female, were given a series of five intraperitoneal injections of crystalline rat hemoglobin as already described. At the time of sacrifice, 12 days following the first injection, gross examination revealed enlargement of the kidneys in all rats, and enlargement of the spleen in several, but did not disclose any other changes. Small blocks from the kidney cortices, and the spleens and livers were fixed and processed for electron and light microscopy as before. Large blocks from all viscera were fixed in neutral formalin, and embedded in paraffin for more extensive surveys with the light microscope.

In all the kidneys of these twelve animals iron-positive granules were readily demonstrable in the majority of cells of the proximal convoluted tubules, especially those in the outermost cortex (Fig. 2). In unstained sections examined with the light microscope these granules had the typical brown color and somewhat refractile quality of hemosiderin granules, and they were isotropic. In some cross-sections of convoluted tubules, every cell contained such granules. Using a technique described by Granick (4), bits of fresh kidney cortex were minced on a slide and treated with 10 per cent cadmium sulfate solution, whereupon typical crystals of ferritin formed within ½ hour. Such crystals could not be produced in comparable tissue from control rats. Sections from several of these kidneys examined with the electron microscope revealed aggregates of dense granules, the aggregates measuring up to 2 μ in cross-section (Figs. 10 to 16). The individual granules of which the aggregates were composed varied remarkably little in size. Measurements were made of 200 such particles on photographic enlargements and on lantern slide projections, using an enlarging eyepiece ruled with a micrometer scale. The mean diameter of the particles shown in Figs. 12 and 15 proved to be 55 A, with a standard deviation of 6 A. As shown in Text-fig. 1, graphic representation on probability paper, ruled according to the normal distribution law, clearly indicates that the particle sizes have a "normal" distribution, a straight line being formed. The data in this graph show that 95 per cent of the measurements were "less than" 68.4 A. In fact, because the graph represents only discrete points, 94.5 per cent of the measurements lay between 46 and 57 A. Such "absolute" figures are, of course, dependent on accurate calibration of the magnification obtained in the electron microscope. However, if one compares the measured diameters of the individual particles in relation to each other, calibration errors are constants applying to all the measured particles. In relative terms the data in Text-fig. 1 show that 94.5 per cent of the measurements lie within approximately 20 per cent of each other. If one takes into account the sources of error in making the measurements, it becomes highly probable that

the actual variation in the diameters of the particles shown in Figs. 12 and 15 is considerably smaller. Measurements were also made on pictures of the scattered cytoplasmic particles in other sections from the same kidney, and from kidneys of several of the other treated rats, and the results agreed closely with those just described. It may be added here that these results agree well with the value of 55 A reported for the electron-dense center of the ferritin molecule by Farrant. The implications will be taken up in the discussion.

As can be seen in Figs. 10 to 12, and 15, aggregates in the renal tubular cells are at times delimited by membranes. In Fig. 10 the presence of a "double" membrane around an aggregate is reasonably clear and in Fig. 15 one may suspect the existence



TEXT-FIG. 1. Frequency distribution of particle diameters in rat kidney cells.

of such a membrane. Moreover, Fig. 10 shows "cristae" with double membranes that extend from the periphery towards the inside of the body containing dense aggregates of particles. The disposition of these membranes around and within the body shown in Fig. 10, and the contour of this body suggest that it might represent an altered mitochondrion. The bodies shown in Figs. 11 to 13 also have peripheral membranes (and perhaps cristae), but at various points these appear to be disrupted. Commonly, the bodies containing the aggregates are larger than neighboring mitochondria. It may be recalled, however, that mitochondria can expand and rupture, and lose their cristae as a result of various kinds of treatment. So called "explosions" of mitochondria have also been observed as artefacts of fixation. It is quite possible, therefore, that the bodies shown in Figs. 10 to 13 and 15 represent greatly altered mitochondria. At the present time, and only for the purpose of verbal identification, the author proposes that such membranous bodies containing aggregates of dense particles be called "siderosomes." There is a similarity between the body shown in Fig. 10 in the cell of a proximal convoluted tubule, and those shown in Figs. 7 and 8 in hepatic

parenchymal cells, and for this reason the latter bodies might also be called "siderosomes." In fact, the author has seen similar bodies inside sinusoidal endothelial cells in liver and spleen of hemosiderotic rats, and occasionally in macrophages.

It can further be seen (Figs. 10, 12, 13, and 15) that the small, dense particles occur not only in aggregates, but are sometimes also scattered about the cytoplasm of the tubular epithelial cells, a phenomenon already encountered in the liver cells of rats treated with ethionine. Fig. 14 shows that some aggregates and individual particles were found in close proximity to brush borders of tubular epithelial cells.

In order to systematize these findings in the cells of the proximal convoluted tubules at this point, the following hypothesis is offered:—

The iron-containing aggregates seen in the cells of proximal convoluted tubules of rats treated with hemoglobin may be mainly composed of ferritin molecules. The aggregates are formed within "siderosomes," structures delimited by membranes and possibly derived from mitochondria (Figs. 10–12). The siderosomes later disintegrate, thereby releasing the ferritin molecules into the cytoplasm where they are dispersed both in clumps and singly by the protoplasmic movements (Figs. 13 and 15). Aggregates and individual molecules may then at times be extruded from the cells into the tubular lumen and be excreted in the urine (Fig. 14). In this relation it may be recalled that, by means of light microscopy, iron-containing granules with the appearance of hemosiderin can be demonstrated in the urine of animals injected with hemolysates, and of patients suffering from hemochromatosis.

In the spleens of these rats there were deposits of brown, iron-positive granules that were similar to the granules seen in the cells of the proximal convoluted tubules. These "hemosiderin" granules were situated in macrophages and in sinusoidal endothelial cells, and outside of cells in the sinusoids. On the whole, the brown granules were larger than those in the kidneys and in places clumps of granules were prominent. Again, electron microscopy revealed dense aggregates of small particles as well as scattered individual particles in the cytoplasm of the affected cells; and again some of the aggregates were situated within discrete structures with membranous boundaries. Particle diameters ranged between 40 and 70 A, but the resolution attained was not quite sufficient to warrant a detailed statistical evaluation.

The observations on the livers of these animals did not differ significantly from those just described. Hemosiderin granules were almost entirely confined to the sinusoidal endothelial cells and to scattered macrophages. In the parenchymal cells they were hard to find with the light microscope, even in large sections. Electron-dense particles corresponding in size, distribution, and disposition to those found in the splenic and kidney cells were again present. In the small blocks taken for electron microscopy no particles were encountered inside parenchymal liver cells though this was undoubtedly due to inadequate sampling.

(*c*) *Hemosiderin in a Case of Cooley's Anemia (Thalassemia Major)*.—It is

well known that hemosiderin occurs in different mammalian species, and ferritin has been isolated from several different mammals (6). Inasmuch as both contain protein moieties, and the species specificity of proteins is very general, it can be assumed that there are also species differences in the composition of hemosiderin and ferritin. Indeed, some differences in the form of ferritin crystals obtained from different mammalian species have been observed, and also differences in immunological specificity (5, 7, 18). One may ask whether such differences are confined to the protein moieties or whether they apply to the iron micelles as well. Evidence provided by others (5, 18) indicates that specificity in the composition of ferritin is dependent upon the protein component, apoferritin. Moreover, the apoferritins of different animal species are immunologically related. Much less is known about species differences in the composition of hemosiderin, and since hemosiderin has generally been found to be inhomogeneous, the demonstration of such differences might be quite difficult. Still, the known similarities of hemosiderin and ferritin and their apparent relationship, (6, 19, 20), suggest that differences in the composition of hemosiderin from various mammalian species would not involve the iron moiety.

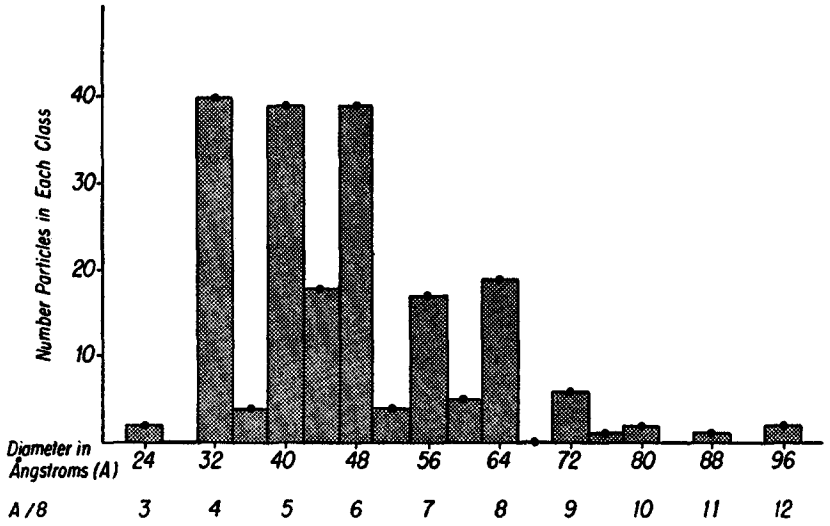
To learn more about the nature of hemosiderin and ferritin in man, tissue was obtained from the liver and spleen of a ten year old boy who was suffering from severe Cooley's anemia, and had received many transfusions of blood. As already indicated, the material was obtained fresh in the operating room. Specimens were prepared for light and electron microscopy as before. Hemosiderin was found in massive quantities in liver and spleen, deposits being present in most of the hepatic parenchymal cells, and in sinusoidal endothelial cells, and macrophages. There were also many extra-cellular accumulations of hemosiderin.

Studies with the electron microscope again revealed dense particles that occurred as aggregates or were individually scattered through the cytoplasm of hepatic and splenic sinusoidal endothelial cells, macrophages, and hepatic parenchymal cells (Figs. 17 and 19). An association of aggregates with membranous bodies is not evident in the pictures shown, though there are features that suggest it in Fig. 19 (see arrows in the pictures).

Most of the scattered particles shown in Figs. 17 and 19 have diameters of about 60 A. Measurements on 200 representative particles gave a mean diameter of 61 A with a standard deviation of 8 A, values that are close to that obtained by Farrant for horse ferritin (10). By contrast, there was considerable variation in the diameters of particles situated within large aggregates that represent the hemosiderin granules proper.

In order to obtain more precise information on the size and shape of these particles in man, pictures were taken with a Siemens Elmiskop 1. Fig. 20 is an example of a hemosiderin granule photographed at a primary magnification of 46,300 times, and subsequently enlarged. The diameters of the particles in this aggregate were measured on suitable photographic enlargements, using a telescopic eyepiece provided with a micrometer scale. In order to take into account deviations of individual particle profiles from circularity in the statistical computa-

tions, two measurements of diameter were made on discrete particles, at 90 degree angles to each other. In addition, the same particles were measured by two different observers, but the differences in measurements did not alter the results significantly. These results are summarized graphically in Text-fig. 2. As can be seen there, the diameters of a great majority of the particles in the hemosiderin granule fall into "classes" that appear to be integral multiples of the same unit. Thus, there are frequency peaks at intervals of approximately 8 A, and only a minority of the diameters lies between these peaks. The periodicity thus revealed is striking, and it suggests that the dense particles in the hemosiderin granule are clusters of much smaller subunits of uniform size, the diameters of which might be in the neighborhood



TEXT-FIG. 2. Frequency distribution of particle diameters in hemosiderin granule in a human liver cell.

of 8 A. The size groups between the various peaks in Text-fig. 2 may represent particles that were sectioned with the glass knife or may be due to asymmetry in the particle profiles.

Further information on structure and size of the dense particles was sought through a study of ferritin that had been isolated from the spleen of the same patient.

Crystals of ferritin were prepared and redissolved according to the method of Granick (6), employing cadmium sulfate and ammonium sulfate. The final solutions of ferritin were dialyzed against distilled water for 24 hours to remove as much of the salts as possible. Furthermore, the mother liquor from which the crystals had been precipitated was dialyzed in the same way. Specimen grids, coated with carbon films, were then dipped into the dialysates, dried, and used for electron microscopy.

Pictures obtained on samples from the two types of ferritin solutions were essentially alike. As can be seen in Fig. 18, they reveal innumerable dense particles in many of which substructure is evident. The appearance of some of these particles indicates

the presence of four subunits, similar to those found by Farrant (10) in horse ferritin. The diameter of these particles is about 60 A, and that of the subunits about 30A. In the light of Farrant's findings the particles depicted in Fig. 18 may be considered to represent clusters of iron micelles in individual ferritin molecules. It should be emphasized here that the protein shells of ferritin molecules are not visible in pictures of "unshadowed" preparations such as that shown in Fig. 18, but their presence was demonstrated by Farrant on material that had been lightly "shadowed" with uranium.

It is noteworthy that the aggregate shown in Fig. 20 contains a substantial number of particles with diameters near 30 and 60 A, and that there are frequency peaks in these regions as shown in Text-fig. 2. This finding supports the view of Granick (6, 19) that the individual iron micelles in ferritin and in hemosiderin are the same. As will be pointed out later, the finding also suggests that ferritin is a constituent of hemosiderin.

DISCUSSION

The observations here recorded provide information on several aspects of hemosiderosis. They demonstrate that hemosiderin granules in different types of cells, occurring under diverse circumstances and in two different species, all contain innumerable particles of great electron density. That these particles represent iron "micelles" seems inescapable in the light of evidence provided by others.

Briefly, studies on the chemical constitution and paramagnetic properties of ferritin and hemosiderin (6–9, 21, 22) as well as x-ray diffraction studies (10, 23, 24) have indicated that the iron in these compounds is present in the form of inorganic micelles, that appear to be ferric hydroxide–phosphate complexes. Furthermore, Farrant has confirmed the presence of such micelles in crystalline horse ferritin by means of electron microscopy (10). He found that the ferritin molecule contains a core of closely adjoining, electron-dense particles, commonly four, and a peripheral shell that is not dense to electrons and is detectable only in "shadowed" material.[1] The quadruplets of dense particles proved to have diameters of about 55 A, while the individual particles had diameters of about 27 A. The results of Farrant's experiments, when considered together with the physico-chemical findings of the other workers, provide a powerful basis for the inference that the electron-dense particles seen in electron micrographs of ferritin represent groups of ferric hydroxide–phosphate micelles of ferritin molecules.

In the material from rats treated with ethionine and from those treated with hemoglobin the mean diameter of the scattered cytoplasmic particles was between 50 and 60 A, with a relatively narrow range, and in the human material it was about 60 A. These values agree closely with Farrant's value for the

[1] It should be added here that treatment of crystalline rat or human ferritin with the osmium tetroxide fixative does not render the protein moiety visible, as control studies by the author have shown.

diameter of the electron-dense core of the ferritin molecule (55 A). It must be added, however, that Farrant's measurements were made on horse ferritin, and that particle size distribution data were not included in his report.

One may ask why substructure was not clearly evident in the particles in the sectioned tissues examined in the present experiments. In answer to this it must be pointed out that the best resolution obtained on embedded and sectioned material from rats was not quite sufficient to reveal clear detail in individual particles. On the other hand, the best resolution obtained on the human particles (such as shown in Fig. 20) should have been sufficient. However, the embedding medium (methacrylate) and the depth of the sections (about 200 A) probably account for obscuration of detail.

It has already been mentioned that the dense particles in the packed aggregates (hemosiderin granules) vary considerably in size, and that the evidence at hand indicates that these particles represent clusters of smaller units; and further, that the diameters of most of these particles are integral multiples of such smaller units. In some hemosiderin deposits, however, *e.g.* in the cells of renal tubules of rats treated with hemoglobin, the particles displayed great uniformity of size. Judging from their mean diameter (55 A), these particles may be the cores of ferritin molecules. Indeed, it has been found by Hampton and Mayerson (25) that ferritin is readily obtainable from the kidneys of mice following intraperitoneal injections of hemoglobin, and in amounts proportional to the injected dose of hemoglobin. Furthermore, tissues rich in hemosiderin are also rich in ferritin (6, 7, 19). However, it is also known that the composition of hemosiderin is inconstant (21, 22, 26, 27). The present findings are compatible with the inference that ferritin is one of the constituents of hemosiderin, and that in some instances granules of hemosiderin as seen with the light microscope may be predominantly composed of ferritin. The finding that the dense particles in hemosiderin granules are often closely packed is in accord with evidence that hemosiderin often contains more iron, gram for gram, than does crystalline ferritin (6, 22).

The observations that some hemosiderin deposits are contained in membranous bodies ("siderosomes"), and that these bodies commonly show disruptive changes will now be briefly considered again. It has already been suggested that hemosiderin granules may be formed in such bodies. Perhaps the synthesis of hemosiderin takes place within them. Another possibility is that hemosiderin is catabolized within such bodies. However, if this were true, it would be difficult to account for the fact that a single cell may contain large dense aggregates inside as well as outside the membraneous bodies (Figs. 9, 15). It seems likely that aggregates are liberated from these bodies by virtue of disintegration of the membranes, and that variation in the appearance of the bodies represents developmental stages. Furthermore, particles may also be extruded singly (Figs. 10, 12, 15).

Is there any relation between the "siderosomes" and the "lysozomes" (peribiliary bodies)? It seems probable that the dense particles that are often seen in the so called lysozomes (11, 31) and whose diameters are somewhere between 50 and 70A, are of the same nature as those within the siderosomes. Indeed they are now thought to represent ferritin (11). The findings of Novikoff *et al.* (11), and of Palade and Siekevitz (12) indicate, however, that lysozomes are more heterogeneous than are the siderosomes. Moreover, lysozomes have thus far been identified only in liver cells, and the term "peribiliary bodies" used by Palade and Siekevitz implies this. The author has also seen such bodies, and they were encountered in the liver cells of rats with hemosiderosis but not in renal tubular cells or in macrophages. Their appearance is so variable, however, that it seems likely that these bodies are not structures of one kind at all. They may in fact be variable derivatives of common cytoplasmic organelles—perhaps the mitochondria—and the finding that they are rich in hydrolytic enzymes does not preclude this possibility. Much further information is required to elucidate the nature of the "lysozomes."

The hypothesis that hemosiderin is formed within discrete cytoplasmic organelles dates back many years. It has been repeatedly proposed that hemosiderin may be formed by the mitochondria of cells (28, 29). In recent years the chief proponents of this view have been the Gilmans (29). From careful cytologic studies employing light microscropy these workers have concluded that there is a close relationship between hemosiderin granules and mitochondria in liver cells of patients with nutritional deficiencies. They have applied the term "cytosiderosis" to this phenomenon. In the light of more recent work with electron microscopes, it has become clear that mitochondria have a characteristic internal structure, and that there are other cell organelles that have a similar size range and might not be distinguishable from mitochondria if only light microscopy were employed (30). The present findings demonstrate that some hemosiderin does indeed occur inside of discrete organelles, and that sometimes these organelles resemble mitochondria. Moreover, the presence of such organelles in parenchymal cells of liver and kidney, and in cells of the reticulo-endothelial system suggests that the mechanism whereby hemosiderin deposits are formed is similar in diverse types of cells. Although many of these bodies have features in common with mitochondria, it seems wisest to reserve judgment on their nature and origin. Hence the operative term "sidersomes" is proposed for them.

SUMMARY

Hemosiderin deposits in rats and in man were studied and compared by means of electron and light microscopy. Typical, isotropic, iron-positive hemosiderin granules were found to contain innumerable, closely packed, electron-dense particles, embedded in matter that was much less dense to electrons. Similar dense particles were often scattered diffusely through the cytoplasmic matrix of cells containing hemosiderin granules.

In cells of proximal convoluted tubules of rats given repeated intraperitoneal injections of hemoglobin the hemosiderin granules contained dense particles with a mean diameter of 55 A, and with a size-frequency distribution that indicated uniformity. These particles corresponded in size to the iron micelles of ferritin molecules. There was less uniformity of particles in hemosiderin granules situated in liver and reticulo-endothelial cells of rats that had been given a diet containing ethionine.

The dense aggregates representing hemosiderin granules were often situated inside discrete cytoplasmic organelles that were bordered by membranes, and sometimes contained "cristae"; and often the membranous borders were markedly disrupted. The term "sidersomes" is proposed for these specialized cytoplasmic structures which may be derivatives of mitochondria, and apparently play a part in the formation of hemosiderin.

Ferritin was crystallized from the livers and kidneys of the hemosiderotic rats with ease, but could not be crystallized from comparable quantities of liver and kidney tissue of untreated control rats.

Specimens from the liver and spleen of a patient with advanced hemosiderosis, obtained at an operation, were also studied. In liver and reticulo-endothelial cells many particles with diameters of about 60 A were scattered through the cytoplasmic matrix. By contrast, hemosiderin granules in the same cells contained particles that varied considerably in size. In representative granules, examined at high resolution, the size-frequency distribution of particle diameters displayed a periodicity consistent with the presence of small, uniform subunits. Electron micrographs of ferritin, isolated from the spleen of the same patient, provided confirmation for the inferences that the dense particles observed inside cells are iron micelles, and that ferritin is probably a component of hemosiderin.

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EXPLANATION OF PLATES

The photographs for Figs. 1 to 4 were taken by Mr. Julius Mesiar. All others by the author.

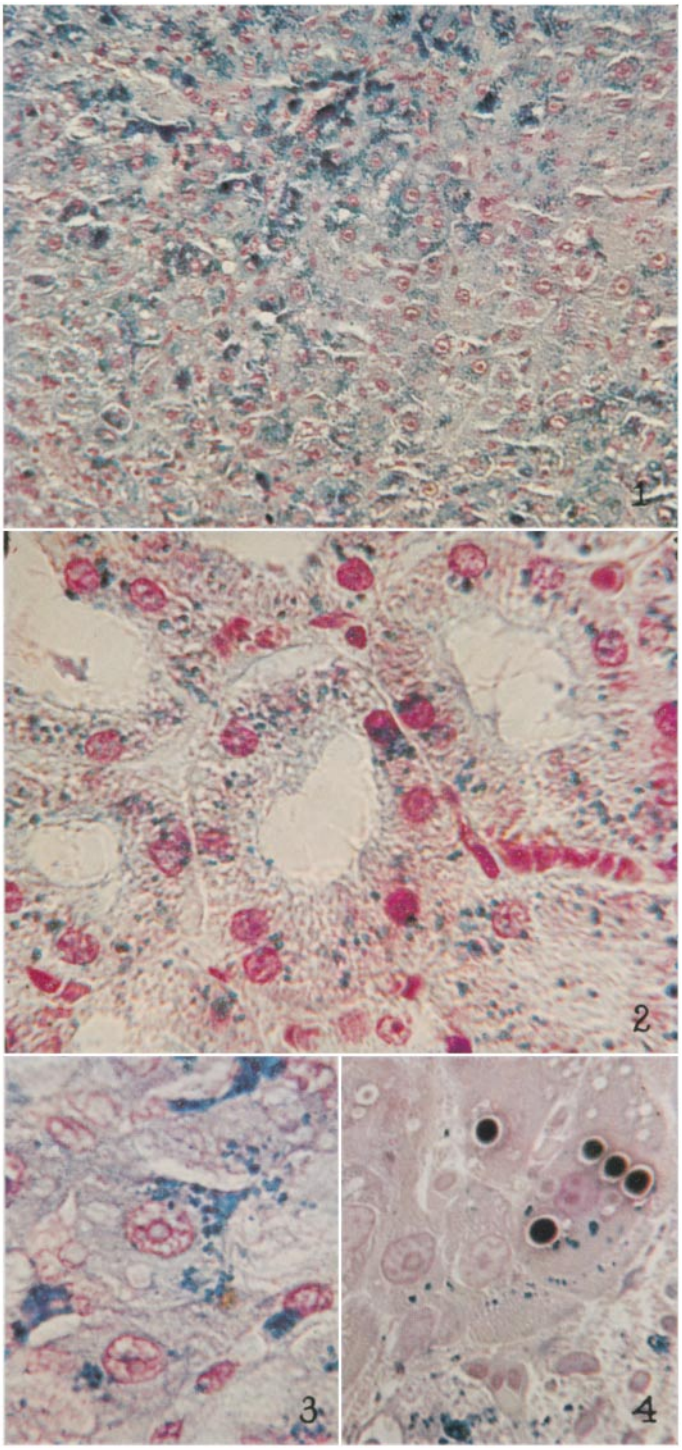
PLATE 24

FIG. 1. Photomicrograph of liver section from a rat fed ethionine. Iron-positive (blue) deposits of hemosiderin are present in the cytoplasm of the majority of liver cells. Fixed in neutral formalin and embedded in paraffin. Potassium ferrocyanide reaction with basic fuchsin counterstain. × 180.

FIG. 2. Photomicrograph of proximal convoluted tubules of rat after 5 intraperitoneal injections of hemoglobin. Many iron-positive cytoplasmic granules are present. Same fixation, embedding, and stain. × 810.

FIG. 3. Detail of Fig. 1. Note the iron-positive (blue) cytoplasmic granules, the bluish hue of the cytoplasm, the vacuoles, and loss of cytoplasmic detail. In the lower right portion of the picture is a Kupffer cell containing hemosiderin. × 810.

FIG. 4. Photomicrograph of liver section from rat fed ethionine. Fixed in buffered osmium tetroxide solution and embedded in methacrylate. There are iron-positive granules (blue), fat globules (brown), and vacuoles in the liver cells. Necrobiotic cells are also present. Same stain as above. × 810.

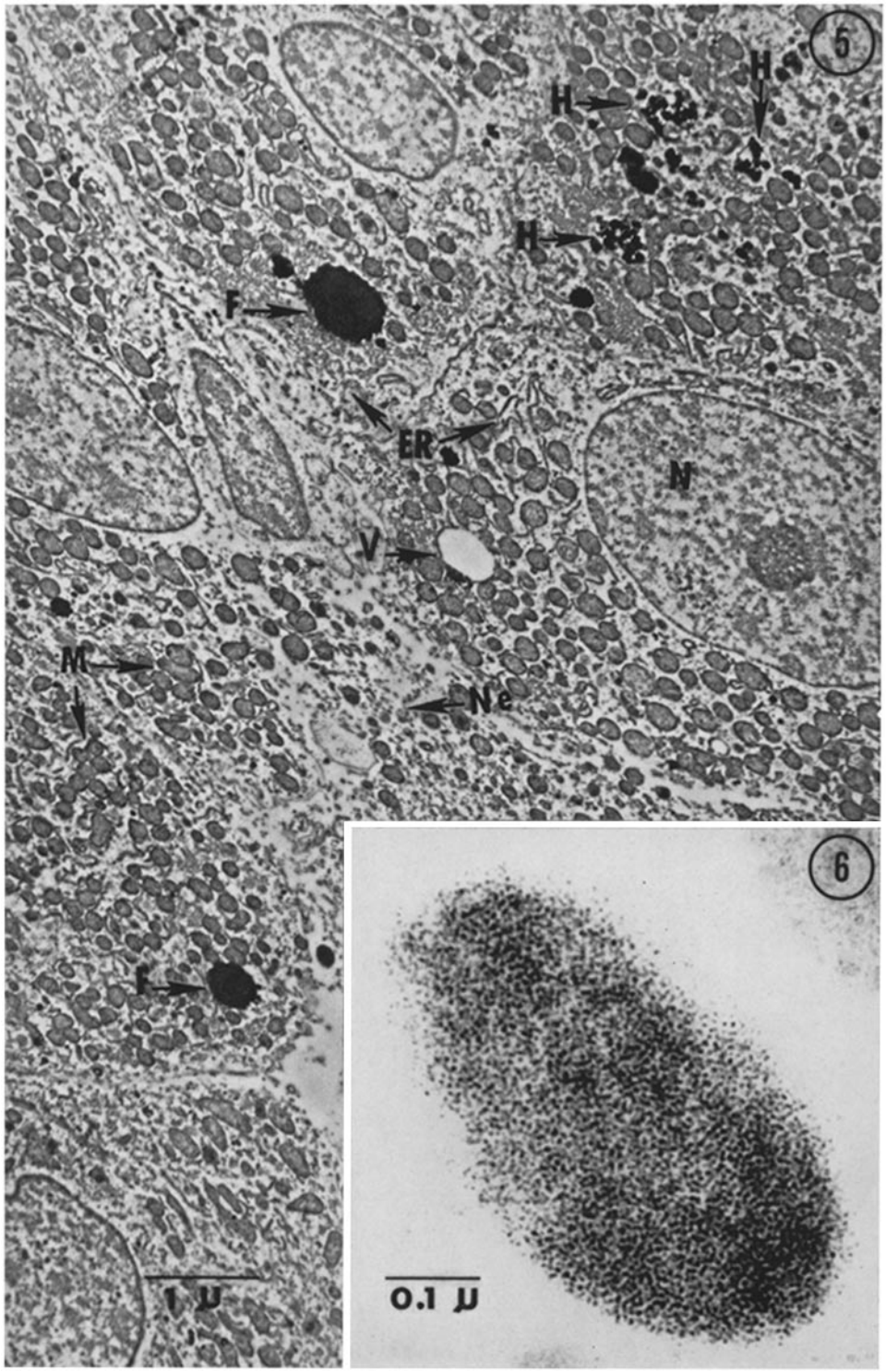


(Richter: Electron microscopy of hemosiderin)

PLATE 25

FIG. 5. Electron micrograph of several liver cells from a rat fed ethionine (same liver as in Figs. 1, 3, and 4). The dense aggregates, marked *H*, represent hemosiderin deposits, and under higher magnification have a distinctive appearance (Fig. 6). There are several other abnormal alterations. At *Ne* the cytoplasm of a liver cell appears disrupted and there is loss and distortion of mitochondria; *F* denotes fat droplets and *V* is a vacuole. ER represents endoplasmic reticulum, *M* mitochondria, and *N* is a nucleus with a prominent nucleolus. × 5000. (The scale now appearing on the figure should read 3μ instead of 1μ.)

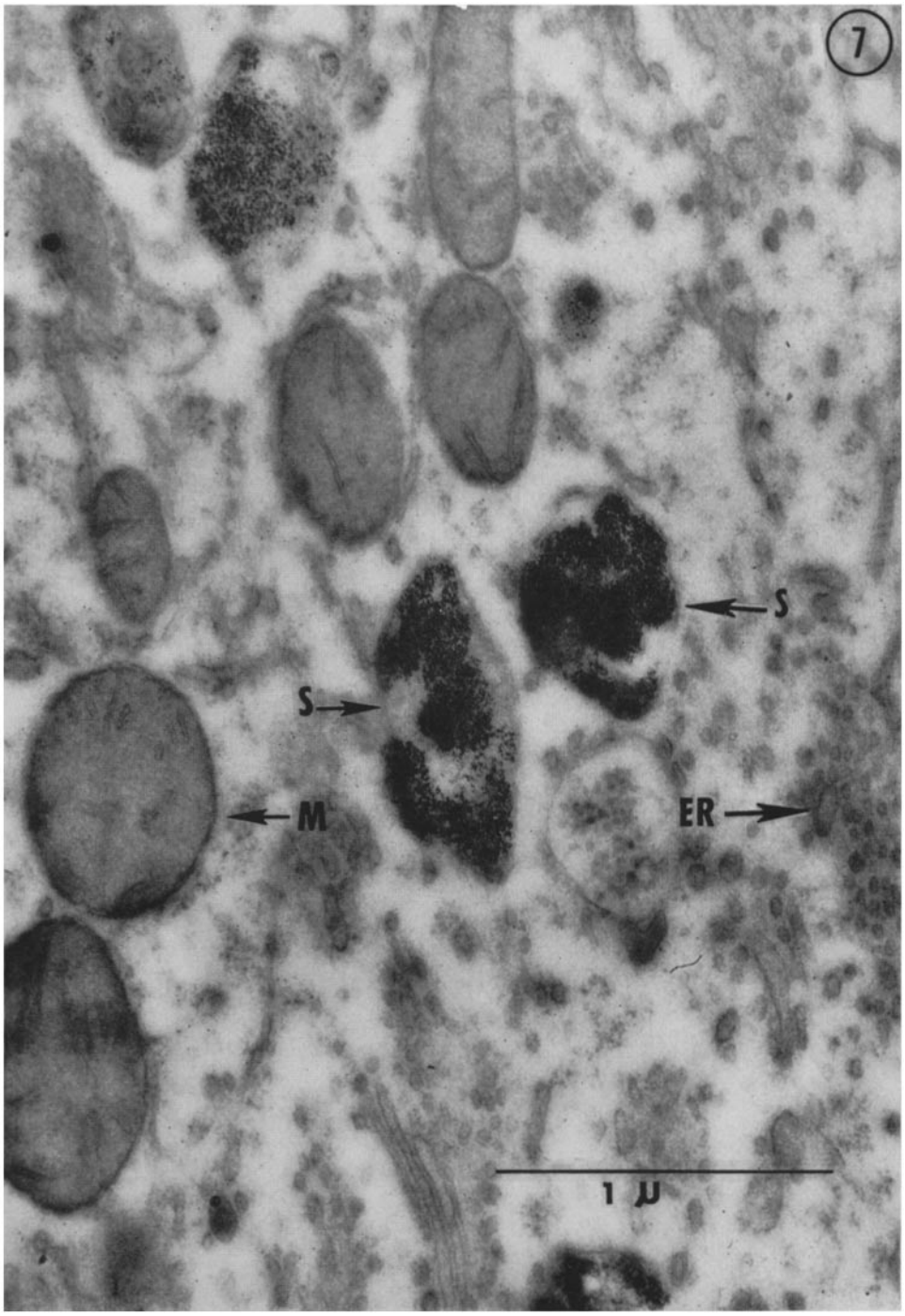
FIG. 6. A typical hemosiderin granule in the same liver. Note the innumerable electron-dense particles that are embedded in a seemingly non–electron-dense matrix. Most of these particles have diameters between 40 and 60 A. × 130,000.



(Richter: Electron microscopy of hemosiderin)

PLATE 26

FIG. 7. Part of liver cell of rat fed ethionine. Several aggregates of dense particles are present in discrete bodies, the "siderosomes." Two aggregates, marked *S*, have peripheral double membranes. Mitochondria (*M*) are prominent as is the endoplasmic reticulum (*ER*). × 47,000.

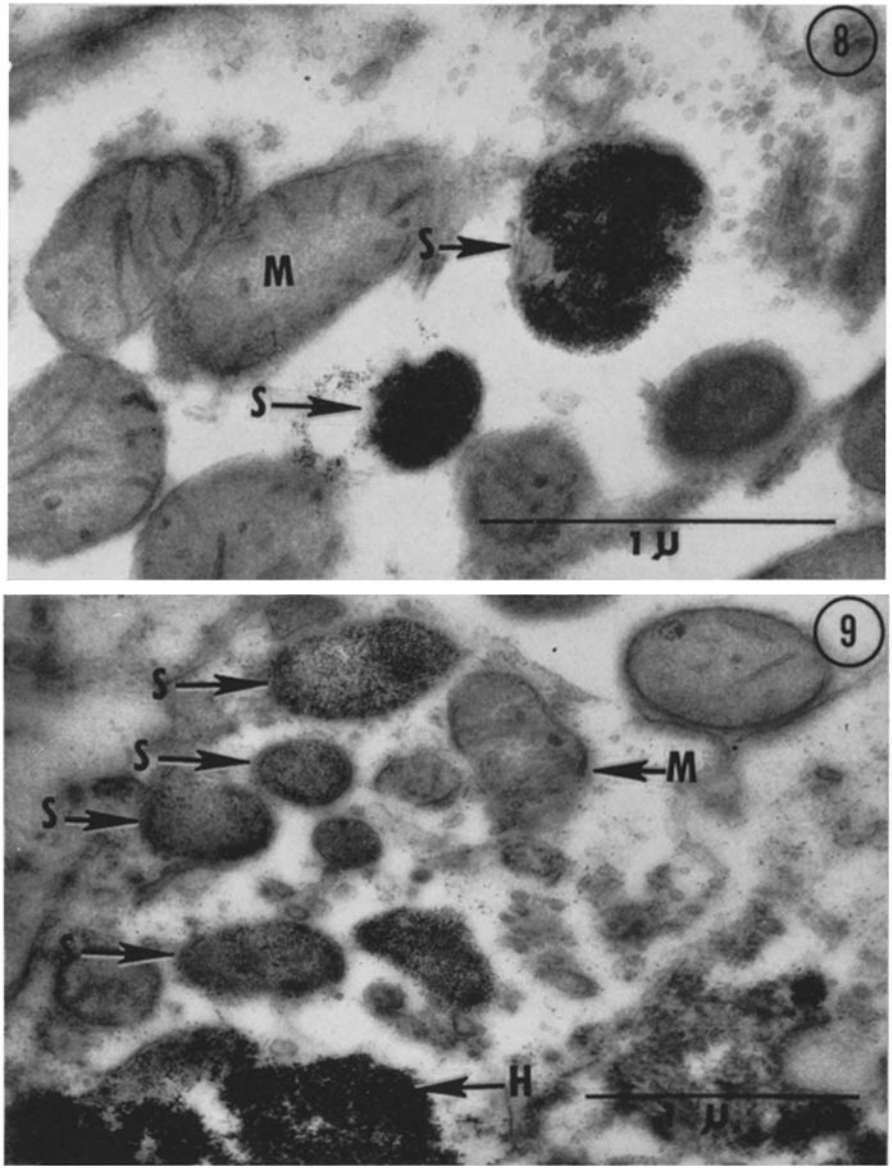


(Richter: Electron microscopy of hemosiderin)

PLATE 27

FIG. 8. Part of liver cell of another rat fed ethionine. There are two siderosomes (*S*). In the upper one the enclosing membrane is clearly visible, and two "cristae" project into the interior of the body near the arrow. Note that the membranous structures of this body are similar to those of neighboring mitochondria (*M*). × 47,000.

FIG. 9. Part of liver cell from the same rat. Loosely packed aggregates of dense particles are present in several siderosomes (*S*). *H* is a large aggregate that is free of membranous structures. *M* is a mitochondrion. Note scattered particles in cytoplasmic matrix. × 36,000.



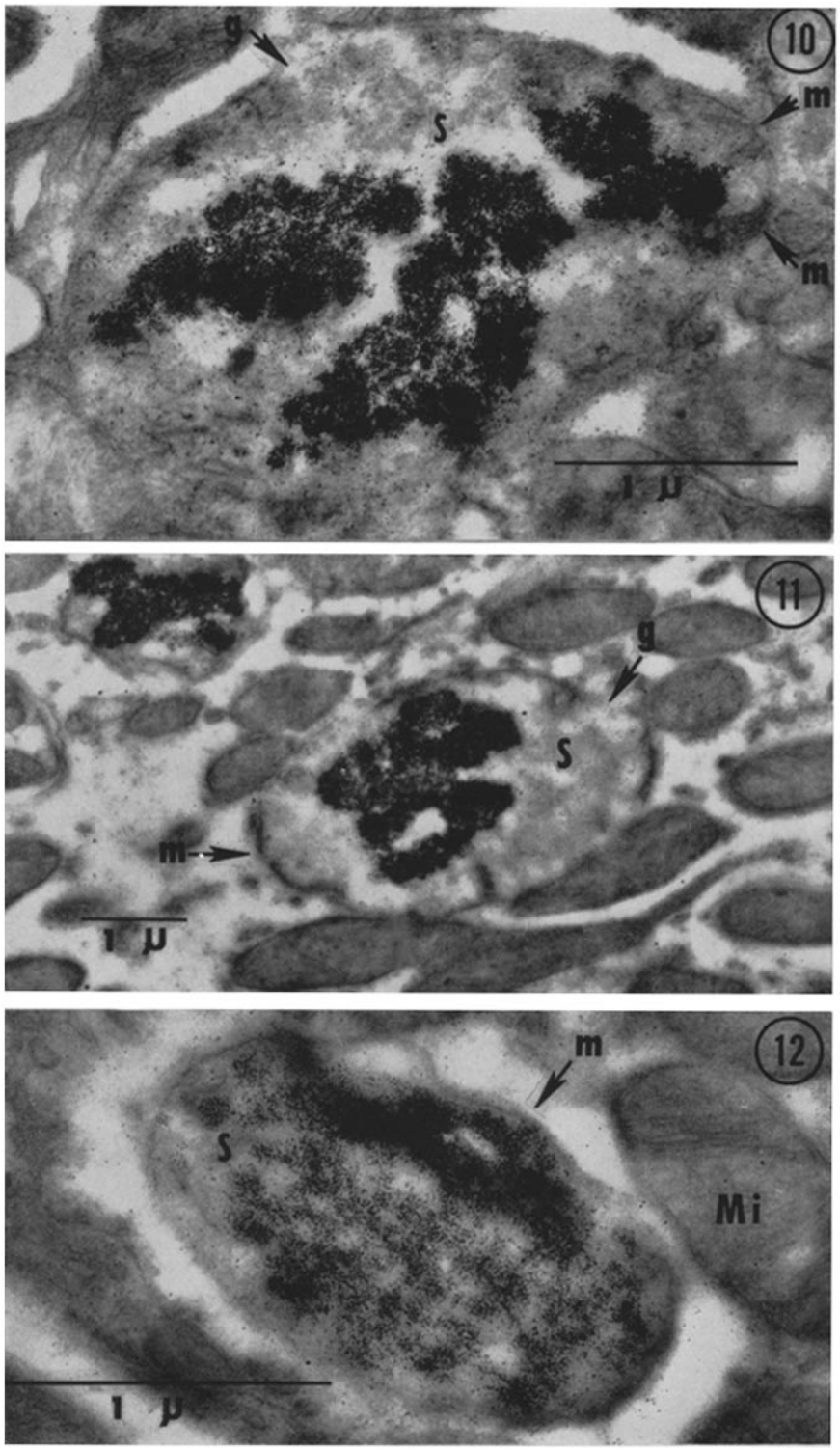
(Richter: Electron microscopy of hemosiderin)

PLATE 28

FIG. 10. Part of cytoplasm of cell in proximal convoluted tubule of a rat treated with intraperitoneal injections of rat hemoglobin. Most of the picture is occupied by a siderosome (*S*) that is delimited by a double membrane. In regions marked *m*, cristae project into the interior. There are gaps (*g*) in the limiting double membrane, and it appears that dense particles are being extruded from the siderosome. × 33,000.

FIG. 11. Siderosome (*S*) in another cell of a proximal convoluted tubule of the same kidney. The membranous border (*m*) is interrupted by gaps (*g*), and seems to have expanded away from the dense aggregate in the center. Note that the siderosome is considerably larger than the surrounding mitochondria. In the upper left corner is another aggregate that is surrounded by a membrane. × 14,600.

FIG. 12. Siderosome (*S*) in a cell of a proximal convoluted tubule of another rat that had been given intraperitoneal injections of hemoglobin. This body has a somewhat folded peripheral membrane (*m*), and contains a rather homogeneous "population" of dense particles that measure about 55A in diameter. Similar particles are scattered through the cytoplasm. There is a mitochondrion (*Mi*) near the right border. × 45,000.

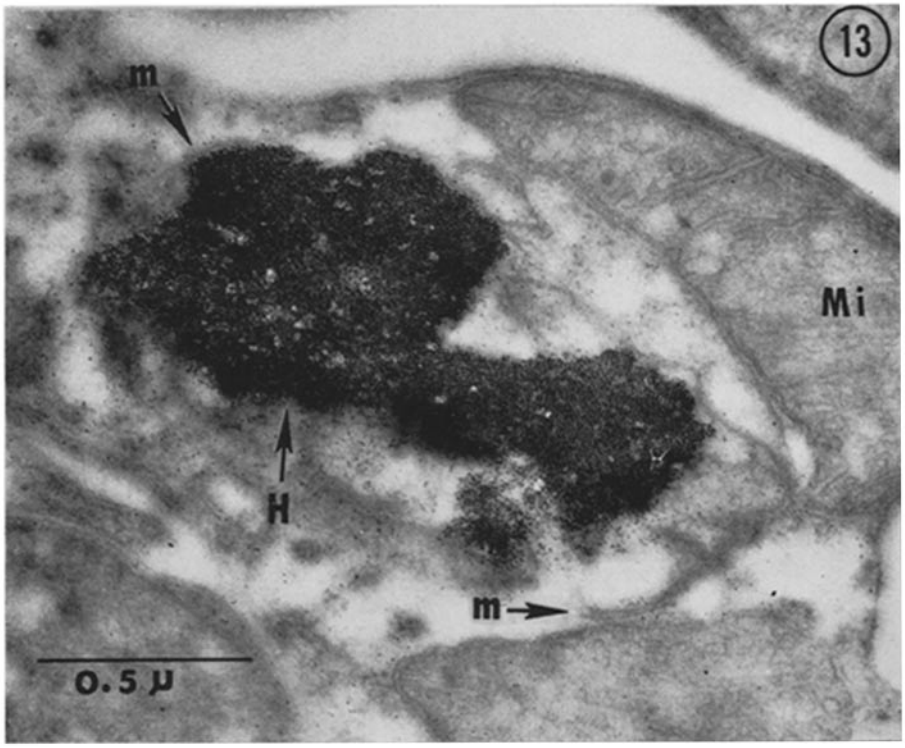


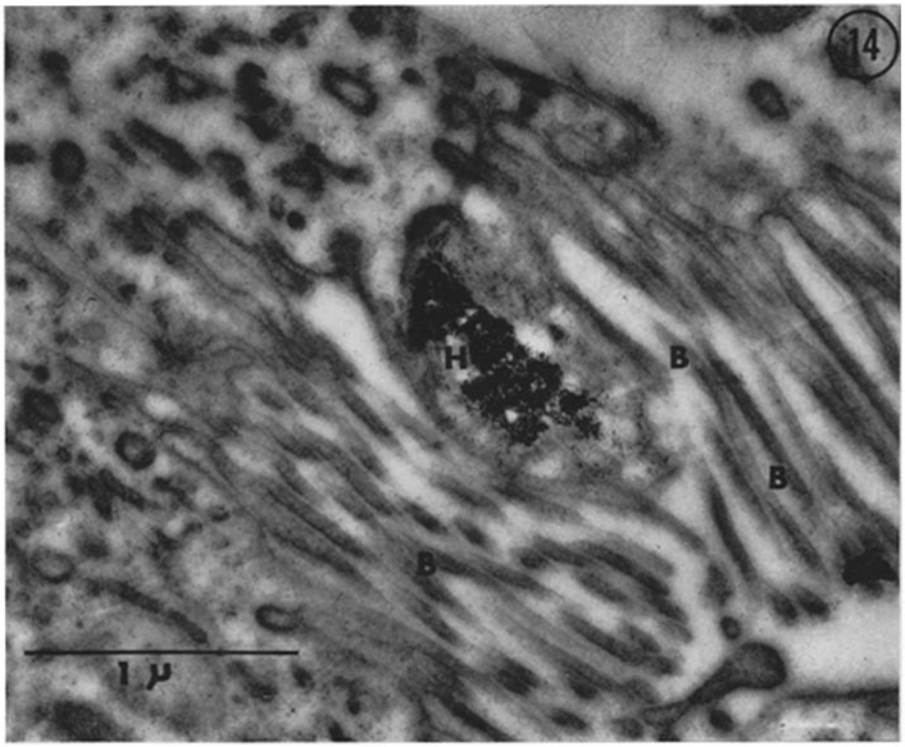
(Richter: Electron microscopy of hemosiderin)

PLATE 29

FIG. 13. Part of cytoplasm of cell of a proximal convoluted tubule from the same rat. Disrupted "membranous" structures are situated about a compact aggregate (*H*). Possible membranous borders (*m*) are indicated in two places. Mitochondria (*Mi*) are situated nearby. × 56,000.

FIG. 14. Brush border of cell of proximal convoluted tubule from the same rat. In close proximity to the brush border (*B*) is an aggregate of dense particles (*H*). At the lower left, individual particles are scattered about villi of the brush border. × 36,000.

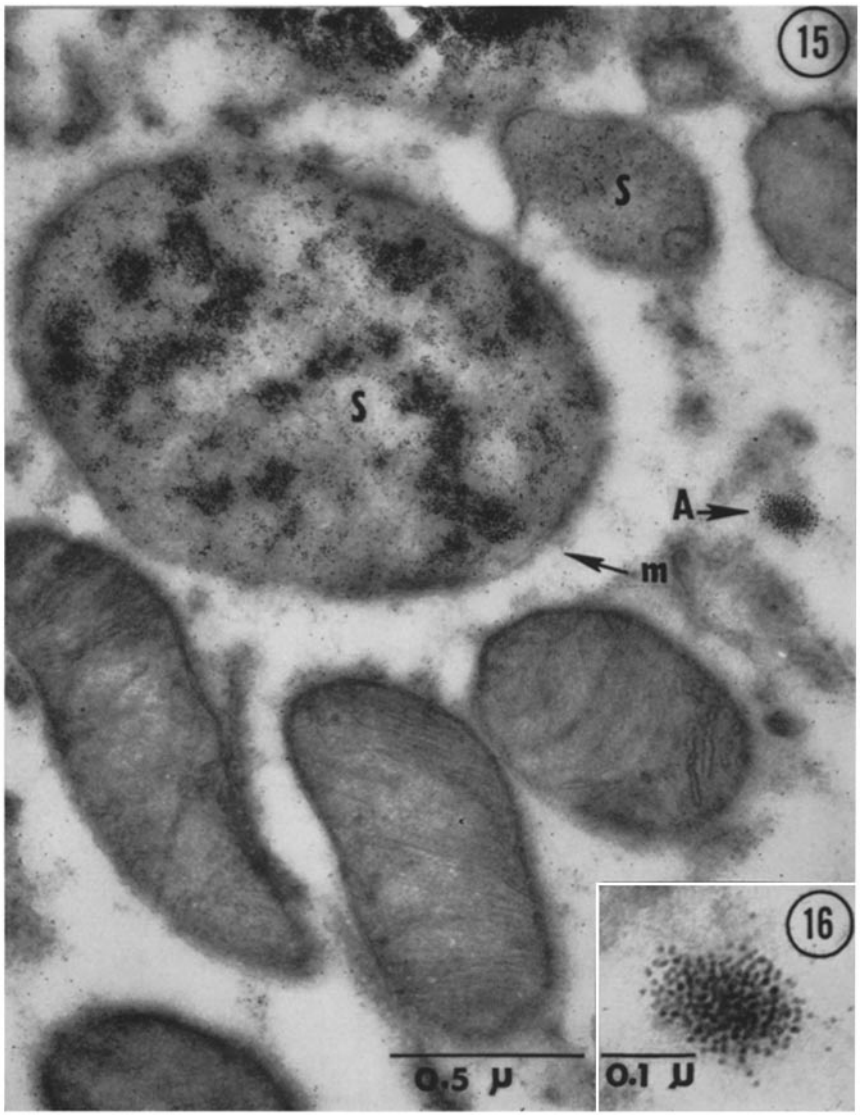




(Richter: Electron microscopy of hemosiderin)

PLATE 30

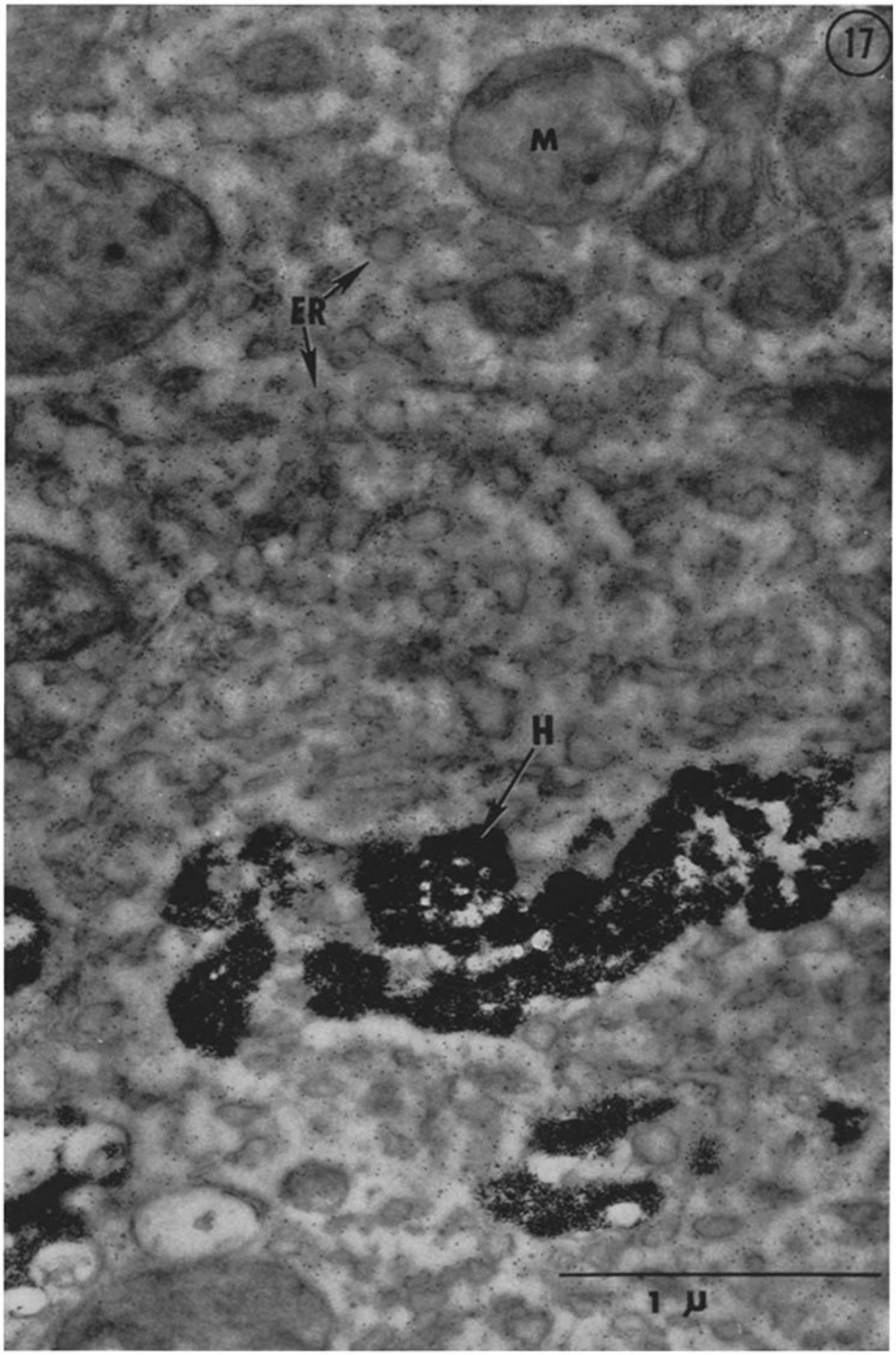
FIGS. 15 and 16. Part of cytoplasm of another cell in a proximal convoluted tubule of the same rat. There are two siderosomes (*S*) that differ in size but contain similar dense particles with a mean diameter of 55 A (see text). There is also an aggregate (*A*) that has no limiting membrane and is shown at higher magnification in Fig. 16; *m* is the limiting (double ?) membrane of the larger siderosome. Several typical mitochondria are evident. Fig. 15: × 50,000; Fig. 16: × 125,000.



(Richter: Electron microscopy of hemosiderin)

PLATE 31

FIG. 17. Part of cytoplasm of a liver cell in a biopsy specimen from a 10 year old boy. The patient, who is suffering from Cooley's anemia, was proven to have massive hemosiderosis, presumably due to transfusions of blood. Aggregates of dense particles (*H*) represent hemosiderin granules. Innumerable particles, about 60 A in diameter, are scattered through the cytoplasmic matrix. *M* is a mitochondrion. Vesicles such as those denoted by *ER* represent endoplasmic reticulum. × 45,000.

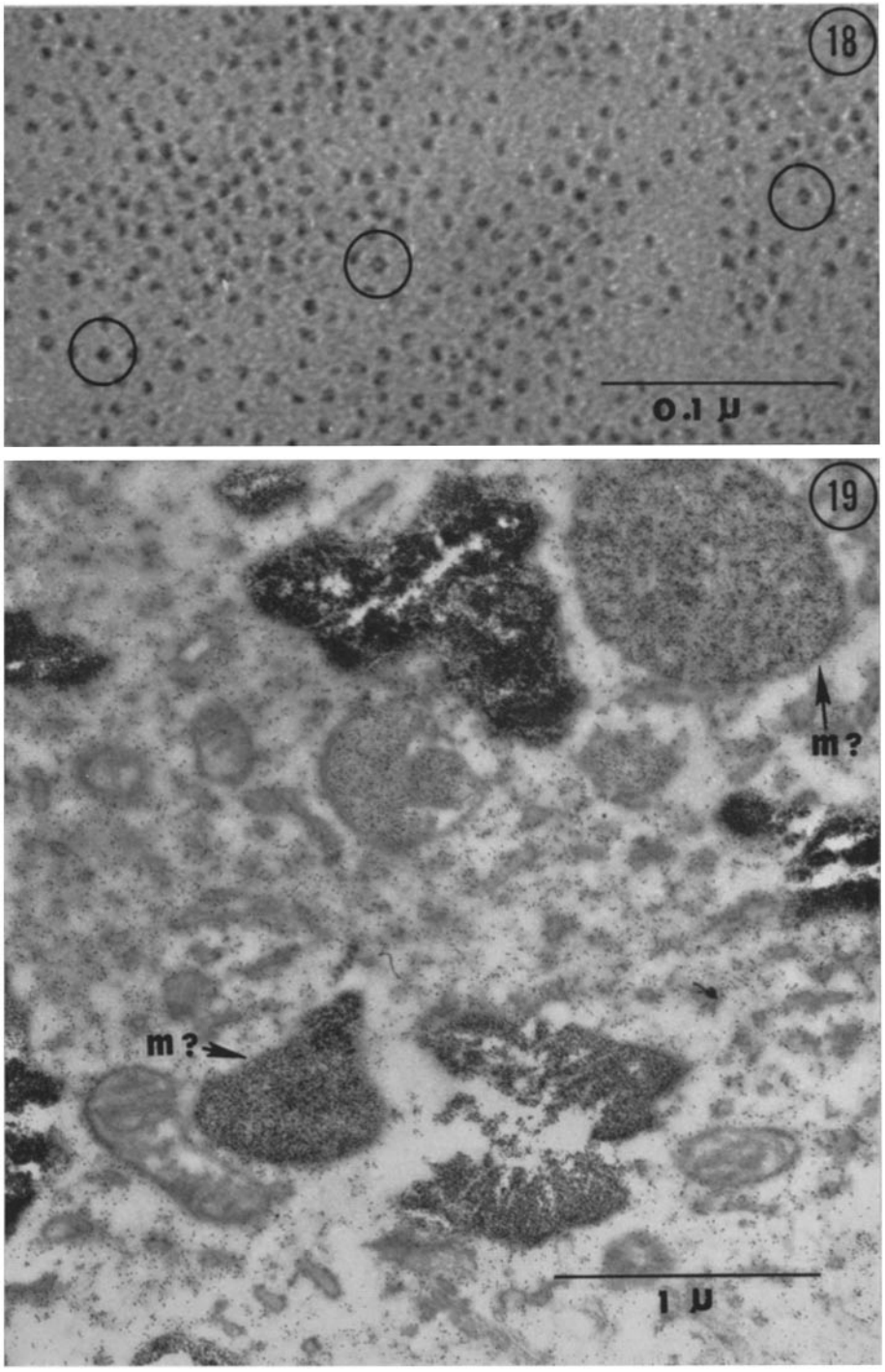


(Richter: Electron microscopy of hemosiderin)

PLATE 32

FIG. 18. Electron micrograph of ferritin from a solution prepared with crystals isolated from the spleen of the same patient. The electron-dense clusters presumably represent the iron micelles of individual ferritin molecules. Many of them show substructure; *i.e.*, appear to be composed of smaller units. Those enclosed in circles are similar to the quadruplets of horse ferritin molecules described by Farrant (10), and the diameters of the whole quadruplets correspond approximately to those of the individual, scattered particles seen in Fig. 19. × 321,000.

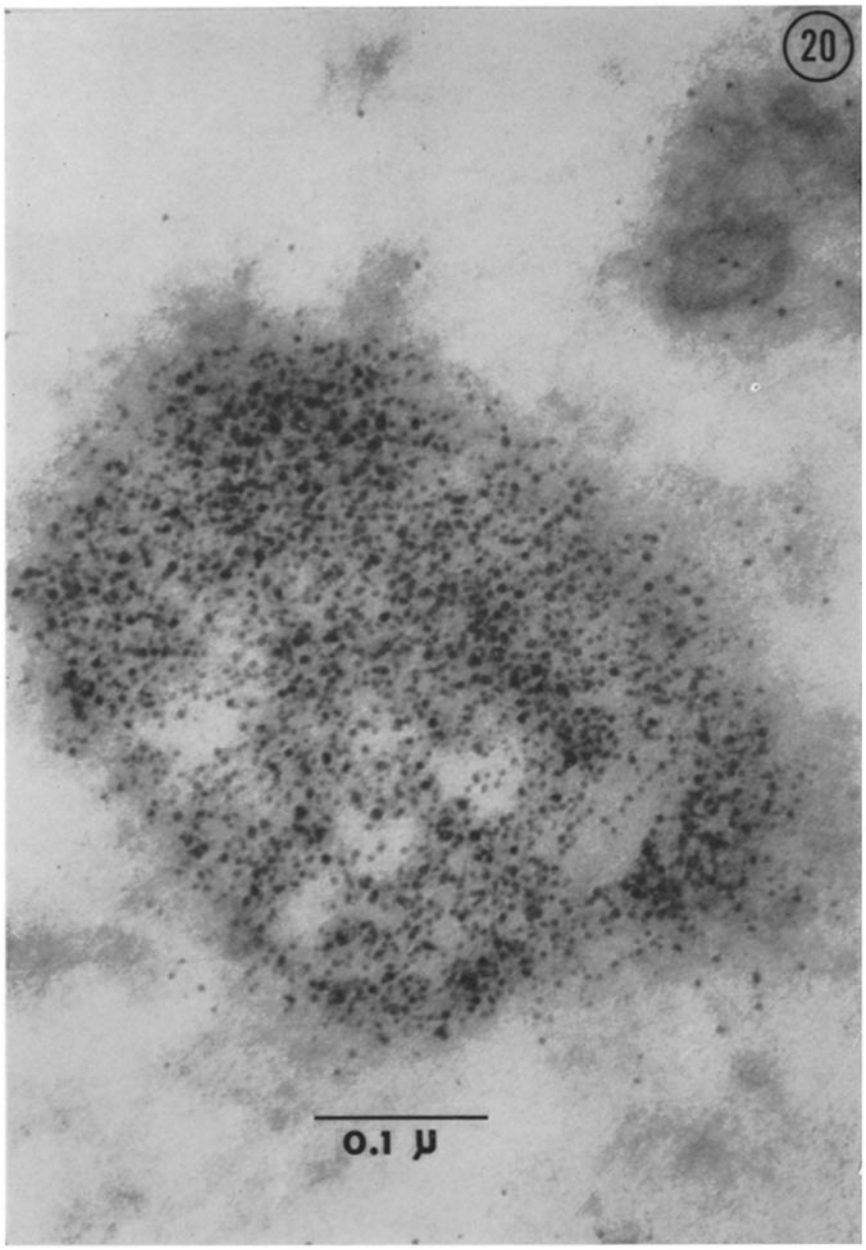
FIG. 19. Part of a reticulo-endothelial cell from the spleen of the same patient. There are massive deposits of hemosiderin (aggregates of dense particles), and a tremendous number of dense particles are scattered through the cytoplasm. Perhaps some of the aggregates are delimited by membranes (*m ?*). × 36,000.



(Richter: Electron microscopy of hemosiderin)

PLATE 33

FIG. 20. Electron micrograph of an aggregate of dense particles in liver cell from the same patient. Note the variation in size of particles in the aggregate. The scattered particles show less variation in size. × 231,000.



(Richter: Electron microscopy of hemosiderin)