REVIEW ARTICLE

THE IRON-LOADED CELL— THE CYTOPATHOLOGY OF IRON STORAGE

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A Review

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"The viability of the tissue in which the red blood corpuscles are transformed into pigment does not alone determine whether the granular, iron-containing, or the crystalline, iron-free, material will be produced. Continuing active metabolism is required to bring the newly generated pigment to its final chemical state, in which it reveals itself as hemosiderin by its microchemical reaction."

M. B. Schmidt, 1889;[1]
author's translation

Storage Iron—What is it?

General usage, amounting to custom, has determined that cellular "storage iron" is of two kinds: ferritin and hemosiderin. Ferritin can be defined unambiguously; hemosiderin cannot. Both contain trivalent iron in the form of hydrous ferric oxides or oxyhydroxides. When present in sufficient amounts, ferritin and hemosiderin can be recognized grossly or in the light microscope by their rusty or yellow-brown color. As might be expected, both give the Prussian (Berlin) blue test* for trivalent iron.[2,3]

The concept of "storage iron" is rooted in the idea that iron in ferritin and hemosiderin is reserve iron, by contrast to, for example, functional iron of heme prosthetic groups in various enzymes or in hemoglobin. The assumption implied by this point of view, ie, that ferritin has no directly essential metabolic role, is no longer justified,[4-6] and various forms of what has been termed "hemosiderin" are cell waste products as well as stores of iron. There does not seem to be cellular reserve iron that is not part of either ferritin or hemosiderin, and in disorders involving excessive storage of iron, cells become laden with these materials. Accordingly, we must

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* The old concept that ferritin iron does not react with ferrocyanide in acid solutions has always been untenable on chemical grounds.[2,3] It should be remembered, however, that if molecules of ferritin are diffusely but sparsely scattered in cytoplasm, only a diffuse blue hue may result from application of the ferrocyanide test.

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characterize them and consider their normal distribution before dealing with pathologic states.

Ferritin

This protein was isolated from horse spleen by Laufberger in 1936 [7] and is a soluble iron storage compound with specific attributes. Early studies, principally by Granick,[8,9] made it clear that ferritin occurs in many sorts of cells and in diverse vertebrate species. Much later, it was also identified in invertebrates [10-13] and in various plants, down to fungi [14,15] (Table 1). Although it does not seem to be present in the simplest kinds of cells, ie, in prokaryotes, ferritin is an archeoprotein, a protein that originated early in the evolution of life. Some of the species in which ferritin has been identified are listed in Table 1. The chemical and physical properties of various ferritins have been reviewed several times in the past decade.[16,17,28] In this review only mammalian ferritins are considered. A number of attributes of mammalian ferritin, which are especially relevant here, will now be summarized briefly.

a) Ferritin is a complex macromolecule, with protein subunits that form a shell around an inorganic core of iron oxyhydroxide, abbreviated here as

Table 1—Occurrence of Ferritin in Diverse Species

Common name	Species	Organs or tissues	Reference
Man	*Homo sapiens*	Liver, spleen, heart, kidney, duodenum, lung, many others including neoplasms	16, 17
Horse	*Equus caballus*	Spleen, liver, kidney, others	16, 17
Rat	*Rattus norvegicus* (several strains)	Liver, spleen, kidney, heart, hepatomas, others	16, 17
Rabbit	*Oryctolagus cuniculus*	Liver, spleen, others	16, 17
Dolphin	*Delphinus cetacea*	Spleen	18
Frog	*Rana pipiens*	Eggs	19
Bullfrog	*Rana catesbeiana*	RBC, liver (larvae and adults)	20,21
Tuna fish	*Thunnus obesus*	Spleen	22
Octopus	*Octopus vulgaris*	Hepatopancreas	13
Earthworm	*Lumbricus terrestris*	Chloragogen cells	12
Marine chiton	*Cryptochiton stelleri*	Radular "teeth"	23
Apple tree	*Malus* species (golden delicious)	Pericarp of apples	24
Sycamore tree	*Acer pseudoplatanus*	Cambium, xylem, phloem	25
Cocklebur plant	*Xanthium pensylvanicum*	Leaves	26
Bean plant	*Phaseolus vulgaris*	Seedlings, cotyledons	14
Pea plant	*Pisum sativum*	Seedlings, cotyledons	14
Fungus	*Mortierella alpina*	Mycelium	27
Fungus	*Phycomyces blakesleeanus*	Mycelium, sporangiophores, spores	15

[FeOOH]x. The core also contains some phosphate (up to one eighth of its own mass).

b) The amount of [FeOOH]x and, therefore, the amount of core material varies within each sample of ferritin. In a fully loaded molecule of ferritin, core iron constitutes approximately 26% of the dry molecule mass. Hence, a single molecule of, for example, horse spleen ferritin can contain more than 4000 atoms of iron (Fe III). By contrast, natural apoferritin (the protein moiety) contains only traces of iron. Ferritin extracted from animal tissues is a mixture of ferritin molecules with differing contents of [FeOOH]x.

c) There is controversy about the size and nature of the protein subunits. According to a conservative view, the combined evidence (eg, x-ray crystallography, ultracentrifugation, SDS–gel electrophoresis, and peptide mapping) indicates that the protein subunits in a molecule of ferritin are of equal molecular weight (~18,500) and constitution, 24 in number, and are arranged in 432 symmetry.[16,17,28] Another view is based on reports of a variety of molecular sizes and weights for ferritin protein subunits, depending on source and on methods of preparation and analysis. Thus, Drysdale and co-investigators [29,30] found that molecules of ferritin extracted from various human tissues are composed of at least two kinds of species-specific subunits; the proportions of these subunits account for patterns produced by isoelectric focusing. Ferritin molecules from human hearts, for example, differ from human liver ferritin in the proportions of heavy (21,000 daltons) and light (19,000 daltons) subunits they contain.[30] Recent reports of carbohydrate in certain preparations of ferritin [31,32] complicate the picture. Subunits substantially smaller than 18,000 daltons in ferritin have also been reported.[33,34] However, a consensus seems to be forming that so-called subunits smaller than ~ 18,000 daltons are likely to be procedural artifacts.[6,30,35] But the question whether the apoferritin protein shell is a heteropolymer or a homopolymer of subunits is unresolved.

d) In solution, the ferritin molecule is nearly isometric and has a diameter of approximately 120 Å, as determined by x-ray scattering.[36] As determined by electron microscopy of negative contrast preparations, the diameter is 105 to 110 Å.[37,38] The [FeOOH]x cores vary in size and shape. In solution, the cores of fully iron-loaded and hydrated molecules have diameters of approximately 70 to 75 Å.[36] By electron microscopy, most cores are smaller, ie, approximately 60 Å.[39,40] What is seen of ferritin molecules in thin sections of cells are the electron-opaque cores, which differ in appearance and size according to the amount of iron they contain, their state of dehydration, and the focus of the electron im-

age.[41-44] Most electron micrographs are underfocused for maximum contrast. Under such conditions phase-contrast images of the cores are obtained. When looking at an [FeOOH]x core of ferritin *in situ* in sectioned cells in the electron microscope, we must imagine that core to be surrounded by a shell of protein subunits.

e) Synthesis of ferritin *in vivo* and *in vitro* has been noted in various cells. The literature dealing with this aspect is voluminous and, therefore, only summary references to it are given at this point.[16,17,45] Taken by itself, the presence of ferritin in a cell is not synonymous with synthesis in that cell. Vascular endothelial cells, for example, can take up circulating ferritin by pinocytosis. Some of the sorts of cells that can synthesize ferritin are hepatocytes, erythroblasts, fibroblasts, columnar absorbing cells in the duodenum and jejunum, proximal renal tubular cells, macrophages, the stellate Kupffer cells, heart muscle cells, and many kinds of neoplastic cells. The genomic information that controls synthesis of ferritin is expressed against a gradient of cell differentiation: even when vigorously stimulated by iron, cardiac myocytes do not make nearly as much ferritin as hepatocytes, and hepatoma cells do not make as much as hepatocytes.[45]

Hemosiderin

This term was proposed by Neumann [46] in 1888 to denote Berlin-blue–positive intracellular and extracellular granules. Virchow [47] had recognized and described such granules as brown pigment, particularly in old areas of hemorrhage or extravasation of blood. He distinguished these granules from yellow hematoidin (biliribin), also present in hemorrhagic lesions, which does not contain iron. Perls [48] had demonstrated the iron-positive granules histochemically in 1867, showing that after application of $K_4Fe(CN)_6$ and hydrochloric acid they gave the Prussian (Berlin) blue reaction (hereafter referred to as PB reaction). The wide occurrence of such pigment in man and animals was then reported in numerous publications. Neumann proposed the term "Hämosiderin" (hemosiderin) to denote hematogenous origin (from hemoglobin) and at the same time to differentiate this material from the iron-negative hematoidin. Neumann concluded that activities of live cells are required to bring about the formation of hemosiderin. Shortly thereafter, Schmidt [1] showed experimentally that this is indeed the case in areas of extravasated blood.

The "pigment" which develops in cells that have phagocytized extravasated erythrocytes or picked up hemolysate is preeminently ferritin, often in molecular aggregates or clusters inside secondary lysosomes. Such collections appear as brown granules in the light microscope and give the

PB reaction.[2,3,40,41] Near the end of the 19th century, Schmiedeberg [49] extracted a brown pigment, which he named ferratin, from pig livers, but this was not specifically identified or purified until the classic crystallization experiments of Laufberger [7] in the 1930s, which marked the discovery of ferritin. Much of what Neumann, Schmidt, and other investigators termed "hemosiderin" on the basis of histologic observations must have been ferritin. Crystals of ferritin have been identified in various sorts of cells in animals and plants. The light microscopist sees these as brown "granules" that are PB-positive.[2,3,40,41] Occasionally, a crystalline habit of such granules may be evident by light microscopy. It is clear that PB-positive material, denoted by the term "hemosiderin" in many publications, includes both water-insoluble material that is currently termed "hemosiderin" by biochemists as well as ferritin. Efforts to isolate hemosiderin and to analyze it have concentrated on methods of extracting the water-insoluble granules; little or no attention has been paid to the likelihood that water-soluble components might be lost in the process of extraction. The definition of hemosiderin as water-insoluble granules, preferred by biochemists, had its origin in this way.[50-54] Even so, "hemosiderin" is chemically heterogeneous. The common feature is the presence of hydrous ferric oxide or oxides, which are brown and give the positive PB reaction. Apart from that, the composition of hemosiderin granules varies, probably in relation to their mode of origin.[50-60] At one extreme are the granules that develop in phagocytic cells after uptake of colloidal ferric hydroxides, eg, as supplied by iron–dextran.[55,59,61,62] In aggregate form, such material is usually situated in secondary lysosomes. It may become admixed with products of lysosomal digestion, including those resulting from autophagic hydrolysis of effete cell constituents.[63] At another extreme are long-retained residues of digested ferritin, the remnants of its [FeOOH]x cores, presumably representing the insoluble remains after lysosomal digestion of ferritin protein; but these, too, may be associated with carbohydrate, heme, and lipid.[50,53,54,58,60,62]

Electron microscopy has revealed much structural heterogeneity in hemosiderin [51,55,57,59] (Figure 1). Size and fine structure of the hydrous ferric oxide in hemosiderin vary considerably, from very fine particles, < 10 Å, to spicular crystals, several hundred Ångstrom units long.[59] Even in advanced cases of hemosiderosis or hemochromatosis, particles which fall into the size-distribution class of ferritin [FeOOH]x micelles may often be found. Membranes, amorphous osmiophilic bodies, and other ultrastructurally heterogeneous material have often been noted in electron micrographs of hemosiderin deposits. Hemosiderin granules isolated by differential centrifugation from liver or spleen are ultrastructurally

heterogeneous.[51,57] It seems likely, therefore, that the sugars and lipids which have been identified chemically in preparations of hemosiderin are not intrinsic to it but represent contamination, such as commingled products of lysosomal digestion and fragments of lysosomal membranes. Old hemosiderin, eg, from horse spleens, has the highest content of iron, in some cases more than 30% by dry weight.[50,53,54] The term "aposiderin"[58] has been used to denote a "carrier" substance with which the iron oxide mineral of hemosiderin is associated. But the composition of aposiderin is undefined. In some preparations, relatively minimal amounts of protein were detected; substantial quantities were found in other preparations.[50,53,54] Because of the limited resolution of the light microscope, histochemical studies uncontrolled by electron microscopy cannot distinguish clearly between the [FeOOH]x-containing substance termed "hemosiderin" and other components of cells or their breakdown products. One should differentiate between hemosiderin of endogenous and exogenous origin. For example, exogenous hemosiderin in various kinds of cells results from injections of preparations containing colloidal ferric hydroxide, such as iron–dextran; in lungs, it can result from inhalation of iron oxide mineral dust. Endogenous hemosiderin is wholly a product of metabolic processes within cells, as by synthesis and degradation of ferritin.

Normal Intracellular Distribution and Origin of Ferritin: The Protein Subunits

Ferritin occurs in many sorts of cells of higher and lower animals and plants (Table 1). Cells which have major functions in the organismic economy of iron metabolism normally contain much more ferritin than do other cells. Thus, maturing erythroblasts (or normoblasts) regularly contain ferritin in quantities sufficient to be visible by transmission electron microscopy, as do the reticuloendothelial cells of the spleen in which effete erythrocytes are destroyed. Hepatocytes, too, regularly contain ferritin, as do many plant cells. There are few quantitative data on the relative abundance of ferritin in the major categories of cells in any animal or plant. Most of the extant information relates to organ or tissue ferritin.[cf45] Some interesting data on the content of ferritin protein in human leukemia cells were published recently (Table 2).[64] The development of

Table 2—Ferritin in Leukemia Cells*

Type of leukemia	No. of cases	Leukocyte ferritin (fg/cell)
Acute myeloblastic	4	9–34
Chronic myeloid (blast phase)	1	13
Acute myelomonocytic	6	27–189

* Data from Cragg et al[64]

sensitive methods for immunoradiometric assay (IRA) and applications of radioimmunoassay (RIA) to biosynthetically labeled ferritin have given quantitative studies of intracellular ferritin a new footing.

Knowledge of the disposition of ferritin *in situ* in cells was first gained by electron microscopy,[40,65] after Farrant's[39] pioneering investigation of horse spleen ferritin. Intracellular locations of ferritin commonly seen on electron microscopy are summarized in Table 3. In assessing distribution and origin one should remember that cells capable of pinocytosis or, more generally, endocytosis can and do take up ferritin from extracellular fluid. Ferritin inside a cell does not tell whether it was synthesized or taken up by that cell.

The first indication of an intracellular site containing ferritin was obtained in analyses of lysosomal fractions from rat livers.[66] It became clear that ferritin is frequently situated in secondary lysosomes and autophagosomes of normal cells, such as hepatocytes or macrophages, and that its quantity in these organelles increases greatly after loading with iron.[55] It was also noted in early experiments that ferritin injected into the bloodstream of experimental animals, or added to media of cell cultures, was taken up by pinocytosis.[67] In metabolically iron-loaded cells, eg, in hemochromatosis, there is ferritin in autophagosomes, often mixed with non-

Table 3—Common Locations of Ferritin in Components of Cells *In Vivo**

	Hepatocytes	Macrophages	Normoblasts
Normally			
Nucleus	Rarely	Rarely	—
RER	+	—	—
SER, prelysosomal	+	+	—
Golgi	—	—	—
Lysosomal system	+++	+++	+
Cytosol	+++	+++	++
Plasma membrane (external surface)	—	—	+
Pathologically†			
Nucleus	+ disperse (rarely crystalloids)	+ disperse	—
RER	+	+	—
SER, prelysosomal	++	+++	—
Golgi	Rarely	Rarely	—
Lysosomal system	++++	++++	+
Cytosol	++++	++++	++
Plasma membrane (external surface)	+	+	++

* This table is an approximation to which there are exceptions, principally with regard to quantities of ferritin in normal cells.

† Iron-loaded hepatocytes and macrophages; normoblasts in refractory sideroblastic anemias

ferritin [FeOOH]x.[cf 68,69] Certain physical properties of the iron in endogenous hemosiderin and in ferritin appear to be similar.[17,57] Results of tracer experiments in rabbits have been interpreted as indicating a transition from ferritin to endogenous hemosiderin.[70]

Early electron microscopic studies of fixed, embedded, and sectioned cells located ferritin in the cytoplasmic matrix, as well as in lysosomal derivatives, particularly in hepatocytes; later it was noted that in rat livers "free" polyribosome fractions and cytosol regularly contained some ferritin.[71-73] On the other hand, the cisternae of the rough endoplasmic reticulum (RER) were, as a rule, virtually devoid of ferritin in electron micrographs of fixed, sectioned cells. When these findings were considered in the light of the prevalent hypothesis that peptides or proteins destined to be exported by cells are synthesized on the RER, it seemed likely that ferritin protein was synthesized by free polyribosomes. Redman [72] found this to be the case in rat liver, and in later experiments Puro and Richter [73] noted that *in vivo* approximately 85% of rat liver ferritin was synthesized by free polyribosomes and that the remainder was synthesized by polyribosomes attached to membranes (RER). The latter result was confirmed by others who studied ferritin synthesis in cell-free systems *in vitro*.[74,75] It has been suggested that ferritin protein synthesized by RER polyribosomes differs qualitatively from that produced by free polyribosomes in the same cells.[76] But why does one not regularly find characteristic [FeOOH]x cores of ferritin molecules within cisternae of the RER in iron-loaded cells?[77] If one accepts as a premise that subunit polypeptide(s) of ferritin, synthesized by membrane-bound polyribosomes, are released into cisternae of the RER, then it seems likely that formation of ferritin, including [FeOOH]x cores, occurs after transport of the protein subunits from cisternae of RER to those of SER. Detailed investigations on the incorporation of ^{14}C-leucine and ^{59}Fe into ferritin in rat livers *in vivo* and in rat hepatoma cells *in vitro* support this inference.[78,79]

Extant evidence indicates that there is but a single peptide chain in each subunit.[16,80] It has been proposed that subunit polypeptide synthesized by attached polyribosomes (RER) differs in amino acid sequence from that synthesized by free polyribosomes in a given cell and that this dichotomy accounts at least partly for electrophoretic microheterogeneities of ferritin.[35,75,76,81] Yet the evidence in support of this view is inconclusive and other factors could account for the observed electrophoretic microheterogeneities. This problem has important genetic implications which will be discussed later.

The presence of protein subunits, separate from completed molecules of ferritin or apoferritin, has been demonstrated in several kinds of cells

(rat hepatocytes, Kupffer cells, hepatoma cells, heart muscle cells, fibroblasts, macrophages) by immunofluorescence [82,83] and by radioimmunoassay, with the aid of subunit-specific antibodies.[78,79] In rat hepatoma cell cultures (Morris-5123-C1 clone) scrutinized at intervals after loading with iron,[83] immunofluorescence revealed that protein subunits were diffusely scattered in the cytoplasm for many hours, with a tendency toward circumnuclear localization. Ferritin, on the other hand, although at first also diffusely scattered in the cytoplasm, was concentrated mostly in clumps in cells examined 17 to 24 hours after iron loading. Loading of media with iron increased the subunit-positive immunofluorescence only moderately and transiently within 2 hours but increased ferritin-positive immunofluorescence throughout the experimental period of 24 hours. On quantitative investigation by radioimmunoassays of various cell fractions (see *Biosynthesis of Ferritin*), these observations were confirmed. The evidence obtained suggests the following sequence of events:

1) Cells + Fe $\longrightarrow$ synthesis of protein subunits $\xrightarrow{\text{Fe II}}$ assembly of apoferritin

2) Apoferritin + Fe II $\xrightarrow{\text{Fe II} \longrightarrow \text{Fe III}}$ ferritin

Incorporation of iron into preformed apoferritin or unsaturated ferritin proceeds immediately and rapidly in hepatocytes and hepatoma cells, principally in the cytosol, and in components of the smooth endoplasmic reticulum. Since turnover of ferritin in rat hepatocytes and hepatoma cells is slow relative to the rate of synthesis,[45,84,85] the rise in ferritin that follows an increase in the extracellular concentration of iron must result from two effects: a) stimulation of synthesis of protein subunits and b) catalysis of incorporation of iron into apoferritin or into iron-poor ferritin. Accumulation of ferritin in cells exposed continuously to excessive concentrations of iron† may be the consequence of a disproportion between the rates of ferritin synthesis and turnover. That it is not simply a consequence of diminished catabolism of ferritin or of diminished release of ferritin from cells is clear from biochemical data.[45,84-86]

Relatively enormous amounts of ferritin may accumulate in hepatocytes or in macrophages in the spleen or lymph nodes after repeated injections of massive amounts of colloidal ferric hydroxide preparations, eg, in rats

† We define as "excessive" a concentration of iron above the range that is normal for extracellular fluid. In the case of cell cultures, the serum component of the medium may be considered the principal source of iron. Thus, for cells cultured in medium containing 10% fetal calf serum, a range from approximately 25 to 100 μg/liter might be taken as a norm.

or rabbits. Depending on dose and time, more than three fourths of injected iron may be incorporated into [FeOOH]x cores of ferritin.[87] Although the proportion of the excess iron going into ferritin diminishes as iron overloading proceeds,[87] one may wonder why cells continue to respond to excess iron by making more ferritin. There is no published evidence for a mechanism of feedback inhibition due to rising concentrations of intracellular iron or ferritin. The capacity of some sorts of cells, eg, hepatocytes, to synthesize and accumulate ferritin exuberantly has important pathobiologic consequences, for if ferritin is not metabolized or secreted at a sufficient rate, cytosiderosis will develop. In 1957 the author proposed the term "siderosome" for membrane-delimited bodies packed with ferritin or any kind of hemosiderin. It has been clear for many years that these bodies are *not* related to mitochondria as he originally thought, but are mostly parts of the cytocavitary system, ie, are secondary lysosomes, autophagosomes, or heterophagosomes, or may be derived from cisternae of smooth endoplasmic reticulum.

Hemosiderin as a Normal Cell Product

Certain cells normally contain some water-insoluble hemosiderin. This is particularly evident in tissues in which iron is being recycled. The phagocytic breakdown of effete erythrocytes in macrophages and reticuloendothelial cells leads to the production of hemosiderin, which might be considered a waste product; but it is also an "iron reserve." The occasional presence of small quantities of hemosiderin in normal hepatocytes is not surprising in view of the turnover of iron-containing enzymes and of the production of ferritin in these cells. In normal individuals, some Kupffer cells regularly contain hemosiderin. Morphologic and biologic data indicate that, in states of iron overload, the [FeOOH]x component of hemosiderin can be retained by cells for years.[50,51,53,54,88,89] The oldest deposits of hemosiderin may be an indigestible or undigested residue of autophagy. Such deposits are particularly prominent in advanced hemochromatosis. Normal levels for tissue hemosiderin in man or animals have not been established.

The General Cytopathology of Iron Storage as Defined Morphologically

Certain features characterize the excessive accumulation or storage of iron in diverse cells. The details of a rat macrophage shown in Figures 3 and 4 tell us that a single cross section of a cell may reveal thousands of ferritin molecules in the cytoplasmic matrix. Also characteristic (Figures 2, 3, 5, 6, and 9) are cytoplasmic bodies, delimited by single ("unit") membranes, within which there are either densely packed collections of

ferritin molecules or of undefined electron-opaque material, presumably micellar [FeOOH]x. Sometimes ferritin occurs in crystalline arrays (Figures 3 and 4). As shown in Figure 1, some membrane-enclosed cytoplasmic bodies in iron-loaded macrophages (or other highly phagocytic cells) may contain heterogeneous dense particles and little, if any, ferritin. Various forms of hydrous ferric oxides have been found in such material, as well as lipids, heme, and undefined protein.[50,51,53-55,57-60] The entire organic matrix of such aggregates has been termed "aposiderin."[58] These heterogeneous electron-dense materials, characteristically gathered in complex cytoplasmic domains ("residual bodies"), may also contain lipofuscin, eg, in hemochromatotic hepatocytes. The ultrastructural variations of hemosiderin-laden cytoplasmic bodies may reflect different ages of the deposits or may indicate mineralization of old hemosiderin.

Accumulation of ferritin or other material containing [FeOOH]x in cell nuclei is uncommon, although paracrystalline arrays have been noted in hepatocytes and in certain cells of invertebrates.[90-92] Intranuclear collections of ferritin molecules could result from inclusion of cytoplasmic material after mitosis, but synthesis of ferritin in the nucleus [90,91] is no longer a tenable inference in the light of modern cell biology.

What methods are available for identifying electron-dense particles as ferritin or nonferritin [FeOOH]x micelles *in situ* in cells? Identification of ferritin by transmission electron microscopy alone is probabilistic. The substructure of the electron-dense cores seen in light-field electron micrographs is largely, if not entirely, a phase contrast phenomenon.[42-44,93-95] The range of appearances in through-focus series of images, the size–frequency distribution, and the arrangement of dense particles must be taken into account. When close-packed paracrystalline arrays are present, the regularity of center-to-center spacings (approximately 110 Å) is helpful. Dark-field electron microscopy of the [FeOOH]x cores of ferritin molecules can reveal a characteristic periodic lattice, but this method has been successful only with unembedded material spread on supporting films.[96] Selected area electron diffraction can yield identification of material containing one of the forms of [FeOOH]x, and electron microprobe x-ray analysis can be used to identify the element Fe in cross-sectional areas as small as approximately 100 nm.[96] At the level of light microscopy, the identification of trivalent iron by the Prussian (Berlin) blue test is helpful. If ferritin is present in collections or crystals sufficient in size to be visible by light microscopy, Perls' Prussian blue (PB) reaction will produce blue granules;[2-3] if ferritin is scattered in the cytoplasm, diffuse, pale-blue staining may be noted after the PB reaction.[2,3,41] It is important that the PB test be properly controlled for acid hydrolysis of iron as well

as for diffusion of reaction product. Immunofluorescent staining of ferritin, either by the direct or, more conveniently, the indirect (sandwich) method, is a specific and sensitive way of detecting ferritin or its protein subunits.[82,83]

Examples of Cytosiderosis

Localized Siderosis

In an organizing localized hemorrhage, such as a contusion or a hemorrhagic infarct, PB-positive material is typically present in the cytoplasm of macrophages and in fibroblasts near the site of injury. In these cells, much ferritin is scattered through the cytoplasmic matrix, and much of it is situated in siderosomes. Not until weeks after extravasation do the [FeOOH]x micelles in the siderosomes take on the appearance of amorphous hemosiderin; but even then ferritin may be abundant in the cytoplasmic matrix, perhaps because its synthesis is continuously stimulated by iron.[97] The literature includes some examples from cases of idiopathic pulmonary hemosiderosis.[96,99]

Generalized Siderosis

The textbook differentiation of hemosiderosis from hemochromatosis rests mainly on the degree of involvement of parenchymal cells in various organs and associated changes, such as fibrosis. Yet the siderosis does not seem to have specific features that could serve as a basis for making this differentiation. Nor are there features by which the siderosis of hepatocytes in primary ("idiopathic") hemochromatosis may be differentiated from the siderosis of secondary ("acquired") hemochromatosis. In advanced cases of both kinds, there are large (>1 μm) deposits of hemosiderin in the cytoplasm. Much of this hemosiderin contains little or no ferritin [51,68,97] and is usually, although not invariably, situated in siderosomes. However, some siderosomes may be packed with ferritin. The amounts of ferritin in the cytoplasmic matrix vary greatly.[100]

It is of interest to take note here of ultrastructural findings in siderotic synovia. Schumacher [69] found that in idiopathic hemochromatosis electron-opaque particles, presumed to represent ferritin and hemosiderin, occur in large quantities in synovial lining cells of Type B, principally within siderosomes. Type B synovial lining cells [101] may be sites of synthesis and secretion of constituents of synovial fluid. They have a better developed rough-surfaced endoplasmic reticulum, fewer pinocytic vesicles and cytofilaments, and less developed Golgi apparatus than synovial lining cells of Type A. The latter may be phagocytic cells that police the synovial fluid. Figure 10 is an electron micrograph made by the author

from material taken from the elbow joint of a 54-year-old male with idiopathic hemochromatosis. The cell shown appears to be of Type A. Most of the dense particles are contained in siderosomes. Since pinocytic vesicles are abundant at the cytoplasmic margins of this cell, one may guess that ferritin was earlier taken up from the joint cavity. Type B cells in the same specimen also contained ferritin. It seems possible that ferritin was released into the joint cavity by Type B cells and reabsorbed by Type A cells. Physical trauma may also be an important factor in the release of ferritin into joint spaces, particularly in patients with hemophilia. Findings reported by Ghadially et al [102] support this assumption.

Zeitoun and Lambling [103] have reported on the fine structure of gastric mucosa, obtained as biopsy specimens, from 2 patients with alcoholic siderotic cirrhosis and 1 with idiopathic hemochromatosis. They noted scattered molecules of ferritin in the cytoplasm of all types of gastric glandular cells as well as in siderosomes. Chief, parietal, mucous, and enterochromaffin cells were heavily involved. In the mucous neck cells, dense particles presumed to represent ferritin were often bunched up at the peripheries in siderosomes. The siderosomes in chief cells were more pleomorphic than those in the other types of cells. Mucous surface cells contained little ferritin. Zeitoun and Lambling also noted much ferritin in the lumens of gastric glands.

Electron microscopy of siderotic heart muscle cells has not yet revealed attributes specific for idiopathic hemochromatosis.[104] Whether pathognomonic ultrastructural changes occur in siderotic heart muscle cells of patients with thalassemia is also unknown.

The genesis of cellular siderosis in human beings can only be conjectured, mainly by reference to studies in animals. Since asymptomatic relatives of patients with idiopathic hemochromatosis often have increased amounts of PB-positive iron in hepatocytes,[105,106] systematic ultrastructural studies of liver biopsy specimens from such persons might be informative (Figures 2 and 8).

Thalassemia

Iron overload in patients with thalassemia major, eg, β-thalassemia, also manifests itself as accumulations of ferritin and nonferritin hemosiderin in various sorts of cells. Accumulation of storage iron in advanced cases of thalassemia major far exceeds the amount of iron transfused in the course of treatment.[107,108] The tendency to accumulate iron in parenchymal cells seems to be as great as it is in idiopathic hemochromatosis. Light microscopy has revealed no differentiating features in the affected parenchymal or reticuloendothelial cells with respect to deposition of PB-

positive iron. The occasional presence of PB-positive bodies in normoblasts and reticulocytes is a consequence of the hemoglobinopathy. At the level of fine structure, the hepatic siderosis in thalassemias seems to have no specific attributes, at least not in material examined by the author. Others have reported dimensional changes in ferritin [FeOOH]x cores and intercore spacings (Figure 11).[109,110] Whether there are specific ultrastructural changes in any cells (eg, in pancreatic acini or islets, adrenal cortex, myocardium) remains to be determined. Accumulations of PB-positive nonferritin iron have been noted in mitochondria of normoblasts from patients with thalassemia major.[111-113] The nature of these electron-dense, intramitochondrial aggregates has not been established. They resemble the "ferruginous" material in the mitochondria of ringed sideroblasts in patients with sideroblastic anemias (discussed later). The cirrhosis of thalassemia major cannot simply be attributed to transfused iron [107-109] or to hepatitis, despite the high incidence of infection with hepatitis viruses as a complication of extensive transfusion therapy. The histopathologic pattern of this cirrhosis is not of the posthepatitic or postnecrotic type. One can assume that the cirrhosis is preceded or accompanied by loss of hepatocytes in consequence of unknown factors, but acute necrosis of hepatocytes has not been evident in the study reported recently by Iancu et al.[109] Thus, damage to hepatocytes is likely to be gradual and slowly progressive, but it could have specific attributes that are associated with altered cellular iron metabolism. Although evidence for this possibility is lacking, it is of more than theoretic interest to investigate it biochemically, perhaps with hepatocytes obtained from young patients with thalassemia major and from their kindred.

Sideroblastic Anemias

In this heterogeneous group of anemias the cytoplasmic matrix of erythrocyte precursors (erythroblasts, normoblasts, reticulocytes) contains more ferritin than is normal. The mitochondria in those cells frequently contain an undefined "ferruginous" material. Such cytosiderosis of normoblasts in various stages of maturation ("erythroblasts" and "normoblasts" in Sabin's terminology) has been noted in primary acquired sideroblastic (normoblastic) anemia, in congenital sideroachrestic anemia, in pyridoxine-responsive anemia, in the anemia associated with lead poisoning, in alcoholic sideroblastic anemia, and in erythremic myelosis (Di Guglielmo's syndrome).[65,112-117] Bessis and Breton-Gorius [118] have reported similar findings in erythroblasts from normal guinea pig bone marrow. Cartwright and Deiss [119] distinguished between two series of siderotic erythroid marrow cells: a) cells with cytoplasmic ferritin, which also occur normally, and b) abnormal cells with "ferruginous" material (not ferritin)

in mitochondria (Figure 12). A classification according to these criteria seems premature, however. Trump et al [117] found both hallmarks in sideroblasts from patients with sideroblastic anemia of unknown origin and from patients with Di Guglielmo's syndrome. A useful definition of sideroblasts is that given by Cartwright and Deiss:[119] normoblasts (Wintrobe's classification) containing at least one PB-positive granule. Some "sideroblasts" may be relatively young erythroblasts, as Goodman and Hall [113] have observed. The term "ring sideroblasts" is often used to denote those sideroblasts in which mitochondria with ferruginous material are arranged around the nucleus (Figure 12). To be distinguished from these cells are "reticulated siderocytes" (R-S cells) and "siderocytes" (S cells). The former are reticulocytes with at least one PB-positive granule, usually composed of ferritin (Figure 7); the latter are mature erythrocytes with at least one PB-positive granule but without ribosomes or mitochondria.

In sideroblasts of erythroid marrow, molecules of ferritin are sparsely scattered through the cytoplasmic matrix and may also be on spots of the external cell surface and in caveolae.[112,116,117] PB-positive granules in the cytoplasm of these cells represent collections of ferritin molecules with or without a membrane envelope. Enveloped collections might result from fusion of vesicles that have taken up ferritin from extracellular fluid by the caveolar mechanisms (ropheocytosis); but unenveloped collections are probably products of ferritin synthesis on free polysomes in erythroblasts (a distinct isoferritin occurs in such cells and in reticulocytes).[120-122] Synthesis of ferritin protein in reticulocytes *in vitro* was first reported by Matioli and Eylar in 1964.[123] As a rule, collections of ferritin are sparse in normal sideroblasts and the mitochondria do not contain ferruginous, electron-dense material. These sideroblasts are normoblasts in various stages of maturation, in which synthesis of hemoglobin has commenced.

Normally, only a small fraction of circulating reticulocytes contains ferritin, viz, the R-S cells of Cartwright and Deiss. These investigators have found a mean value of 0.03 R-S cells per 100 circulating red blood cells in human beings.[119] The mean percentage of circulating nonreticulated siderocytes (S cells) is even smaller (0.01%). Under pathologic conditions these percentages can become markedly increased. In hemolytic anemias, up to 31% R-S cells and up to 7% S cells have been noted.[119]

Two Experimental Models

Nutritional Iron Overload of Rat Liver

It is possible to overload rat livers with iron by various dietary manipulations. Feeding of certain toxic agents, eg, ethionine, will produce sider-

osis of hepatocytes, even if no extra iron is added to the feed.[124] Another example is the method of MacDonald [125] in which rats are given a choline-deficient diet enriched with iron. However, in these and other procedures, excess iron is an accessory agent. Several years ago [126] the writer reported a method by which massive hepatic siderosis was produced in rats without resort to toxic agents or to diets deficient in an essential constituent such as choline. This is the method of cyclic starvation and feeding. The diet is nutritionally complete but enriched with ferric ammonium citrate (up to 1.23% Fe). The procedure is to alternate feeding of the diet *ad libitum* (2 or 3 days) with starvation (2 days) for 6 months or longer; distilled water is always allowed *ad libitum*. In consequence of this treatment, hepatocytes, Kupffer cells, and sinusoidal endothelial cells became heavily laden with PB-positive iron. In female rats, concentrations of iron in the liver rose from control values of approximately 0.14 mg/g wet weight to a mean of 4.8 ± 0.2 mg/g wet weight over periods ranging up to 245 days. The approximate rate of accumulation of iron in the livers of the rats that were cyclically starved and fed the iron-enriched diet was calculated to be 43.9 ± 1.3 μg Fe/g liver/day of feeding. Cirrhosis did not develop. As seen by electron microscopy, siderosis of hepatocytes and Kupffer cells was severe, resembling that in cases of idiopathic hemochromatosis. Lipofuscin-like material was often associated with the hemosiderin (Figure 9).

In Figures 6 and 9, particles with the dimensions of ferritin [FeOOH]x cores are the most prominent feature, especially in membrane-enclosed collections (siderosomes), many of which may be secondary lysosomes. The peribiliary location of these bodies is characteristic but not exclusive. Some ferritin was also situated in bile canaliculi. A cross section through a hepatic sinusoid is shown in Figure 5, with an endothelial cell containing siderosomes, several of which are paired and connected (detailed in Figure 6). Such siderosomes may have been formed from connected cisternae of the SER. Nearly all of the siderosomes in the hepatocytes next to this endothelial cell are situated at some distance from the space of Disse; mitochondria are interposed. At higher magnification these hepatocytes were seen to contain enormous quantities of ferritin in the cytoplasmic matrix right to the margin at Disse's space. The hepatocytic microvilli that project into Disse's space are packed with molecules of ferritin, which are also scattered in this space and in the sinusoidal lumen. The "myelin" bodies in the space of Disse (Figure 5) and in the sinusoidal lumen may have originated from membranes of cast-off microvilli.

Electron micrographs can hardly be expected to uncover the complex interactions reflected by such findings. Uptake of ferritin from the blood

plasma or lymph by endothelial or reticuloendothelial cells is well substantiated,[127,128] but where did the intravascular ferritin in Figures 5 and 6 originate? If in hepatocytes, was it released into the Disse space? Was it released by exocytosis or from pinched off, disintegrated hepatocyte microvilli? To what extent is ferritin synthesized by sinusoidal endothelial cells? How is ferritin excreted into the bile canaliculi, and what determines the rate of excretion? In essence, these are questions about the pathogenesis of hemochromatosis and should be considered before one attempts to answer whether or how excessive accumulation of iron injures hepatocytes.

Lead Poisoning

It has been noted repeatedly that the number of PB-positive granules in several sorts of cells increases in patients with lead poisoning.[129,130] Ring sideroblasts in the bone marrow and siderocytes in the peripheral blood are common in severe cases.[65,112,114] Less appreciated is the tendency of other sorts of cells to accumulate PB-positive material, principally ferritin, in patients with chronic lead poisoning. In reticuloendothelial cells in the spleen, this accumulation of ferritin may be a consequence of increased destruction of red blood cells; but it has also been noted as an acute effect of a single, small dose of lead in proximal tubular epithelial cells.[130] In rats, characteristic acute lesions consist of collections of ferritin molecules in close proximity to cytoplasmic microfibrillar bodies, produced by a single dose of lead (5 μg/g body weight) within 24 hours. Experiments have shown that this disposition of ferritin is not coincidental;[130] that the ferritin, or at least the ferritin iron, vanishes within 4 days if no additional lead is given; and that the cytoplasmic microfibrillar bodies disappear somewhat later (after approximately 8 days), presumably by autophagy.[130] The reasons for this association of ferritin with acute cytoplasmic microfibrillar bodies are unknown. There may be a relation to diminished utilization of iron for heme synthesis, ie, to one of the major effects of lead. Reduction of heme synthesis and a consequent rise in the concentration of iron in the cytosol might be compensated by increased incorporation of iron into ferritin.

Pathogenetic Considerations

Entry and Exit of Iron

It is not known how iron enters and exits from cells nor what regulates its passage. Of the major conceptual models, those postulating active transport through the membranes have most currency. On the basis of

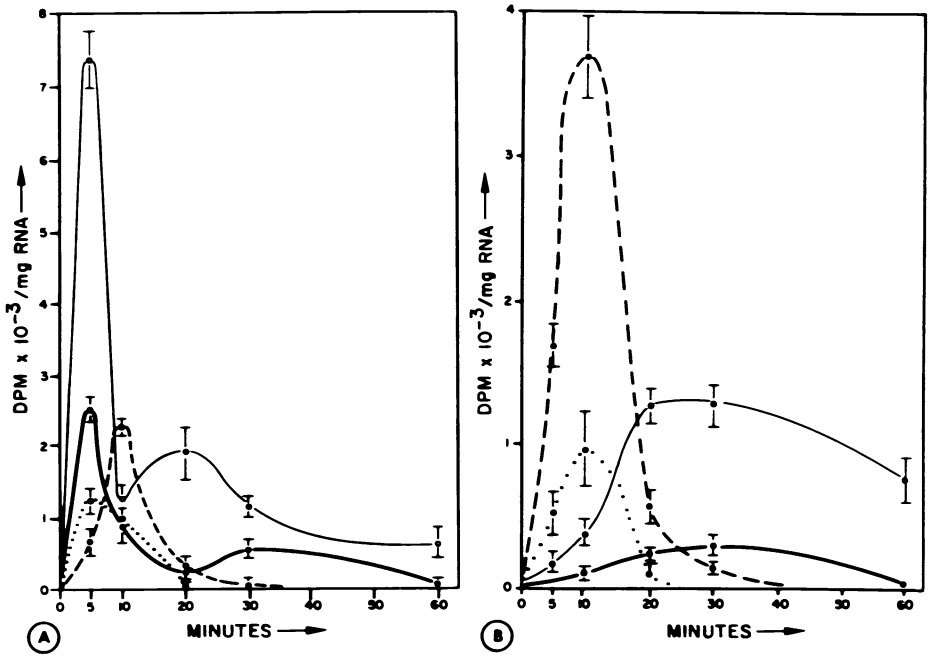
evidence that iron is donated to cells (at least to erythrocyte precursor cells) by transferrin, it is assumed that receptors for transferrin are situated on the outer cell surface and that iron is then detached from its ligand in transferrin and is somehow transported through the plasma membrane, probably after reduction of Fe III to Fe II.[131,132] According to another view,[131,133] transferrin itself is supposed to enter cells by endocytosis, give up iron, and then be released by exocytosis. Kinetic data, however, favor energy-dependent transport *through* the outer cell membrane ("plasma membrane") for entry of iron into cells, eg, reticulocytes, Friend erythroleukemia cells, and isolated hepatocytes.[132,134,135] Several investigators have obtained evidence indicating involvement of ATP in the release of iron from transferrin at or in cell membranes.[136-138] The HCO_3^- anion may be released at the same time as iron. Possibly, release of iron results from prior release of bicarbonate. The nature of the transferrin receptor at the cell surface is in dispute. It may be a glycoprotein.[134] That various sorts of cells take up iron from extracellular fluid by endocytosis, ie, pinocytosis, seems obvious when one considers that solutes and colloidal particles are regularly present in extracellular fluid. On a similarly large scale, exocytosis or reverse pinocytosis might be associated with excretion of iron. However, to what extent, if any, iron is released from the interior of cells by active transport through the plasma membrane is unknown. The existence of such a mechanism, linked to cell metabolism, would seem to be necessary to prevent wide fluctuations in the content of iron of individual cells in populations such as hepatocytes and vascular endothelial cells. It has been suggested that exocytosis of ferritin normally maintains a balance of intracellular iron, but evidence for such a mechanism is lacking. To the contrary, even in cells containing much ferritin, electron microscopy does not often reveal exocytosis of ferritin. On the other hand, the existence of a block of iron transport through the cell envelope (plasma membrane) has often been postulated but never substantiated. Judging from clinical effects of iron chelators, serum transferrin seems to function equally well in primary and secondary states of iron overload.

A special case is that of erythrophagocytosis in macrophages or other cells of the reticuloendothelial system and in certain epithelial cells, eg, follicular cells of the thyroid.[140] Ingestion of erythrocytes, especially heterologous ones, is followed by digestive processes in phagosomes ("phagolysosomes") and liberation of iron from hemoglobin.[139,140] The ferritin that characteristically appears in the phagocytic cells on digestion of erythrocytes is at first scattered in the cytoplasmic matrix and is later concentrated in secondary lysosomes or in multivesicular bodies.[139,140] The pro-

cess proceeds for days, and one can imagine that synthesis of ferritin is stimulated as iron is released from phagosomes into cell sap.

Biosynthesis of Ferritin

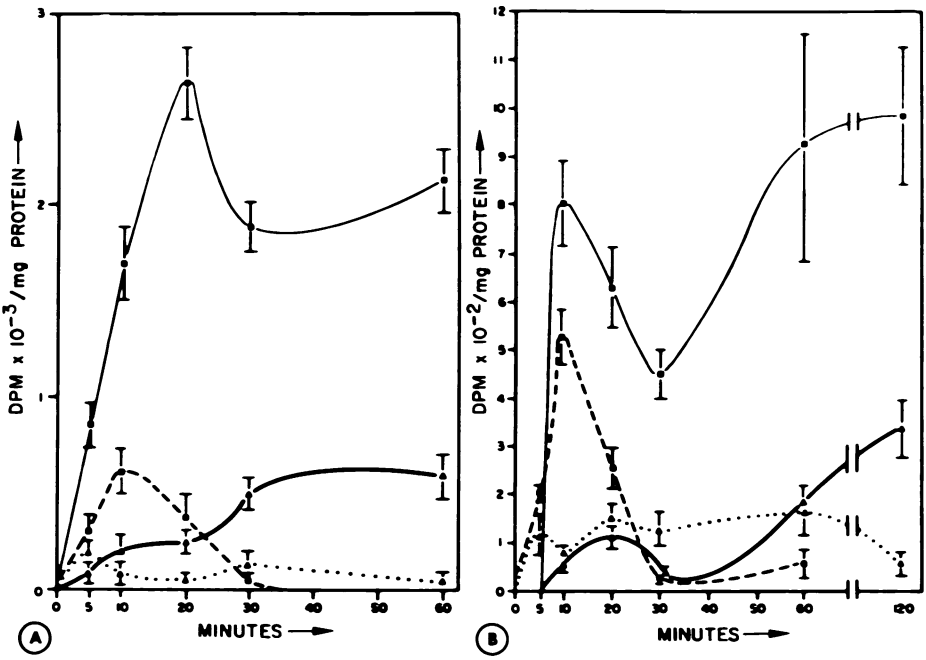
It has been known for many years that iron stimulates the synthesis of ferritin protein and the production of new ferritin molecules in various sorts of cells.[141-143] *In vivo* and *in vitro* this is a specific response to rising extracellular concentrations of iron, either at a posttranscriptional [35] or a posttranslational level.[84,85] There is evidence that posttranslational events have a significant role in the production of new ferritin. Some years ago, Drysdale and Munro [84,85] suggested that iron stabilizes an unstable precursor of ferritin. Zähringer et al [35] have proposed that iron mediates transfer of mRNA, coded for ferritin, from mRNP. Although the synthesis of ferritin protein has been investigated in cell-free systems *in vitro*,[74] the natural sequence of events leading from synthesis of subunits to completed ferritin molecules can only be studied with functioning cells. A first approach to this was Redman's study,[72] which clearly showed that rat liver ferritin protein is synthesized *in vivo* mainly by free polyribosomes. Later, Puro and Richter [73] determined that approximately 15% is synthesized by attached polyribosomes (RER). At the time, it seemed possible, however, that this minor fraction was synthesized by different sorts of cells, ie, Kupffer cells. But in subsequent experiments, Lee and Richter [78,79] showed that in a clonal line of rat hepatoma cells 15 to 20% of ferritin protein was synthesized by attached polyribosomes and 80 to 85% was synthesized by free polyribosomes. These findings are of interest for several reasons. First, they suggest that some ferritin protein may be routed through the cell by specialized cisternal pathways of the endoplasmic reticulum (possibly without the intervention of the Golgi apparatus) and may, after assembly of ferritin molecules from protein subunits and iron, be secreted. Second, these findings could account for some molecular heterogeneities in ferritin prepared from various sources and for differences between serum ferritin and liver cytosol ferritin.[30,144-146] Normally, serum ferritin could be a product of secretion. It has been reported that sugars may be attached to ferritin.[82] Since glycosylation in the Golgi zone is well established for other proteins, ferritin protein might be glycosylated during vectorial transport in Golgi vesicles or cisternae. Yet the association of sugars with ferritin could follow, not precede, transport into secondary lysosomes. A basic question yet to be answered is whether the genome of a given cell is coded for only one sort of ferritin protein subunits, or for two, or for more. At least until the primary structure of subunits in at least one kind of ferritin, extracted from cells of



TEXT-FIGURE 1—Time course of appearance of ^{59}Fe-labeled and (^{14}C)leucine-labeled protein subunits and ferritin in the free polyribosomal and the membrane-bound polyribosomal fractions of M-5123-C1 hepatoma cells after addition of ^{59}Fe and (^{14}C)leucine to culture medium in pulse–chase experiment. Chase with "cold" precursors started 3 minutes after pulse. **A**—^{59}Fe radioactivity. **B**—(^{14}C) leucine radioactivity. Each point represents the mean of two independent experiments. *Bars* indicate standard deviations. ○——○, protein subunits in free polyribosomal fraction; ○———○, ferritin and apoferritin in free polyribosomal fraction; ●···●, protein subunits in membrane-bound polyribosomal fraction; ●———●, ferritin and apoferritin in membrane-bound polyribosomal fraction. (From Lee and Richter,[79] by permission of Journal of Biological Chemistry)

one sort (eg, from clonal cell cultures) is known, this question will remain open. It is really broader: what are the cellular determinants of the observed heterogeneities of ferritin? A corollary is: what modifications of ferritin occur during extraction from cells and purification?

Even without reference to these questions, it has been possible to get information about the way in which the ferritin molecule is assembled in cells. Lee and Richter [78,79] investigated the biosynthesis of ferritin protein subunits and the intracellular sites of assembly of ferritin from subunits and iron in rat livers and rat hepatoma cells by combining cell fractionation with radioimmunoassay after labeling with radioactive precursors. It can be seen in Text-figure 1 that synthesis of protein subunits by free and, to a less extent, membrane-bound polyribosomes peaked within 10 minutes after initiation of a pulse in cells stimulated by iron. By contrast, labeled apoferritin (assembled protein subunits) and ferritin appeared and



TEXT-FIGURE 2—Time course of appearance of ^{59}Fe-labeled and (^{14}C)leucine-labeled ferritin and protein subunits in smooth membrane and supernatant fractions of M-5123-C1 hepatoma cells after addition of ^{59}Fe and (^{14}C)leucine to culture medium in pulse–chase experiment. Chase with "cold" precursors started 3 minutes after pulse. A—^{59}Fe radioactivity. B—(^{14}C)leucine radioactivity. Each point represents mean of two independent determinations. *Bars* indicate standard deviations. ■—■, protein subunits in smooth membrane fraction; ■———■, ferritin and apoferritin in smooth membrane fraction; ▲···▲, protein subunits in supernatant fraction; ▲———▲, ferritin and apoferritin in supernatant fraction. (From Lee and Richter,[79] by permission of Journal of Biological Chemistry)

peaked significantly later, and their relative concentrations declined much more gradually than those of subunits. It can also be seen in Text-figure 1 that in both polyribosomal fractions, incorporation of ^{59}Fe into ferritin reached a first peak in 5 minutes; this peak was presumed to be due to insertion of iron into preexisting ferritin. Much later, after maximum incorporation of ^{14}C-leucine, a second ferritin peak appeared in both polyribosomal fractions, indicating insertion of iron into newly synthesized and assembled apoferritin. Yet some ^{59}Fe became attached to protein subunits rather early in the free and attached polyribosome fraction (peaks at 5 and 10 minutes, respectively). Since only "unweighable" quantities of labeled iron were involved, attachment to unassembled subunits may have been random rather than a reaction essential to ferritin synthesis. In the smooth membrane (mainly SER) and supernatant compartments (Text-figure 2) some ^{59}Fe was bound to newly synthesized

Table 4—$^{59}Fe/^{14}C$ Ratios for Ferritin and Protein Subunits in Fractions of M-5123-C1 Hepatoma Cells*

	Protein subunits				Ferritin			
Time	FP	BP	SM	CY	FP	BP	SM	CY
5	0.48 ± 0.036	2.4 ± 0	1.5 ± 0.34	1.6 ± 0.06	6.3 ± 1.8	10.6 ± 1.19	(875 ± 150)/0	0.76 ± 0.23
10	0.60 ± 0.016	0.84 ± 0.08	0.74 ± 0.17	1.38 ± 0.29	4.6 ± 0.78	4.2 ± 0.25	2.15 ± 0.91	4.8 ± 0.06
20	0.59 ± 0.14	0.4 ± 0.03	0.5 ± 0.01	0.42 ± 0.08	2.5 ± 0.15	2.2 ± 0.07	0.73 ± 0.034	4.3 ± 0.5
30	0.62 ± 0.015	0	0.51 ± 0	0.24 ± 0.015	2.1 ± 0.15	1.7 ± 0.097	0.5 ± 0.07	11.1 ± 0.71
60	0.64 ± 0.011	0	0.48 ± 0.04	0	1.23 ± 0.025	1.08 ± 0.17	0.51 ± 0.003	4.5 ± 0.76

* Means and standard deviations; pulse-chase experiments
Cell fractions: FP = free polyribosomes; BP = membrane-bound polyribosomes; SM = smooth membranes; CY = cytosol.
Adapted from Lee and Richter[79]

subunits and to apoferritin or ferritin, although such binding occurred later than in the two polyribosomal compartments. It appears, therefore, that the capacity of ferritin protein to bind iron is not compartmentalized.

Further information on incorporation of iron by assembled apoferritin or by incompletely filled ferritin and on the attachement of iron to subunits was obtained by calculating $^{59}Fe/^{14}C$ ratios. In the experiments just mentioned, the $^{59}Fe/^{14}C$ ratios were considerably higher for labeled ferritin than for subunits in the two polyribosomal fractions (Table 4). The data indicate movement of subunits and ferritin from the two polyribosomal compartments, respectively, to the cytosol and smooth membrane compartments. Taken together with results of experiments in which ferritin and its protein subunits were located *in situ* in cells by immunofluorescence,[82,83] the tracer experiments can be interpreted as follows:

a) There is a basal concentration of free, unassembled (or only partly assembled) subunits in the cytosol. When that concentration rises in consequence of synthesis of new subunits by free polyribosomes, subunits will be assembled into apoferritin in the cytosol near the polyribosomes.

b) Assembly into apoferritin or iron-poor ferritin molecules is promptly followed by incorporation of iron.

c) The much smaller fraction of subunits synthesized by membrane-bound polyribosomes is released into cisternae of the RER and moved into those of SER, perhaps with intermediate glycosylation in Golgi cisternae. Assembly and insertion of iron would then take place in terminal SER cisternae or vesicles.

Disposition of Completed Ferritin

What happens to ferritin inside cells? The mean turnover time is normally slow. The half-life of ferritin molecules in adult rat liver has been estimated to be at least 2 to 3 days.[45,84,85] Under conditions of iron overload, when massive quantities of ferritin accumulate in cells, there is likely to be long continued "storage." This does not preclude a continuous degradation via autophagy. The degradative process leads to formation of protein-poor hemosiderin, of which [FeOOH]x micelles are the principal component. (See *Hemosiderin* and *Hemosiderin as a Normal Cell Product.*) An intracellular pathway of ferritin that originates from synthesis of subunit peptide by attached polyribosomes and ends in autophagic vacuoles (secondary lysosomes) can be deduced. However, for the major fraction of ferritin that originates from subunit synthesis on free polyribosomes, the intracellular route is far from clear. In cells overloaded with iron, ferritin molecules are often heavily scattered in the cytosol (the cytoplasmic "matrix" in electron micrographs). A mechanism for gather-

ing these molecules together into compact, at times enormous, collections must therefore exist. It could be via fusion of many small cytoplasmic vesicles that have engulfed a few ferritin molecules. Such fusion might be followed by continued delivery of ferritin by small vesicles to the "siderosomes" formed initially: a sort of "policing" of the cytoplasm by vesicles.

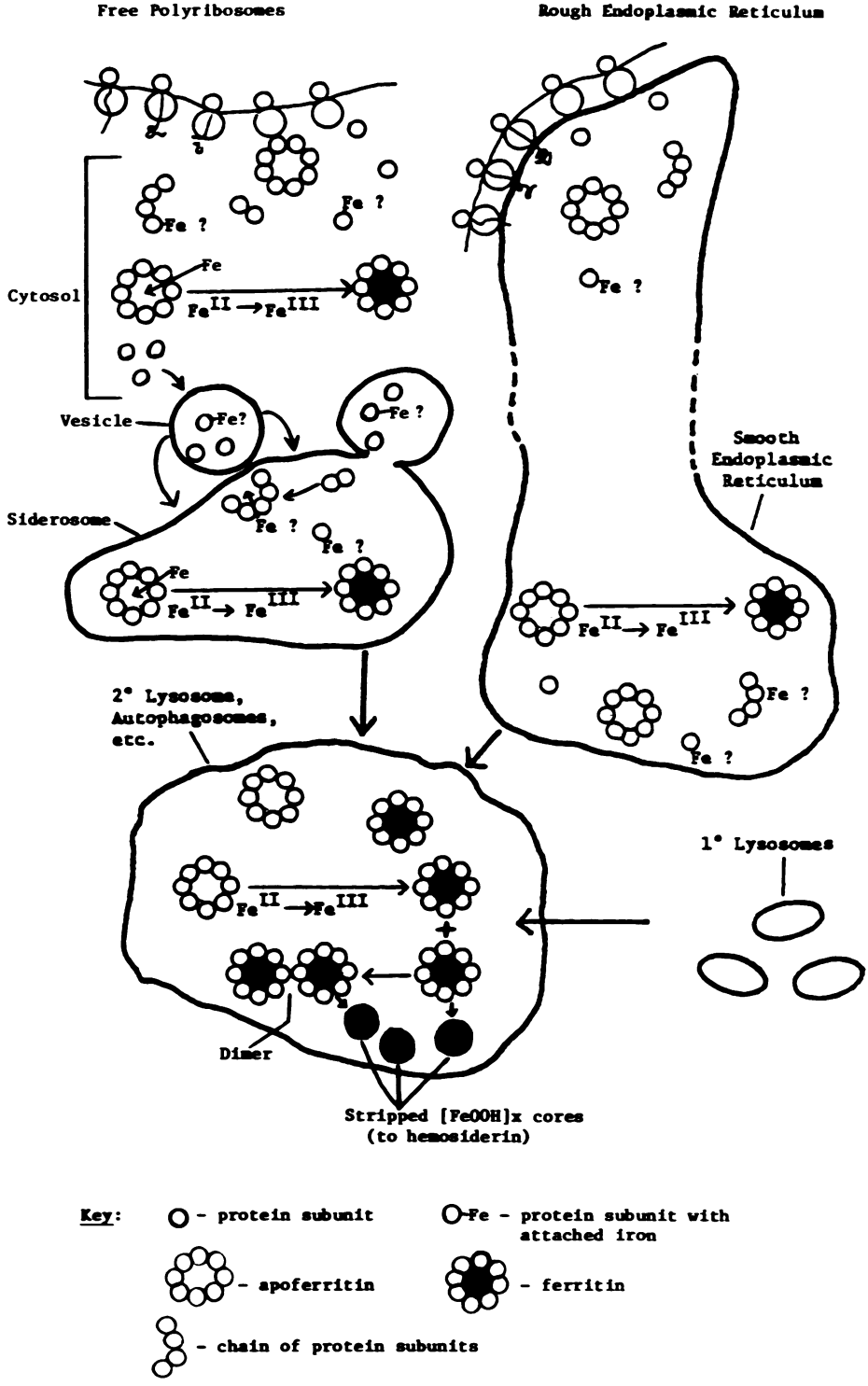
There is some evidence for extrusion of hemosiderin from cells, ie, hemosiderin granules in extracellular spaces. Judging from the infrequency of such extracellular deposits in electron micrographs, this is a minor way of disposal (Figure 5). A large body of clinical-pathologic and experimental observations, however, indicates that enormous quantities of hemosiderin can be stored by cells.

The author's concepts of biosynthesis and storage of ferritin in cells that possess well-developed RER but synthesize ferritin mainly on free polyribosomes are summarized in Text-figure 3.

Storage Iron and Cell Damage

The question of whether excessive amounts of *stored* iron are damaging to cells has been debated for a long time. Applying the word "damage" in the broadest possible sense, ie, to any abnormal alternation of cell function, be it accompanied by definable structural changes or not, one can say that cytosiderosis induced by administration of iron is attended with damage since it is associated with changes not normally seen.

It is well-known that excess ionized, divalent iron (which is invisible in the electron microscope) is toxic to cells and can cause cell death. But the question of interest here is whether storage iron (ferritin, hemosiderin) impairs cells functionally, possibly to the point of necrosis. There is no evidence for functional damage due to storage iron alone. Findings that hepatocytes heavily laden with ferritin and hemosiderin may be biochemically deficient and structurally altered cannot tell us whether or to what extent damage is due to iron overload rather than to other factors, although Peters and Seymour[147] have noted increased lysosomal acid phosphatase activity in liver biopsy specimens from patients with primary or secondary iron overload. They also found an associated decrease in lysosomal *N*-acetyl-β-glucosaminidase. Diseases such as thalassemias, hypertransfusion states, or so-called nutritional pigment cirrhosis are clearly not good examples from which to draw conclusions about damage caused by storage iron itself. Even analyses of the situation in genetically determined hemochromatosis, in which the degree of hepatic damage seems to be related to the quantity of storage iron, may not be relevant since turnover of iron is itself abnormal in this condition. Hereditary hemo-



TEXT-FIGURE 3—Schematic outline of pathways from synthesis of protein subunits of ferritin to formation of endogenous hemosiderin.

chromatosis reflects unknown cellular defects that permit the preeminent involvement of parenchymal cells in storage of iron in many different sites. One should look, therefore, to experimental situations in which the disposition of storage iron can be monitored biochemically, physiologically, and morphologically and in which there are no acute toxic effects of Fe^{++}. Neither experiments with intact laboratory animals nor others with cells grown *in vitro* have yet yielded a proper model. It is surprising that there are so few biochemical data on intermediary metabolism in iron-loaded cells *in vivo*.

Although it is possible that either hepatic sinusoidal endothelial cells or fibroblasts in spaces of Disse are stimulated to secrete excessive amounts of tropocollagen in response to iron overload and can thus initiate hepatic fibrosis, biochemical data on this point are inconclusive.[109,148] One may recall that in most cases of transfusional siderosis (other than those associated with thalassemia) severe siderosis of hepatic sinusoidal cells and of triadic fibroblasts is associated with but minimal fibrosis. One must distinguish between possible chronic effects of the condition for which transfusions are given and possible effects of iron. After rats are overloaded with colloidal [FeOOH]x (as by injections of iron dextran), there is massive deposition of iron in sinusoidal lining cells, macrophages, and fibroblasts in the liver but only a slight increase in collagen.[55,87,97] In a recent study of liver biopsy specimens from infants with thalassemia major, Iancu et al [109] detected no evidence of hepatocellular necrosis in advanced siderosis and found fibrosis to be minimal.

Still, some clinical observations point to a relation between massive iron overload and deviations from normal cell functions. For example, serum enzyme levels indicating hepatocyte damage in patients with iron overload may return to normal after phlebotomy or during treatment with chelators.

Genetics and Siderosis

There should no longer be much doubt about the hereditary nature of at least two forms of idiopathic hemochromatosis.[149-152] One of these appears to have an autosomal recessive character; the other appears to have an autosomal dominant character, perhaps with incomplete expression. The much greater frequency of idiopathic hemochromatosis in males than in females has not been explained. Linkage to HLA-A3 and B14 genes has been noted, but not invariably.[152] Although it has been reported that abnormal isoferritin patterns are present in organs or serums from patients with idiopathic hemochromatosis,[145,146,153] the published evidence seems inconclusive. Other claims for specific defects

in iron metabolism in this disease also remain to be confirmed, as pointed out recently by Jacobs.[148] We are left with speculations about biochemical defects in heritable disorders of iron storage. The mode of inheritance of the ferritin genes is not known for any species, nor is it known how many genes in a cell are coded for ferritin subunits. No subunit of ferritin protein has yet been sequenced in its entirety. Only when the primary structure of subunits in at least one kind of ferritin (or "isoferritin") is known will it be possible to resolve disputes between the adherents of the homopolymeric hypothesis and those of the heteropolymeric hypothesis on the structure of apoferritin. An important question to be answered is how many sorts of ferritin protein subunits are coded in the genome of a cell: one, two, or more than two? If the ferritin polypeptides synthesized by membrane-bound polyribosomes differ from those synthesized by free polyribosomes, then what is the mechanism by which synthesis of the respective mRNAs is controlled? And if the RER–ferritin differs only by being glycosylated, how is the mode of glycosylation controlled in the cell? To answer these questions, it will be necessary to analyze ferritin subunits from cell clones rather than from heterogeneous cell populations. The problem of identifying epigenetic modifications (posttranslational changes) in subunit protein complicates the study of the inheritance of ferritin, but it is surmountable with existing biochemical methods.

Conclusion

In this review the author has considered cytopathologic changes that are clearly related to excessive storage of iron and has purposely not dealt with phenomena whose relation to iron storage is unspecific or is essentially conjectural. Means for gaining better, deeper understanding of both sorts of pathologic alterations are at hand thanks to advances in cell biology and biochemistry over the past 2 decades. These means, and others yet to be discovered, will be seized by enterprising investigators of disordered iron metabolism.

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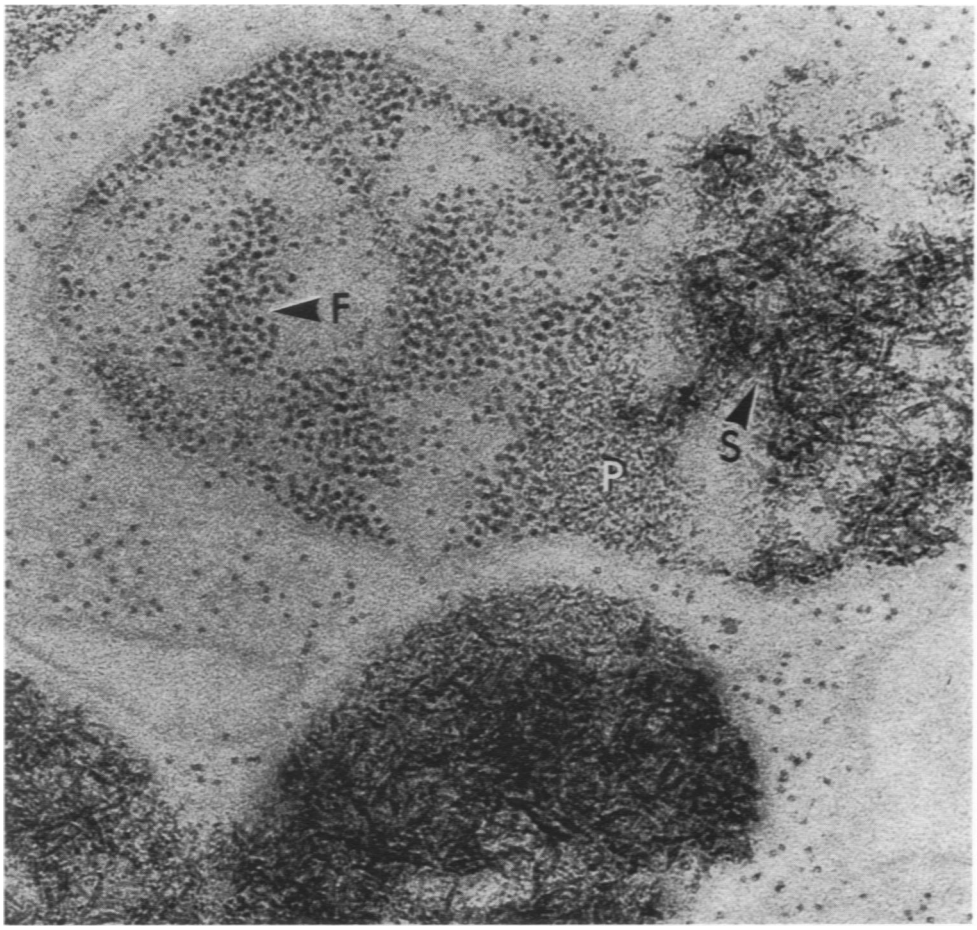

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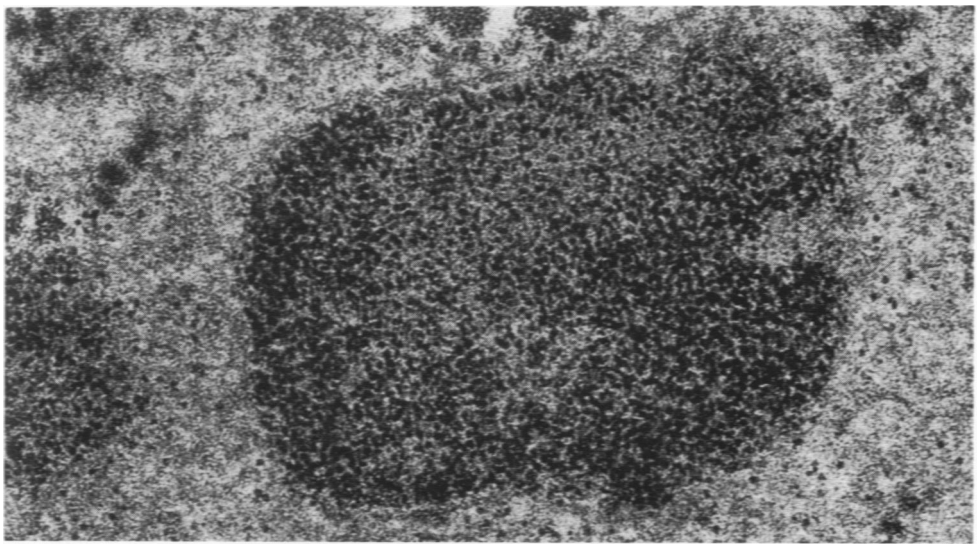
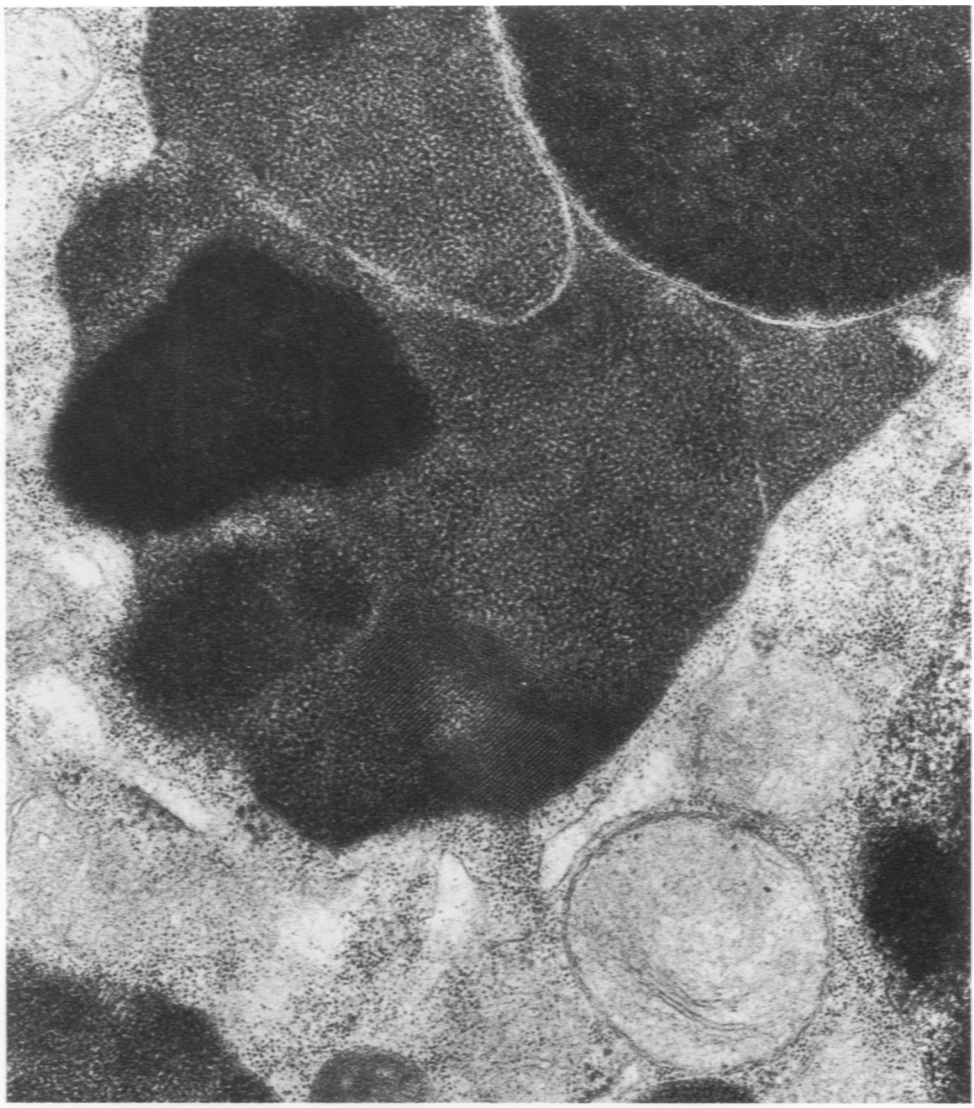


2

Figure 1—Three forms of [FeOOH]x in siderosomes of rat macrophages in lymph node draining foot injected with iron dextran 7 days previously. *F*, cores of ferritin molecules, which are also scattered in the cytoplasmic matrix; *S*, spicular crystals of β-[FeOOH]x; *P*, small particles of [FeOOH]x. Fixation: glutaraldehyde, OsO_4. Unstained section. (× 167,000) **Figure 2**—Siderosome with ferritin in hepatocyte (detail of Figure 8) from 53-year-old brother of patient with idiopathic hemochromatosis. PB-positive iron in liver was 4+. Many of the dense particles in the siderosome resemble those outside it, in the cytoplasmic matrix; others appear to be "amorphous." Fixation: glutaraldehyde, OsO_4. (Uranyl acetate and lead citrate, × 167,000) (Material provided by Dr. John R. Wright, State University of New York, Buffalo, N.Y.)

3



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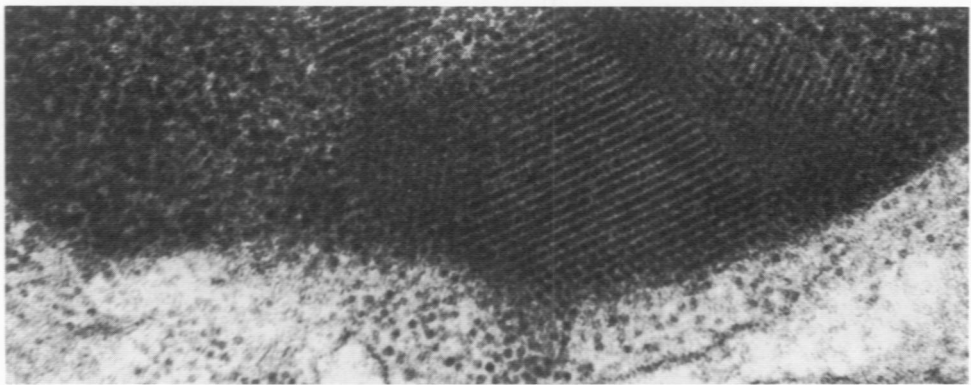


Figures 3 and 4—Ferritin and nonferritin [FeOOH]x ("hemosiderin") in giant compound siderosome. Macrophage in lymph node draining foot pad of rat injected with iron dextran 7 days previously. Note crystalline arrays of ferritin (detail in Figure 4) and the individual ferritin molecules (cores) in the immediate vicinity of the arrays and scattered through cytoplasmic matrix. Fixation: glutaraldehyde, OsO_4. Unstained. (**3**, × 83,000; **4**, × 200,000)

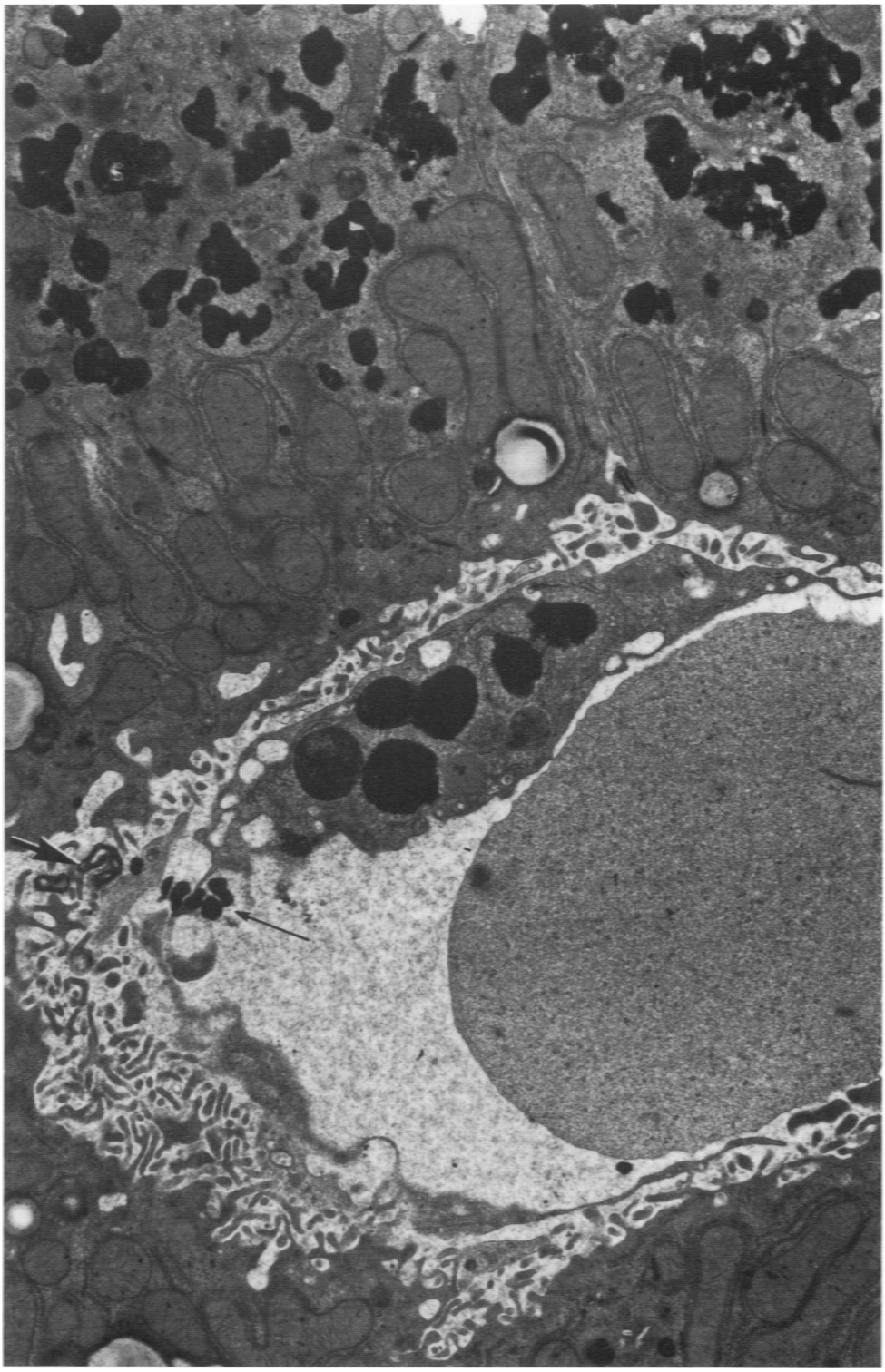
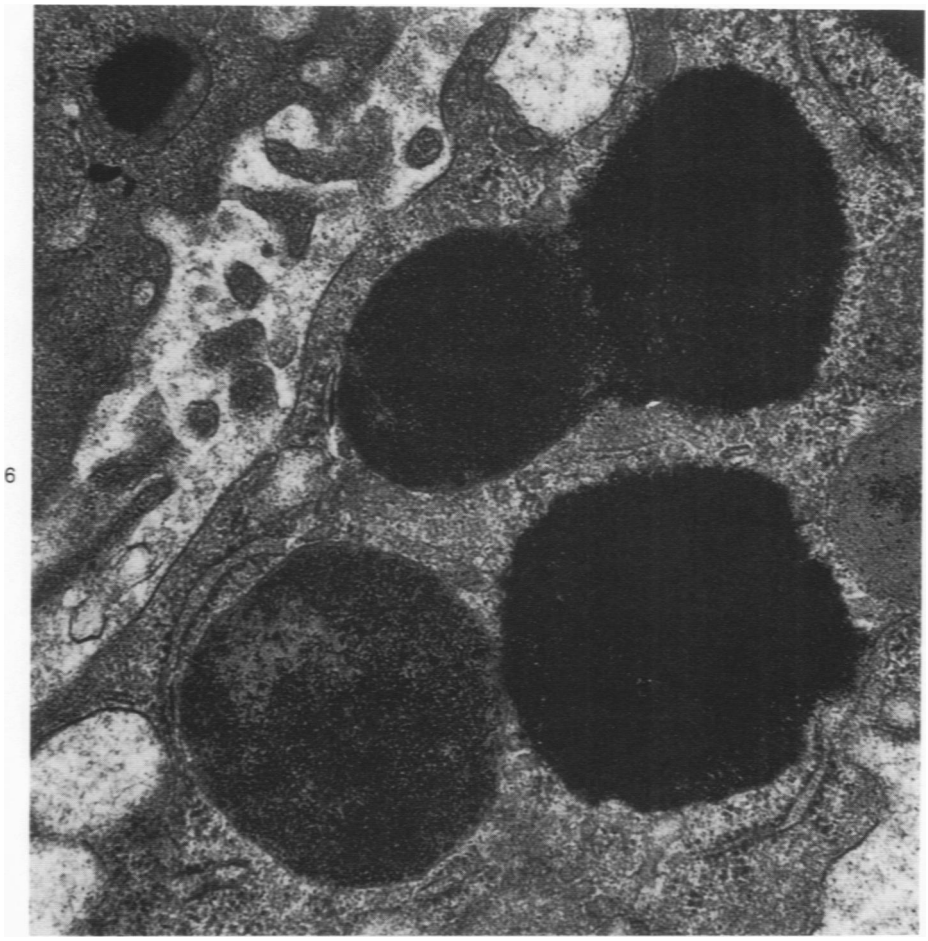


Figure 5—Liver sinusoid with adjacent cells from rat subjected to cyclic starvation and feeding for 232 days. Diet contained 1.23% iron (as ferric ammonium citrate). Note siderosomes in Kupffer cell and hepatocytes, as well as several outside the Kupffer cell in sinusoidal lumen and space of Disse (*small arrow*). There are two myelin bodies in space of Disse (*large arrow*). Cell in the lumen is a reticulocyte. Liver contained 5.2 mg Fe per gram wet weight. Fixation: glutaraldehyde, OsO_4. (Uranyl acetate and lead citrate, × 20,000)



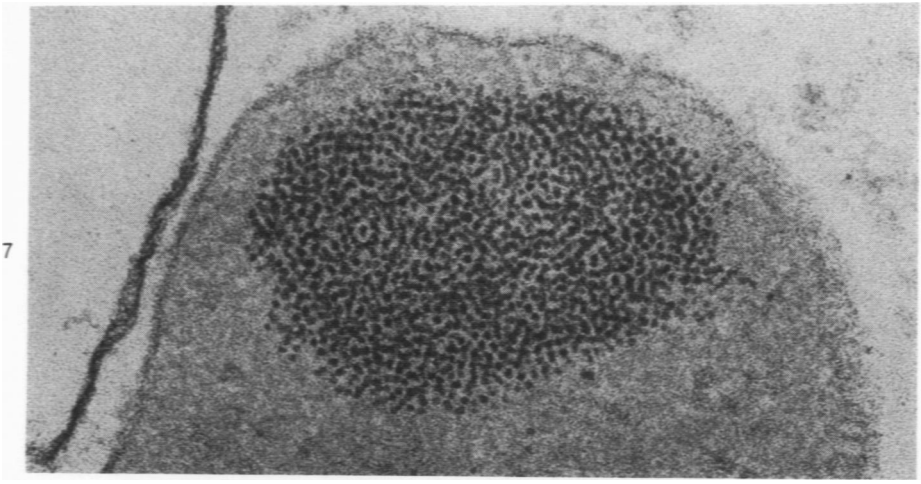


Figure 6—Detail of Figure 5. Ferritin, seen here as dense cores, is scattered in the cytoplasmic matrix of the Kupffer cell and of the hepatocyte at *upper left*, in space of Disse, between Kupffer cell and hepatocyte, and in endocytic vesicles or caveolae. Siderosomes in the Kupffer cell seem to contain much ferritin. Note structural heterogeneity of these bodies and bilobed siderosome in *upper half* of picture. (× 75,000) **Figure 7**—Part of reticulated siderocyte in sternal marrow of rat subjected to cyclic starvation and feeding for 237 days. Note large collection of ferritin molecules in cytoplasmic matrix. Fixation: glutaraldehyde, OsO_4. (Lead citrate, × 183,000)

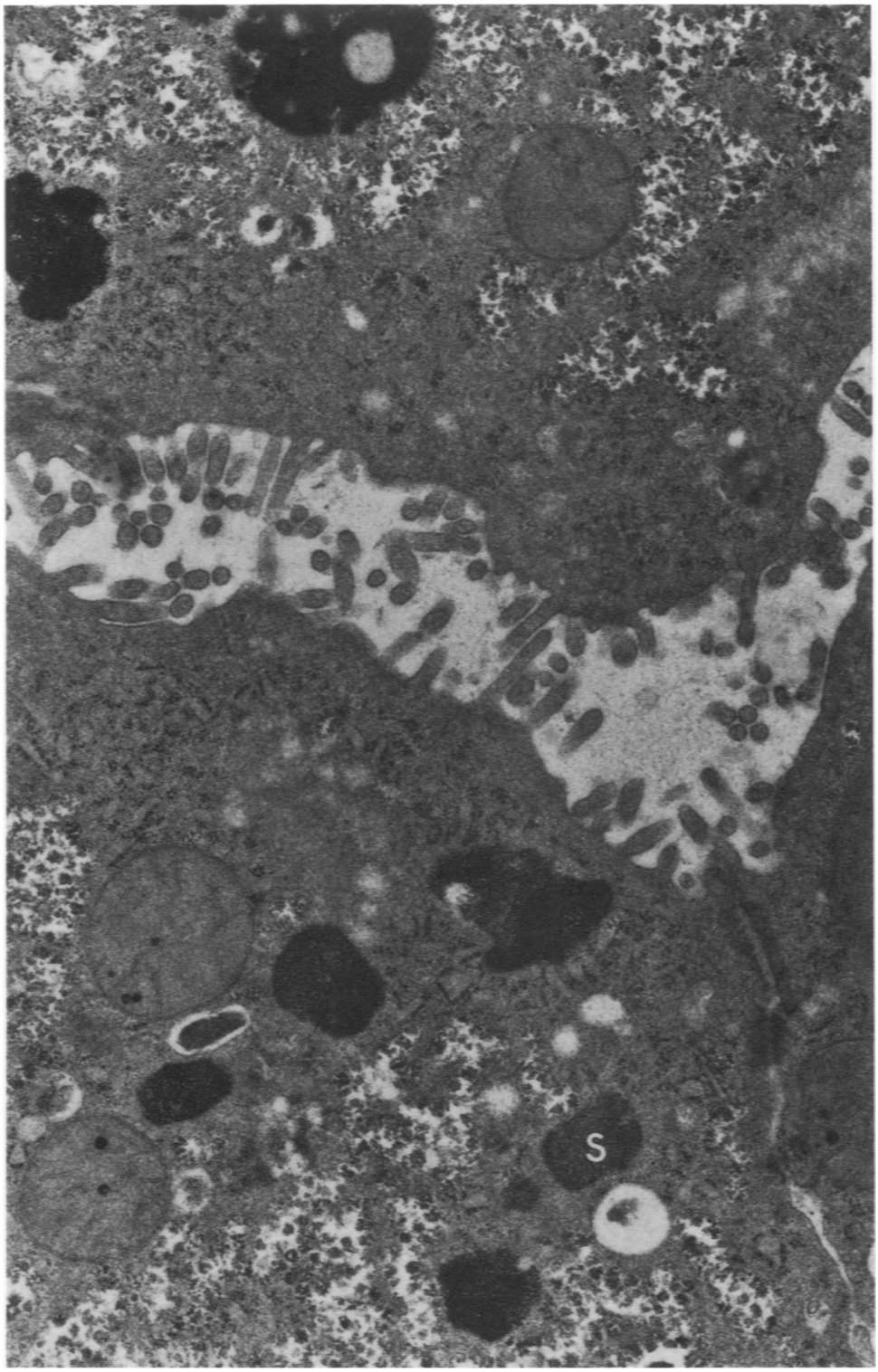


Figure 8—Parts of hepatocytes and bile canaliculus in biopsy specimen from 53-year-old brother of patient with idiopathic hemochromatosis (kindred studied by Rowe et al[185]). Note siderosomes and lipofuscin-containing residual body near top. The siderosome marked *S* is shown at higher magnification in Figure 2. The hepatocytes contain much glycogen. Fixation: glutaraldehyde, OsO_4. (Uranyl acetate and lead citrate, × 33,300) (Material provided by Dr. John R. Wright, State University of New York, Buffalo, N.Y.)

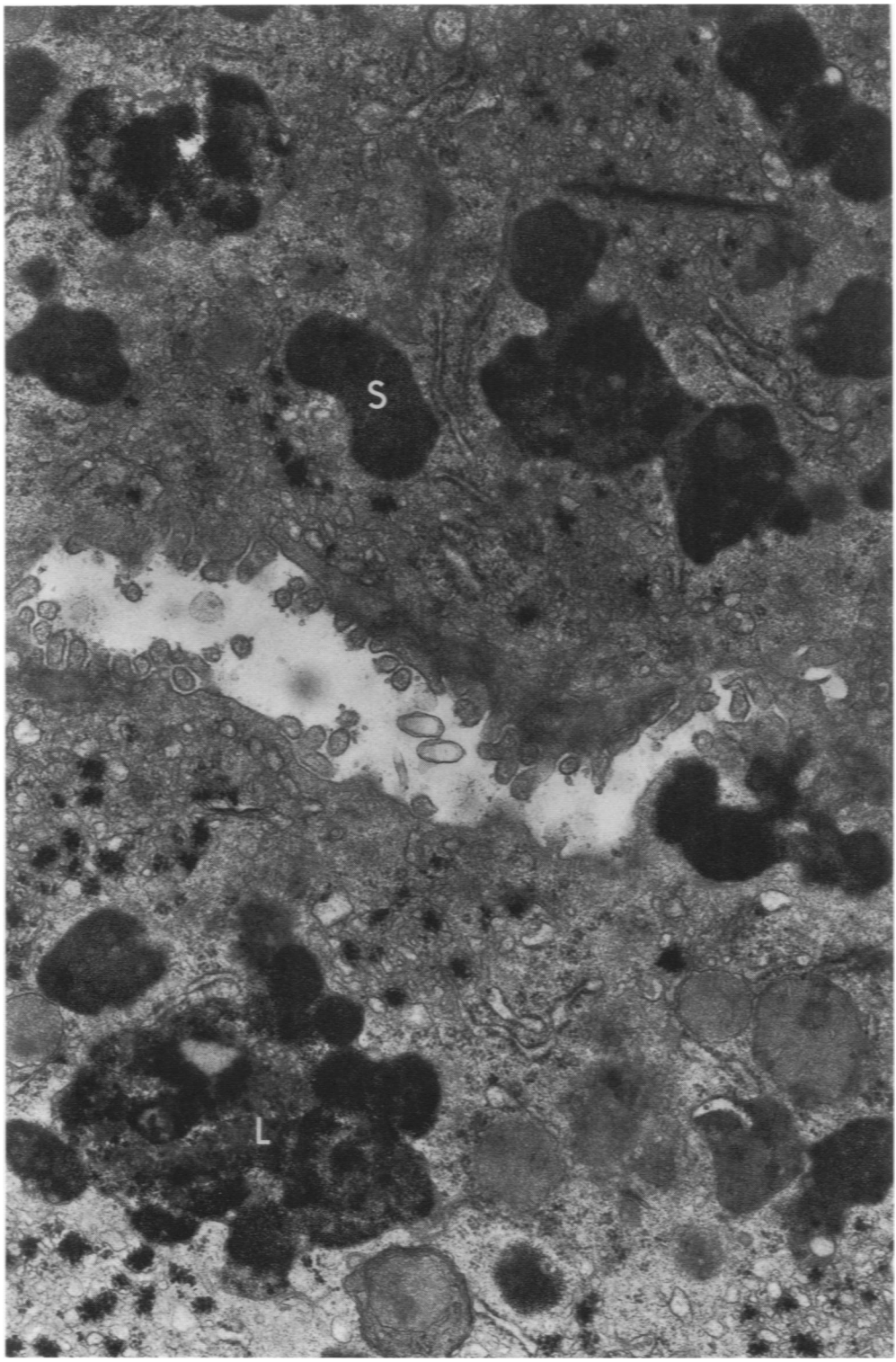


Figure 9—Parts of hepatocytes with bile canaliculus from rat subjected to cyclic starvation and feeding of diet containing 1.23% iron (as ferric ammonium citrate) for 208 days. There are peribiliary siderosomes of secondary lysosome type. Some (*L*) have much, others (*S*) have little or no lipofuscin. Note similarities to Figure 8. Liver contained 6.5 mg Fe per gram wet weight. Fixation: glutaraldehyde, OsO_4. (Uranyl acetate and lead citrate, × 33,300)

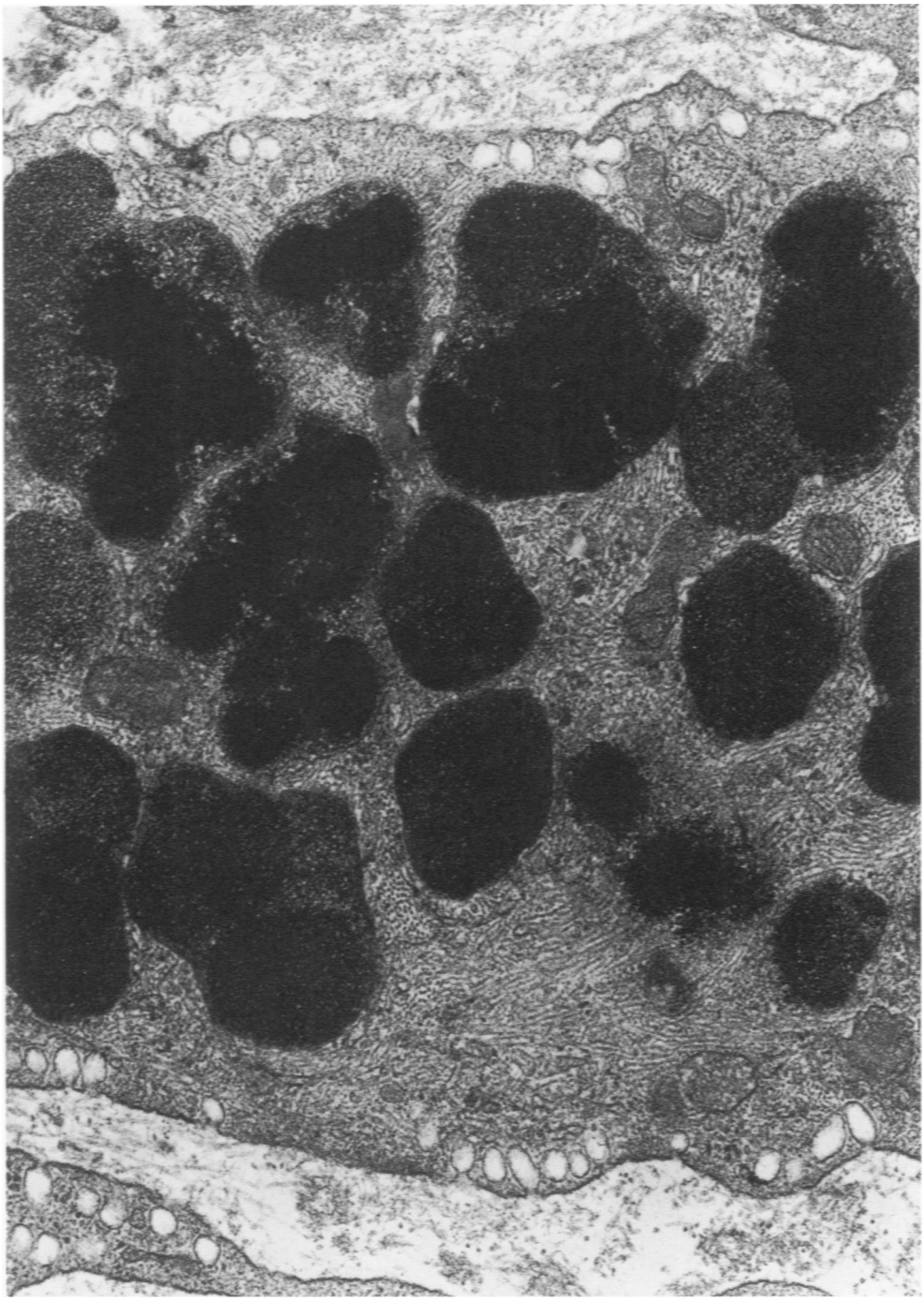
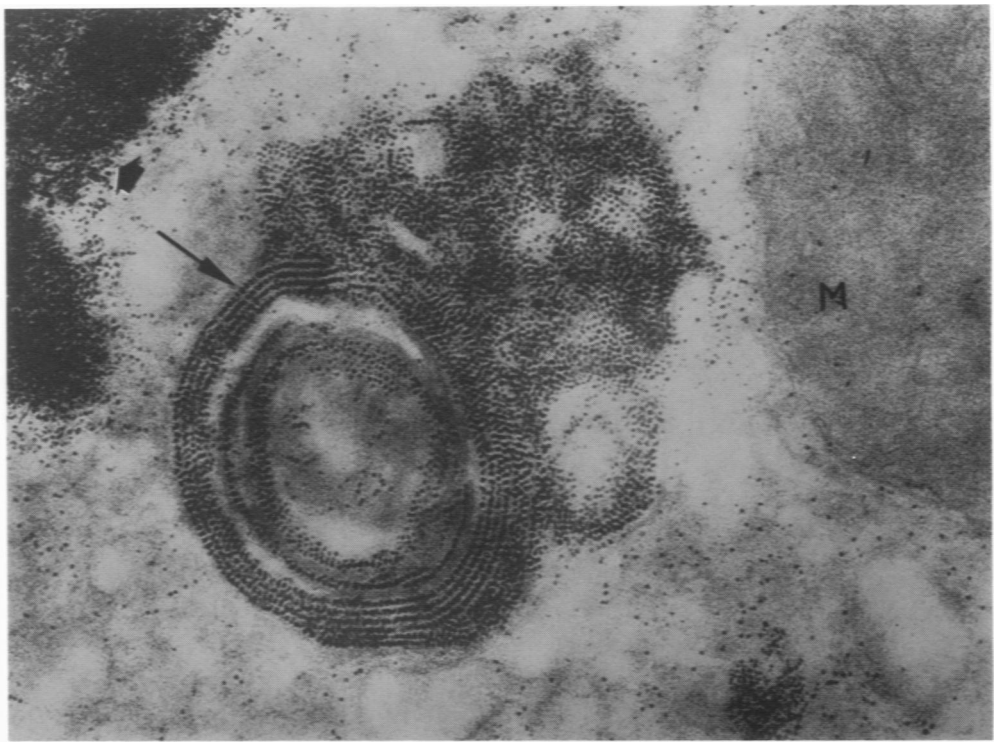


Figure 10—Part of a Type A synovial cell in biopsy specimen from elbow joint of a 54-year-old man with idiopathic hemochromatosis. Many siderosomes of various aspect are packed with dense particles; some also contain more homogeneous, lipofuscin-like material. Note many arrays of cytofilaments (actin?) seen longitudinally and in cross-section. The many coated vesicles and caveolae near the cell surfaces seem to be free of ferritin. Fixation: glutaraldehyde and OsO_4. (Uranyl acetate and lead citrate, × 45,000)



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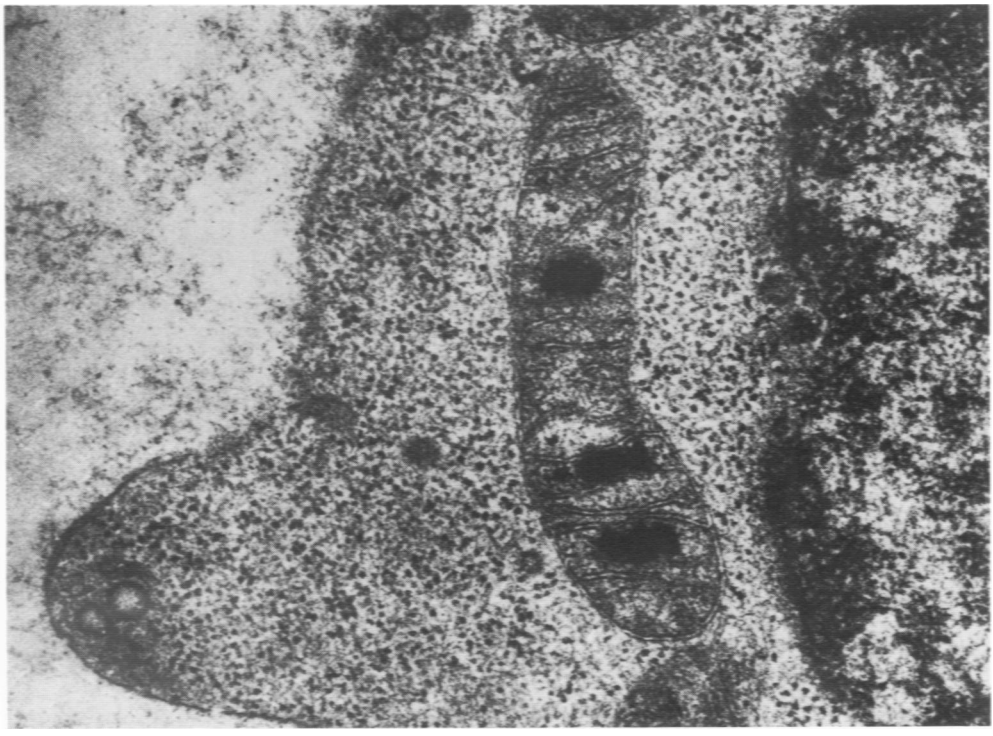

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Figure 11—Part of hepatocyte in biopsy specimen from a 3-year-old boy with thalassemia major. Ferritin (represented by dense cores) is scattered in cytoplasmic matrix and also in arrays delimited by faintly visible membranes (*small arrow*) (presumably part of secondary lysosome). Other dense particles are situated in aggregate at *upper left (large arrow)*. *M*, mitochondrion. Fixation: glutaraldehyde and OsO_4. Unstained section. (× 100,000) (Picture provided by Dr. T. C. Iancu, Lady Davis Carmel Hospital, Haifa, Israel) **Figure 12**—Part of immature normoblast from patient with sideroblastic anemia of undetermined etiology. Note ferruginous deposits in the mitochondrion. These were found to be rich in iron, as determined by electron probe x-ray microanalysis. (× 50,000) (Picture provided by Dr. B. F. Trump, University of Maryland School of Medicine)