THE NATURE OF STORAGE IRON IN IDIOPATHIC HEMOCHROMATOSIS AND IN HEMOSIDEROSIS

Electron Optical, Chemical, and Serologic Studies on Isolated Hemosiderin Granules*

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Although ferritin has long been recognized as an important iron storage compound and as an intermediary in normal iron metabolism, its role in idiopathic hemochromatosis and in secondary hemosiderosis is still unknown. Diverse means have been employed to gain more knowledge on the pathway of iron in these conditions, and numerous publications attest the difficulties inherent in trying to distinguish between normal and abnormal storage of iron in cells of various sorts. Histochemical studies have provided evidence that inorganic compounds of iron stored in cells as hemosiderin are combined with an organic carrier substance that contains variable quantities of protein, lipid, and carbohydrate (1, 2).

Results of chemical analyses led Ludewig to emphasize the heterogeneity of hemosiderin granules (3); his findings have provided qualitative and quantitative data on the carbohydrate, lipid, protein, and iron content of various hemosiderin preparations. The term "hemosiderin" has been used rather loosely; generally it refers to granules that are visible in the light microscope, brown when unstained, and give a positive Prussian blue test. Iron that gives a positive Prussian blue test with potassium ferrocyanide without the previous application of oxidizing agents must be in the trivalent (ferric) state. This is true of the bulk of iron in hemosiderin granules; it is also true of the iron hydroxide present in ferritin (4, 5, 7). X-ray diffraction studies of hemosiderin granules isolated from horse spleen have indicated the presence of α-ferric oxide in various states of hydration (8). Hemosiderin granules isolated by Behrens and Taubert (9) according to the method of Behrens and Asher (10), were shown to be devoid of ferritin. On the other hand, McKay and Fineberg found small quantities of ferritin in hemosiderin granules isolated from horse spleen by a different method (11). Bielig and Wöhler also found some ferritin in human hemosiderin (12).

Recent investigations in which electron microscopy was used in conjunction with other methods have shown that crystals and aggregates of ferritin are commonly present in diverse sorts of cells in cases of hemosiderosis, and that under the light microscope such deposits have the appearance of hemosiderin granules and give the Prussian blue reaction. By means of

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electron microscopy it is possible to distinguish these hemosiderin granules from others which contain little or no ferritin. Furthermore, parenteral injections of colloidal hydrous ferric oxide in various forms will give rise to intra- and extracellular precipitates of hydrous ferric oxide that appear like hemosiderin granules in the light microscope, and that give a positive Prussian blue test. Using electron microscopy and electron microdiffraction one can identify such deposits and distinguish them from endogenous hemosiderin (6).

Numerous authors have stated that ferritin and hemosiderin are distinct, separate entities. With this idea as a premise, theories of abnormal iron storage have been formulated. For example, it has been proposed that the production of hemosiderin reflects an abnormal pathway of iron metabolism, the normal equivalent being the synthesis of ferritin (13, 17). That this hypothesis is inadequate has become clear from the results of recent investigations on the fine structure and disposition of hemosiderin *in situ* in cells (4–6; 14–16 *a*, *b*). These studies have shown that the iron hydroxide contained in hemosiderin can occur in physically different forms, that certain hemosiderin granules are largely or wholly composed of ferritin, while others contain less ferritin or no ferritin; and that still other hemosiderin granules contain iron hydroxide micelles related to those of ferritin, though different in size and shape. Such differences may depend on the age and derivation of the hemosiderin granules; *e.g.*, on whether the hemosiderin resulted from the degradation of hemoglobin in macrophages or from the transformation of other iron compounds.

The work now to be reported was done to learn more about the nature of storage iron in idiopathic hemochromatosis in man, and to make comparisons with the storage iron in secondary hemosiderosis. The question was raised whether there is a distinct form of storage iron in idiopathic hemochromatosis. To seek an answer, a correlative study was made in which cell fractionation, chemical and serologic procedures, electron microscopy, and electron diffraction were utilized.

Materials and Methods

General.—Hemosiderin granules were isolated from tissues of three patients. Liver and spleen were obtained at necropsy in one case. In a second case only the liver was available postmortem. The spleen of the third patient was removed during an operation. Crystalline ferritin was prepared from parts of these organs. In two of the cases biopsy specimens of the liver were available for electron microscopy. A brief summary of each case will now be given.

Case 1.—A splenectomy and liver biopsy were performed on a 10-year-old boy of Greek extraction. He had been suffering from Cooley's anemia (thalassemia major) since infancy and had been given well over a hundred transfusions of whole blood. The diagnosis of thalassemia major was firmly established by extensive hematologic and roentgenologic studies. This patient is still living, and receives transfusions frequently.

Case 2.—A 53-year-old white man developed progressive congestive heart failure, abdominal pain, and diarrhea. Four months before death, examination revealed an enlarged heart

and liver, atrophic testes, excessive brown pigmentation of the skin, and scanty body hair. The serum bilirubin was slightly increased, the BSP (bromosulfalein) excretion time was prolonged. Occasionally, there was sugar in the urine, and the fasting blood sugar was moderately elevated. The plasma iron concentration was elevated to 354 mg. per cent. He developed auricular fibrillation and died in congestive heart failure.

Autopsy revealed changes consistent with advanced hemochromatosis involving heart, liver, spleen, pancreas, lymph nodes, testes, salivary glands, adrenals, thyroid, stomach, and skin appendages. The heart weighed 470 gm.; there was hypertrophy of both ventricles. Other significant findings were edema and passive hyperemia of the lungs, advanced cirrhosis of the liver, and fibrosis of the pancreas. On the basis of clinical and autopsy findings the case was classified as idiopathic hemochromatosis.

Case 3.—A 59-year-old white man developed enlargement of liver and spleen. Laboratory examinations at this time revealed a hemoglobin of 7.3 gm. per cent, normal WBC and differential count, and bone marrow hyperplasia, particularly of the erythroid series. Coombs' tests were negative, the fecal urobilinogen was moderately elevated, but the serum bilirubin concentration was normal. The anemia persisted in spite of several transfusions and of therapy with vitamin B_{12} and iron. The spleen became larger. 20 months before death a splenectomy and cholecystectomy were performed. At this time the spleen weighed 525 gm., and showed reticuloendothelial hyperplasia without specific features. There was cholelithiasis with chronic cholecystitis. The anemia remained; hemoglobin concentrations returned to between 5 and 8 gm. per cent in spite of transfusions. During his final 1½ years, the patient received 27 liters of blood by transfusion. The liver became larger. The patient died of pulmonary embolism.

At autopsy, there were fresh emboli and organizing thrombi in the major pulmonary arteries. There was advanced hemosiderosis of the liver, but without cirrhosis. There was much hemosiderin in macrophages, situated in lymph nodes and bone marrow; and in epithelial cells of the gastric mucosa and of the pancreatic acini. Since histologic examination of the spleen 1½ years before death had not revealed hemosiderosis, the case was classified as secondary hemosiderosis, presumably due to transfusions, and possibly related to the anemia.

Isolation of Hemosiderin Granules:

Method I.—A sample of liver or spleen weighing up to 10 gm. was minced with scalpels in an equal weight of distilled water at room temperature. Care was taken to exclude the capsules of the organs. The tissue was homogenized in a Waring blendor designed for small volumes. The homogenate was ground with a pestle through a fine monel metal sieve to eliminate coarse fibers. The homogenate was then centrifuged for 15 minutes at 700 R.P.M. The supernatant was discarded, and the sediment was resuspended in sucrose solution (sucrose in distilled water, specific gravity of 1.20). The ratio of tissue volume to fluid volume was approximately 1:5. The suspension was ground thoroughly in a Blaessig tissue homogenizer,[1] using the L plunger provided. The homogenate was then centrifuged for 15 minutes at 700 R.P.M. Following this, the supernatant material was discarded, and the sediment thoroughly resuspended in a fivefold volume of sucrose solution (in distilled water) of specific gravity 1.40. This suspension was centrifuged for 30 minutes at 1000 R.P.M. The sediment was resuspended in a fivefold volume of distilled water and centrifuged at 600 R.P.M. for 15 minutes. This last step was repeated three times, and the final sediment was used in the experiments to be described.

After each centrifugation, the sediment was examined in the light microscope, and Prussian blue tests for trivalent iron (see below) were done to check for the presence of hemosiderin. As seen in the light microscope, the final product consisted of golden and brown granules,

[1] Obtained from Blaessig Glass Specialties, Rochester, New York.

measuring up to 6 μ in diameter, and of clumps of such granules. There were no other recognizable cell components. Nearly all the granules gave a strongly positive Prussian blue reaction for trivalent iron.

Method II.—This is essentially the method of Greenberg (18) in which aqueous solutions of KI are used as vehicle for separating hemosiderin granules by means of differential centrifugation. Greenberg's procedure was modified in that the tissue was homogenized in a Waring blendor, strained through a fine monel metal sieve, and subsequently ground in a Blaessig tissue homogenizer.[1]

Method III.—This is the method of Behrens and Asher (10) in which CCl_4 is used as a vehicle for separating hemosiderin granules. Again, the homogenates were prepared with the aid of a Waring blendor, a monel metal sieve, and a Blaessig tissue homogenizer as used in Methods I and II.

Preparation of Ferritin and Apoferritin:

Crystalline ferritin and apoferritin were prepared as described by Granick (19), using samples of liver and/or spleen.

Prussian (Berlin) Blue Tests:

Suspensions of hemosiderin granules, and sectioned tissues (see following) were tested for the presence of trivalent iron. The modification of Perls' Prussian blue test given by Lillie (20) was used. Confirmatory tests were done as described by G. D. Parkes in Mellor's Modern Inorganic Chemistry (21).

Chemical Analyses:

Iron.—Samples of hemosiderin or of crystalline ferritin were dried to constant weight in a desiccator. Weighed quantities were then thoroughly digested in a 1:1 mixture of H_2SO_4 and HNO_3 as described by Lorber (22). Subsequently, quantitative iron determinations were done as also described by Lorber (22). A Coleman spectrophotometer was used to determine optical densities. Lorber's method proved to be sufficiently accurate for the purposes of this study. Under optimal spectrophotometric conditions, we were able to detect concentrations of iron (in solution) as low as 1 μg/ml.

Nitrogen and Protein.—Hemosiderin and ferritin preparations were dried to constant weight. The nitrogen content was determined by means of a micro-Kjeldahl procedure (23).

To determine protein content, a biuret method was used (24). No attempt was made to differentiate peptides from protein. The granules were suspended in measured volumes of 50 per cent NaOH. The suspensions were heated to 75°C. for 5 minutes and subsequently kept at room temperature for 1 to 2 hours. Then the suspensions were centrifuged and a quantitative biuret test was done on the supernatants.

Serologic Procedures:

Hybrid male and female rabbits were given intravenous and subcutaneous injections of graded quantities of human ferritin. This ferritin was crystallized from the spleen of a case of transfusional hemosiderosis not included in the present study. Three recrystallizations with $CdSO_4$ were carried out, and $CdSO_4$ was removed by dialysis (19). The final product contained 22.90 per cent Fe, and 77.05 per cent protein as calculated from nitrogen determinations (on the basis of N $\times$ 6.25 = protein). The biuret method gave a protein content of 76.9 per cent.

Aliquots of ferritin were dissolved in Ringer's solution and used for immunization. The dosage schedule for the injections was changed from time to time. Up to 10 mg. of ferritin per injection were given subcutaneously or intravenously.

The rabbits were bled periodically to obtain sera. Antibody titers were determined by

means of precipitin tests, first by the tube dilution method. Sera with high titers were then used for quantitative determinations of antibody nitrogen according to methods of Kabat and Mayer (23). Appropriate modifications were used since antigens and antibodies were proteins. The concentrations of antibody nitrogen in the strongest antiferritin sera were in the range of 100 to 200 μg./ml.—amply sufficient for agar-gel diffusion tests.

Agar-gel double diffusion tests were performed essentially as described by Ouchterlony (25), but round rather then square wells were used. Further details will be given in the experimental sections.

Preparation of Tissues and of Hemosiderin Granules for Light and Electron Microscopy:

Liver biopsy specimens, obtained from Cases 1 and 2, and tissue from the spleen of Case 1 were cut into tiny blocks (1 mm. in greatest dimension). They were fixed for 1½ hours in Palade's fixative containing 3 per cent sucrose. They were dehydrated in graded concentrations of ethyl alcohol, and embedded in a mixture containing 9 parts of *n*-butyl methacrylate, and 1 part of methyl methacrylate. Luperco was used as a catalyst for polymerization.

Samples of isolated hemosiderin granules and of crystalline ferritin were not fixed in Palade's fluid, but were directly dehydrated in graded concentrations of ethyl alcohol. These granules were embedded (*a*) in methacrylate as described above, (*b*) in araldite (26), (*c*) in methacrylate as described, but with divinyl benzene in a concentration of 15 per cent in the final embedding mixture. In procedures (*a*) and (*c*) luperco was used as a catalyst.

Blocks were sectioned in a Porter-Blum microtome, using glass or diamond knives. The thickness of sections was varied according to requirements. Generally the thickness ranged between 200 and 1000 A.

In a few instances sections of crystalline ferritin with a thickness of one to two lattice planes were obtained. Such sections therefore had thicknesses of about 150 A (27). As a rule, the sections were mounted on carbon-coated specimen grids; for electron microdiffraction they were mounted on formvar-coated specimen grids.

Thick sections (0.5 to 2 μ) were also cut, and were used for light microscopy. Prussian blue tests for hemosiderin were done as already described.

Electron Microscopy and Microdiffraction:

An RCA electron microscope, model EMU-3b, was used for both electron microscopy and electron microdiffraction. The instrument was provided with objective and projector apertures described elsewhere (28), and was equipped for primary magnifications up to 120,000. It was operated at a beam potential of 100 kv. Magnifications were calibrated with a diffraction grating replica, and by means of monolayers of ferritin molecules on carbon substrates.

Transmission microdiffraction was done (*a*) after removal of the lower projector pole piece, and (*b*) with all pole pieces in place. The diffraction leaves provided by RCA were used. Standard calibrations were made (29, 30). The powder patterns obtained were evaluated by means of conventional crystallographic methods (29–32).

EXPERIMENTS AND RESULTS

Chemical Analyses.—Determinations of the iron, protein, and nitrogen content of the hemosiderin granules isolated from the three livers gave the results shown in Table I. The values obtained with samples of crystalline ferritin (prepared from the same livers), and iron to nitrogen ratios, are also shown in Table I. It can be seen that the three samples of ferritin were nearly identical with respect to protein, nitrogen, and iron content, while the samples of hemosiderin varied greatly. The percentage of protein in the hemosiderin

samples as determined by means of the biuret method was less than the product of their nitrogen content and 6.25. This indicates that non-protein nitrogen was present in the hemosiderin. By contrast, when the nitrogen content of the ferritin crystals is multiplied by 6.25, the product closely approximates the value obtained with the biuret method (Table I).

TABLE I

Summary of Findings on Hemosiderin Granules Isolated from Three Livers

Sex and age of patient	Diagnoses* (autopsy)	Sample	Per cent Fe‡	Per cent N‡	Per cent protein‡ (Biuret method)	(N) × 6.25 (per cent)	Fe/N
Male: 10	Thalassemia major; hemosiderosis (secondary)	Hemosiderin (Method I)	36	3.3	6.3	20.6	10.9
		Ferritin	23.1	12.2	76.8	76.3	1.9
Male: 53	Hemochromatosis ("idiopathic")	Hemosiderin (Method II)	7.5	5.5	11.5	34.4	1.4
		Hemosiderin (Method III)	7.6	5.3	13.8	33.1	1.4
		Ferritin	24.5	12.0	75.2	75.0	2.0
Male: 62	Hemosiderosis (secondary); anemia (hemolytic?)	Hemosiderin (Method II)	17.5	1.3	3.1	8.1	13.5
		Hemosiderin (Method III)	12.8	2.1	7.0	13.1	6.1
		Ferritin	22.9	12.3	75.9	76.8	1.9

* Only principal pertinent diagnoses are given.

‡ Per cent of dry weight.

Serologic Tests.—Antiferritin sera with antibody nitrogen concentrations of 180 μg./ml. and 22 μg./ml., respectively, were used in agar-gel diffusion tests against suspensions of hemosiderin granules and of crystalline ferritin in 0.9 per cent NaCl solution. As shown in Figs. 7 to 10, the antisera contained more than one antibody. One of the antisera gave up to three zones of precipitation with each of the three ferritin preparations, while the other two antisera gave at least two zones. These results raise the question whether the antibodies were directed against one or against several antigenic components of the three preparations of crystalline ferritin. Alternatively, one might ask whether the same impurities were present in all three preparations, as well as in the ferritin used to immunize the rabbits from which the antisera were obtained (see Materials and Methods). This problem is now being investigated further.

Fortunately, the brown color of ferritin proved to be an excellent marker of the precipitation zones, indicating that they actually contained ferritin. As is well known, this brown color is due to the presence of the ferric hydroxide in ferritin. Indeed, the precipitation zones gave positive Prussian blue tests. Furthermore, it can be seen in Fig. 7 that the apoferritin and ferritin, prepared from the same livers, gave identical precipitation zones. This finding confirms the evidence presented by Granick (33), and by Mazur and Shorr (34), that the immunologic specificity of ferritin is due to its protein moiety, apoferritin. It may be added parenthetically that the diffusion rates of ferritin and apoferritin in agar-gels are not identical (35). This phenomemon is undoubtedly related to the difference in molecular weight of the two substances, and is being explored further at present.

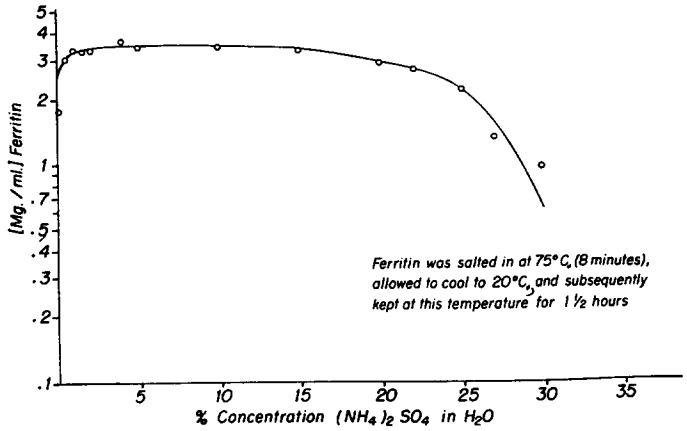
When the antiferritin sera were tested simultaneously (in agar-gel) against suspensions of hemosiderin granules and against suspensions of crystalline ferritin, identical precipitation zones developed (Figs. 7 to 10); but the reactions of the antisera with material diffusing from the hemosiderin wells were weak. Nevertheless, precipitation zones resulted from a reaction of material in hemosiderin granules with antiferritin sera; and these precipitation zones joined adjacent ferritin–antiferritin precipitation zones in the manner presumed to indicate identity of antigens (Figs. 7 to 10). Hence, one might conclude that antigenically active ferritin was present in the hemosiderin granules. To confirm this, the zones of precipitation were cut out of the agar diffusion plates, and prepared for electron microscopy. For this purpose the bits of agar containing the precipitates were dehydrated in graded concentrations of ethyl alcohol, embedded in methacrylate, and sectioned in routine manner (see Materials and Methods). The sections were then examined in the electron microscope. It was found that characteristic ferritin iron-hydroxide micelles were present in clumps in all precipitation zones examined, thus indicating the presence of ferritin molecules. However, in the precipitates derived from hemosiderin and antiferritin serum there were fewer ferritin molecules per area examined than were present in the control ferritin–antiferritin precipitates.

Transmission Electron Microscopy.—The findings on hemosiderin granules from all three cases were essentially similar. Images of representative, sectioned granules are shown in Figs. 1 to 4. As may be seen in Fig. 1, the fine structure of the granules is not uniform. The principal component of the granules that is visible in the electron microscope consists of aggregates of opaque particles ranging from about 10 to 75 A, and varying in shape from spherical or ellipsoid to filamentous. Some of the granules also contain osmiophilic globules and remnants of cytomembranes. A survey of individual granules at higher magnification reveals considerable variation in the structure or substructure of the opaque particles. In some granules (Figs. 3 and 4) many typical tetrads of ferritin iron micelles are apparent, while in other granules such configurations appear

to be absent (Fig. 2). Still other granules have regions with the structure of crystalline ferritin (Fig. 4). The findings are in agreement with evidence previously obtained in studies of hemosiderin *in situ* in cells (4–6; 16 *a*, *b*). They are also consistent with the relatively low protein content of the isolated mixtures of hemosiderin granules (Table I); and they are consistent with the results of the agar-gel precipitin tests that indicate the presence of only small quantities of serologically active ferritin (or apoferritin). It seems surprising, however, that most of the isolated granules contained so little ferritin since large quantities of ferritin are often demonstrable in hemosiderin deposits *in situ* in cells (4–6; 16 *a*, *b*). For example, examination of a biopsy specimen, obtained from the liver of Case 2 revealed many hemosiderin granules that were rich in ferritin iron micelles. These granules were situated in the cytoplasm of liver parenchymal cells (Figs. 11 and 14) and in sinusoidal endothelial cells. While there were also granules that appeared to contain few ferritin iron micelles or none, it was clear that there was considerably more ferritin in hemosiderin *in situ* in cells than there was in isolated granules of hemosiderin. One could surmise, therefore, that an appreciable quantity of ferritin had been lost in the process of isolation. That this surmise is reasonable will be shown in the following section.

Solubility of Human Ferritin.—Aqueous solvents are commonly used in the isolation of hemosiderin granules. Even in the procedure of Behrens and Taubert (9), in which sedimentation of the granules is accomplished with the aid of CCl_4, a preliminary treatment with aqueous solutions is used. Although there are differences in the solubility of ferritin derived from different animal species, it is clear that ferritin is soluble in aqueous solvents (19, 33, 36, 42). To provide more evidence on this point in relation to the present study, the solubility curve of human ferritin in aqueous ammonium sulfate was determined. For this purpose crystals of ferritin were prepared from all three livers, using Granick's methods (19). The $CdSO_4$ was removed by dialysis against distilled water until the dialysate no longer gave a positive sulfate test with $BaCl_2$. The ferritin crystals were then sedimented and resuspended in distilled water. The suspension was shaken thoroughly, and divided into equal aliquots. Care was taken to provide a large excess of undissolved ferritin. Equal volumes of various solutions of $(NH_4)_2SO_4$ in distilled water were added to aliquots of the ferritin suspension so as to produce known final concentrations of $(NH_4)_2SO_4$. To several of the aliquots only distilled water was added. The final volumes of all test suspensions were the same, and a large excess of undissolved ferritin remained in all of the suspensions after maximum solubilization. The final concentrations of $(NH_4)_2SO_4$ ranged from 0 to 30 per cent (Text-fig. 1). The test suspensions were heated to 75°C. and thoroughly mixed for 8 minutes. The solutions were then cooled to 20°C. The suspensions were briefly centrifuged, and the supernatant solutions were used for quantitative spectrophoto-

metric determinations of ferritin. The results of three determinations were the same within the limits of the method. As shown in Text-fig. 1, the solubility of human ferritin in dilute $(NH_4)_2SO_4$ solution is considerable; thus, in 1 per cent $(NH_4)_2SO_4$ it is greater than 3 mg./ml. It is also clear from Text-fig. 1 that characteristic salting-in and salting-out took place, and that the solubility was nearly constant over a wide range of concentrations of $(NH_4)_2SO_4$. It can be shown that solutions of NaCl yield similar effects, and one can infer that at the concentrations of electrolytes in cells the solubility of human ferritin must be considerable. Indeed, the efficacy of the standard method (19, 36) used for the extraction of ferritin is proof of this inference.



TEXT-FIG. 1. Solubility of human ferritin in solutions of $(NH_4)_2SO_4$.

In isolating hemosiderin granules from cells, the concomitant extraction of ferritin has generally been disregarded. Scrutiny of the experimental procedures reported by previous workers reveals that specific attempts to prevent extraction of ferritin were not made. The solubility study recorded in this section indicates that variable quantities of ferritin can be lost from hemosiderin if aqueous solvents are used in the isolation of hemosiderin.

Electron Diffraction Data.—Hemosiderin granules were dried at room temperature and atmospheric pressure for several days. They were then ground to a very fine powder. Dilute suspensions of the powder in distilled water were sprayed onto formvar-coated specimen grids which were then allowed to dry. While most of the powder particles were too thick for transmission electron diffraction, some of them gave adequate powder patterns (Fig. 13). In addition to this procedure, granules dehydrated in ethyl alcohol, embedded in methacrylate, and sectioned for electron microscopy were studied by means of selected area microdiffraction. The sections were generally about 1000 A thick,

and were mounted directly on specimen grids. Most of the methacrylate was then removed as best as possible by soaking in warm acetone during 24 hours. Then the grids were coated with formvar. This method of preparation permitted examination of the fine structure of individual granules on which diffraction patterns were obtained. Fig. 15 shows the results obtained with this combined procedure.

TABLE II

*Results of Electron Diffraction on Hemosiderin Granules**

$Fe_2O_3.xH_2O$		Hemosiderin	
Intensity‡	d_{hkl} (A)	Intensity	d_{hkl} (A)
		40	3.10
80	2.59	100	2.54
100	2.24	80	2.21
		40	1.82
100	1.58	80	1.57
70	1.36	70	1.35
70	1.30	50	1.30
		30	1.20
40	1.12		
		30	1.11
80	1.00	50	1.01
70	0.910	40	0.906
40	0.857	30	0.855
20	0.789		
40	0.745	30	0.745
30	0.707		
30	0.673		

* Isolated from liver of a 62-year-old male with acquired (?) hemolytic anemia and hemosiderosis.

‡ Estimated.

The spacings calculated from the pattern shown in Fig. 13 are given in Table II. For comparison, spacings determined for partly hydrous alpha ferric oxide (α-$Fe_2O_3 \cdot H_2O$) are also given in Table II (see also Fig. 12). It has been demonstrated before that the electron diffraction patterns of hydrated ferric oxides vary with the state of hydration (37). In the anhydrous state α-Fe_2O_3 gives many sharp rings, commensurate with the presence of a high degree of crystalline order (6, 38). When partly dehydrated colloidal gel of hydrous α-Fe_2O_3 is bombarded by an intense, finely collimated beam of electrons for some time (*e.g.* 100 kv. during 20 minutes), the diffraction patterns become progressively sharper until eventually the pattern of anhydrous α-Fe_2O_3 is obtained. Similar results can be achieved if the colloidal gel is dried at room temperature for

several days, and dehydrated by heating for 20 minutes at 200°C. (6). The results given in Table II clearly indicate the presence of hydrated α-Fe_2O_3 in the hemosiderin granules. Whether only α-$Fe_2O_3 \cdot H_2O$ is present, or a mixture of α-$Fe_2O_3 \cdot H_2O$, α-$Fe_2O_3 \cdot 3H_2O$, and α-Fe_2O_3, cannot be stated with certainty at this time.

Extraction of Ferritin and Apoferritin from Hemosiderin Granules: Demonstration in the Electron Microscope.—Confirmation of the presence of ferritin and its protein moiety, apoferritin, in isolated hemosiderin granules was sought by applying solubility properties of ferritin previously determined by Granick (19). Small quantities (200 mg. wet weight) of hemosiderin granules obtained from all three cases were suspended in 5 ml. of 2 per cent aqueous $(NH_4)_2SO_4$, and kept in a water bath at 70°C. for 2 hours. Following centrifugation at low speed (2000 R.P.M.), the supernatants were removed and dialyzed in cellophane bags against distilled water during 18 hours. The contents of the bags were then concentrated to 0.5 ml. by evaporation *in vacuo* in a desiccator. The concentrates had a light brown color. Drops of each concentrate were then spread upon carbon-coated specimen grids, dried in air, and doubly shadowed with chromium according to the method of Kahler and Lloyd (39). Using this preparative procedure the presence of ferritin molecules in the extracts of hemosiderin granules could be unequivocally proven as is demonstrated in Fig. 6. The remaining portions of the concentrated extracts were used for the demonstration of apoferritin by means of the "negative staining" technique. As previously reported by the author (41), apoferritin molecules and their internal structure can be demonstrated with this technique—a technique invented by Brenner and Horne (40). The concentrated extracts of hemosiderin granules were mixed with 3 ml. of 2 per cent phosphotungstic acid that had been previously neutralized with N KOH. To separate ferritin from apoferritin, the mixtures were centrifuged in microtubes in a Spinco ultracentrifuge (model L) at 142,000 *G* during 1 hour. This treatment results in nearly complete sedimentation of ferritin, whereas most apoferritin remains suspended immediately above the sediment. Following ultracentrifugation, the tubes were handled without agitating their contents. In each tube fluid was carefully removed from the top downwards, leaving the layer immediately above the bottom undisturbed. Samples from this layer were spread upon carbon-coated specimen grids. After drying for 24 hours at room temperature, these grids were examined in the electron microscope at high magnifications, and under conditions suitable for work requiring high resolution. The images clearly showed outlines of particles corresponding in size, shape, and internal structure to the apoferritin molecule as previously demonstrated (41). As seen in Fig. 5, there are innumerable non-opaque circular profiles, 105 A in diameter, that enclose opaque cores with diameters of up to 60 A. As has been shown before, the contours of the cores correspond to the central cavities of apoferritin mole-

cules, while the non-opaque circular profiles that surround the cores represent the protein shells of apoferritin molecules. In the complete ferritin molecule the central cavities of apoferritin are filled with ferric hydroxide micelles in a characteristic configuration. These micelles can be removed by means of reducing agents (*e.g.* dithionite solution), leaving behind intact apoferritin protein shells situated around central cavities.

In sum, both ferritin and apoferritin were shown to be present in hemosiderin granules isolated from a case of hemochromatosis, and from two cases of secondary hemosiderosis. But it must be emphasized that only a fraction of the material contained in the isolated granules was actually extracted, and shown to be ferritin or apoferritin.

The implications of the findings will now be discussed.

DISCUSSION

The findings set forth above clearly indicate that the composition of hemosiderin granules from the three cases varied considerably, but the results were qualitatively similar with respect to inorganic iron and to ferritin and apoferritin. A detailed and comparative study of organic components of hemosiderin is necessary before wider generalizations can be made. Although some evidence on the composition of organic components has been reported by other investigators (1–3), it is not known whether there are significant differences between the organic components of hemosiderin in idiopathic hemochromatosis and in hemosiderosis.

A number of workers have investigated the chemical composition of horse spleen hemosiderin. Thus, it was shown by Behrens and Asher that the iron content of horse spleen hemosiderin can be as high as 35.9 per cent of dry weight (10). In this work CCl_4 was used as the vehicle for separating hemosiderin from other cell components. In later work Behrens and Taubert refined the procedure for separating horse spleen hemosiderin (9). They did not find ferritin in the hemosiderin which they isolated from horse spleen, but stated that some protein was probably present. Scrutiny of their experimental protocol gives reason to believe that ferritin was extracted during treatment of the tissue homogenate preceding the separation of hemosiderin granules. Thus, the tissue homogenate was heated in aqueous solution with and without $(NH_4)_2SO_4$. The findings of Granick on the solubility of horse ferritin (36), and the shape of the solubility curve of human ferritin shown in Text-fig.1, indicate that ferritin is extracted from hemosiderin granules obtained from cell homogenates by the method of Behrens and Taubert.

Bielig and Wöhler have reported that apoferritin is present in horse spleen hemosiderin (12). Similar observations were recorded by Greenberg (18) and by McKay and Fineberg (11), who used aqueous KI as a vehicle for separating hemosiderin granules. These workers also found protein other than apoferritin

in the granules. In granules isolated from horse spleen the protein content has been relatively low, and the iron content high. This also applies to data published by Ludewig, who found that horse spleen hemosiderin granules, separated by means of differential centrifugation in solutions of KBr or of KI, had an iron content of 24 to 36 per cent, a nitrogen content of 3.7 to 5.4 per cent (3). Ludewig demonstrated the presence in hemosiderin of hexosamine, galactose, mannose, and fucose. He concluded from his data that there are different kinds of hemosiderin or "hemosidera" (*sic*).

A different light on the constitution of hemosiderin was cast by recent work of Shoden and Sturgeon (17) which has extended earlier observations by Shoden, Gabrio, and Finch (42). Shoden and Sturgeon have insisted upon a clear separation of hemosiderin from ferritin. They studied the distribution of ferritin and "hemosiderin" in rabbits that had been given intravenous injections of saccharated iron oxide, iron-dextran, or ferrous sulfate. In their experimental procedure ferritin was first extracted from liver homogenates; any remaining iron-rich granules were separated by means of differential centrifugation or with a magnet. It is this residue of granules to which Shoden and Sturgeon have applied the term "hemosiderin." Under the circumstances, one would not expect these granules to contain ferritin. Although immunochemical analyses were not reported, the data of Shoden and Sturgeon would seem to indicate absence of ferritin from the hemosiderin granules which they isolated. Examination of some of their material in the electron microscope has confirmed this impression (35). It should be added, however, that the parenteral injection of saccharated iron oxide or of iron-dextran results in the formation of peculiar intracellular precipitates throughout the reticuloendothelial system. Such precipitates resemble true endogenous hemosiderin when viewed in the light microscope, but have been shown to consist largely of hydrous α-Fe_2O_3 with a characteristic structure. These precipitates are only gradually transformed, partly into ferritin (6).

Precipitates of the hydrous ferric oxide, present in saccharated iron oxide and in iron-dextran, differ from ferritin in their magnetic susceptibility as do the colloidal ferric hydroxides prepared by standard laboratory methods. Iron in the synthetic compounds has a magnetic susceptibility equivalent to 2 or to 5 unpaired electrons (43, 17). By contrast, the iron atoms of ferritin have magnetic susceptibilities indicative of 3 unpaired electrons; and hemosiderin granules of the type here termed "endogenous" were found to contain iron in the same magnetic state (43). One would expect, therefore, that "hemosiderin" granules resulting from the parenteral administration of synthetic iron compounds would contain trivalent iron in several states of magnetic susceptibility unless a complete physical transformation from the 2 or 5 state to the 3 state had taken place. From previous reports one can infer that hemosiderin derived from endogenous metabolic processes (*e.g.* hemochromatosis, transfusional

hemosiderosis) would contain predominantly iron atoms with 3 unpaired electrons. It is clear that electron microscopy permits selective study of individual hemosiderin granules, and by virtue of this fact one can discriminate between different types of granules. On the other hand, chemical and serologic examination of granules in bulk can yield only statistical averages. For example, if in a sample that contains many hemosiderin granules a few granules differ markedly from all the others, the difference would be distributed over the entire sample in the results of bulk chemical and serologic studies. In this case, the difference might be inconspicuous, or might not be detected at all.

It has been shown previously that in experimental hemosiderosis of short duration, induced by injecting hemoglobin into rats, a large proportion of hemosiderin granules are rich in ferritin; and that certain cell organelles, for which the term "siderosomes" has been proposed, are intimately connected with the formation and turnover of ferritin in diverse types of cells (4, 5). At present, it appears that siderosomes are of two general varieties. One variety, the most common, is characterized by its heterogeneity in electron micrographs. Typically, these siderosomes vary greatly in size, shape, and symmetry; they may have diameters up to 2 or 3 microns though most of them are smaller than this. These cytoplasmic bodies are delimited by membranes which may be single or multiple. Similar membranes, irregularly disposed, are often present in the interior of these siderosomes. Less often, these membranes form orderly layered arrays. However, the distinctive morphologic feature of these siderosomes is their content of iron compounds that possess characteristic structure. The derivation of this type of siderosome is still a matter of conjecture. While it may be derived from mitochondria, it seems more likely that it originates from pinocytic vacuoles.

The second type of siderosome is seen especially well in maturing erythroblasts, and is clearly an organelle derived from mitochondria (14–16 *a*). As a rule, this variety contains iron mainly in the form of ferritin, although under certain circumstances, other iron compounds appear to be present (4, 5, 14–16 *a*, *b*).

In cases of hemosiderosis and hemochromatosis of long duration most of the hemosiderin granules are not bounded by membranes. In such cases the cytoplasm of liver cells, and of macrophages in the spleen, may contain many large hemosiderin granules that are free of membranes; and siderosomes may be scarce or absent. On the basis of previous studies it seems likely that the formation of siderosomes precedes production of large non-membranous hemosiderin granules. Perhaps the latter granules result from degenerative changes in siderosomes.

For purposes of discussion, the trivalent iron in hemosiderin of endogenous origin can be grouped into two categories. The first of these categories includes iron that is clearly a part of molecules of ferritin, while the second category

comprises all other trivalent iron in hemosiderin. In explaining the pathogenesis of hemochromatosis and of secondary hemosiderosis one of the principal problems is that of accounting for these two forms of iron. Although the deposition of two forms of trivalent iron may be the result of wholly distinct pathways in cell metabolism, the sum of experimental evidence indicates that this is not the case. On the contrary, the electron optical studies, and the magneto-, bio-, and immunochemical analyses of endogenous hemosiderin all point to a close relationship between the two categories of iron; and it appears likely that all of this trivalent iron originates during ferritin synthesis.

The established facts on the nature of iron in endogenous hemosiderin can be adequately explained if one assumes that denaturation and degradation of ferritin can and do take place inside of cells. On this assumption the trivalent non-ferritin iron in hemosiderin would result from denaturation and degradation of aggregated ferritin. Such an hypothesis accounts for the presence of variable quantities of ferritin and apoferritin in hemosiderin. It also accounts for the presence in hemosiderin of iron atoms with 3 unpaired electrons. It accords with the statistical studies on the sizes of iron-containing particles in hemosiderin granules (4). Furthermore, this hypothesis is fully compatible with the presence of carbohydrates, lipids, and various proteins in some varieties of hemosiderin granules. Such organic substances may be remnants of structural elements of siderosomes, of other cytoplasmic constituents, or they may be decomposition products of material taken into cells from the extracellular environment. The last mentioned mechanism was demonstrated in histiocytes in bone marrow by Bessis and Breton-Gorius (16 *a*), who found that in macrophages phagocytized erythrocytes are transformed into granules containing a mixture of ferritin, lipid, and unidentified material. It is also well known that various forms of hydrous ferric oxides, especially their colloidal gels, are excellent adsorbents, and can bind organic substances.

The proposed hypothesis takes into account the fact that, as a general rule, the ferritin content of liver, spleen, and other organs is vastly increased in hemochromatosis and in hemosiderosis.

Any hypothesis on the pathogenesis of hemochromatosis and of secondary hemosiderosis must account for the physical state of the iron hydroxide in these conditions. It can hardly be an accident that the iron hydroxide contained in molecules of ferritin is in a specific state of physical order, and possesses iron atoms with a characteristic magnetic susceptibility. Recent physico-chemical investigations have made it plain that the physical state of ferric hydroxide in ferritin is the result of interaction between apoferritin and iron (44, 45). The formation of ferric hydroxide of a particular physical type within molecules of the protein, apoferritin, must involve more than a simple chemical reaction, *e.g.* more then oxidation of Fe^{++} to Fe^{+++}, and subsequent production of (FeOOH) complexes. There must also be a mechanism for the change in the

quantum state of the iron, and for the physical ordering of the (FeOOH). One could, of course, assume that various sorts of cells can bring about such transformations without the intervention of apoferritin. At present we have no evidence that this is so. We do, however, have much evidence that indicates a close relationship between the cellular production of ferritin and the development of hemosiderin deposits. If there is a mechanism for the production of endogenous hemosiderin that is unrelated to the metabolism of ferritin, the demonstration of its existence has still to be provided. The reasons for iron storage in hemochromatosis or hemosiderosis are still unknown. The evidence points to metabolic changes in cells; in idiopathic hemochromatosis genetic determinants may be implicated (46, 47), but in secondary hemosiderosis inheritance does not seem to play a role. Whatever the metabolic events, the implication of ferritin in the pathogenesis of iron storage diseases seems to be established.

SUMMARY

Using three different methods of cells fractionation, hemosiderin granules were isolated from tissues (liver and/or spleen) of three patients. The samples were obtained from a case of idiopathic hemochromatosis, a case of thalassemia major with secondary (transfusional?) hemosiderosis, and a case of transfusional hemosiderosis associated with an unclassified anemia.

Iron, nitrogen, and protein content of the hemosiderin granules varied over a wide range. Electron microscopy of sectioned granules revealed aggregates of dense particles of different shapes, with diameters ranging from 10 A to about 75 A. In some of the granules dense particles corresponding to the iron hydroxide micelles of ferritin molecules were abundant. But many of the granules contained very few of these molecules.

The presence of ferritin and apoferritin in the samples of hemosiderin granules was demonstrated by means of precipitin tests in agar-gel, using rabbit antiferritin sera with known antibody nitrogen concentrations. At least three antigenic components were detected in highly purified crystalline ferritin prepared from tissues of the three patients; the hemosiderin granules contained the same antigens, but probably in much smaller quantities. Both ferritin and apoferritin molecules were extracted from hemosiderin granules, and were demonstrated in the electron microscope after suitable preparation.

The solubility curve of human ferritin in solutions of $(NH_4)_2SO_4$ was investi gated. The results indicate that substantial quantities of ferritin or apoferritin can be lost in saline, aqueous media during isolation of hemosiderin granules from cells.

It was shown by means of electron microdiffraction on selected hemosiderin granules that the dense particles represent forms of partly hydrated α-Fe_2O_3. The conditions necessary for electron microdiffraction in an electron micro-

scope precluded an exact determination of the state of hydration of the α-Fe_2O_3 or of its structural relation to (FeOOH) micelles of pure ferritin in its undenatured state.

The findings were considered in the light of evidence on the structure and disposition of hemosiderin *in situ* in cells, and on the structure of ferritin. Differences between endogenous hemosiderin and hemosiderin derived from injections of colloidal iron compounds were pointed out. The evidence indicates that in hemochromatosis and in secondary hemosiderosis much of the inorganic storage iron in liver and spleen is derived from degraded ferritin.

The findings suggest that an abnormal cellular metabolic pathway of ferritin is implicated in the pathogenesis of hemochromatosis and transfusional hemosiderosis.

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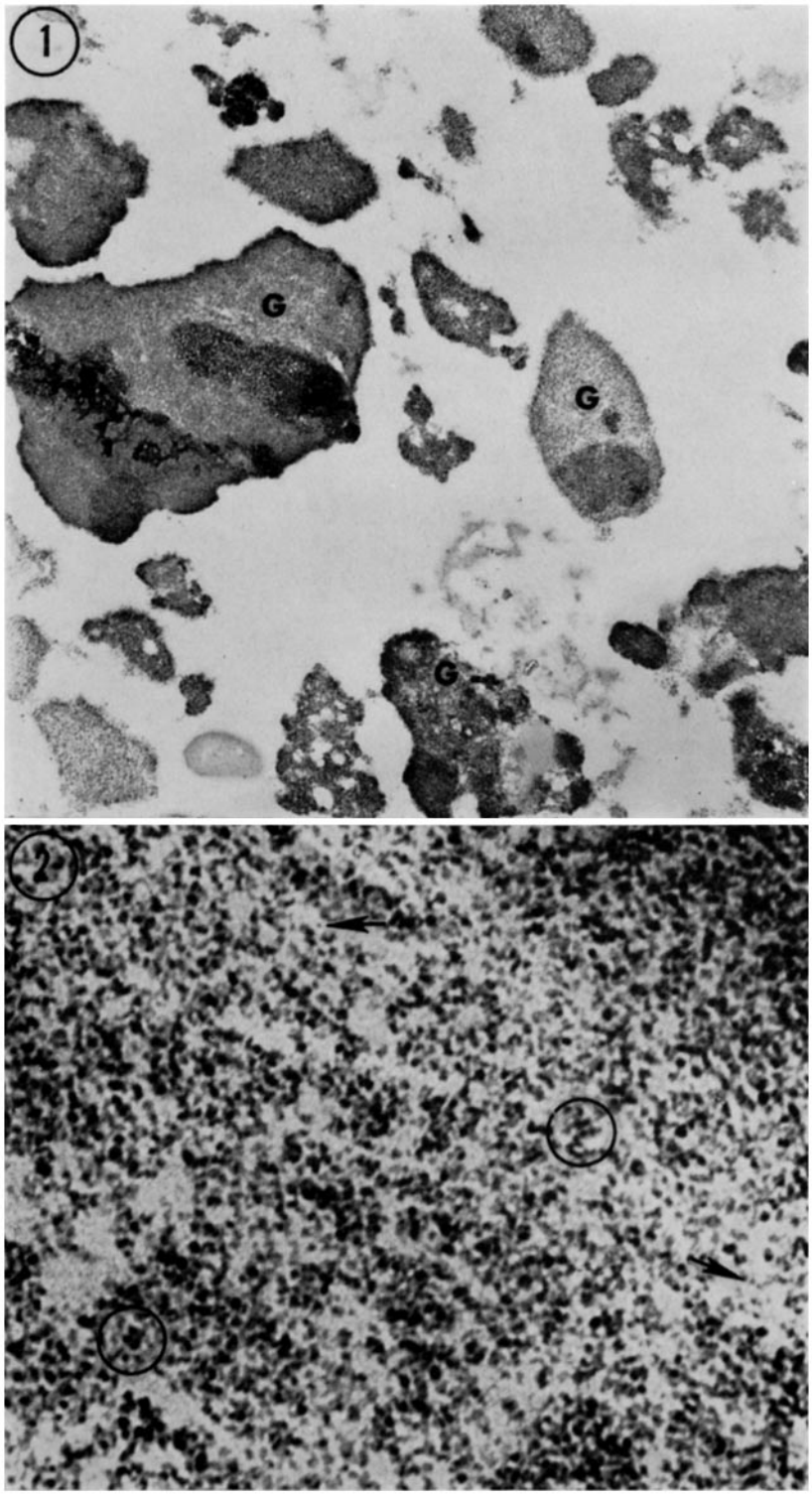
EXPLANATION OF PLATES

PLATE 47

FIG. 1. Electron micrograph of hemosiderin granules isolated from Case 2 (idiopathic hemochromatosis) by means of differential centrifugation in sucrose solutions (see text). Although the granules (*G*) are not homogeneous, they appear to be largely composed of closely packed aggregates of dense particles, less than 100 A in diameter. × 33,000.

FIG. 2. Part of one type of hemosiderin granule isolated from Case 2. At this magnification the smallest visible particles measure about 10 A in diameter (arrows). Owing to the dense packing of particles in this picture, one cannot be certain that ferritin molecules are present. However, there are some square arrays, 60 A in diameter, with four visible subunits, 30 A in diameter (circles). × 400,000.



(Richter: Nature of storage iron)

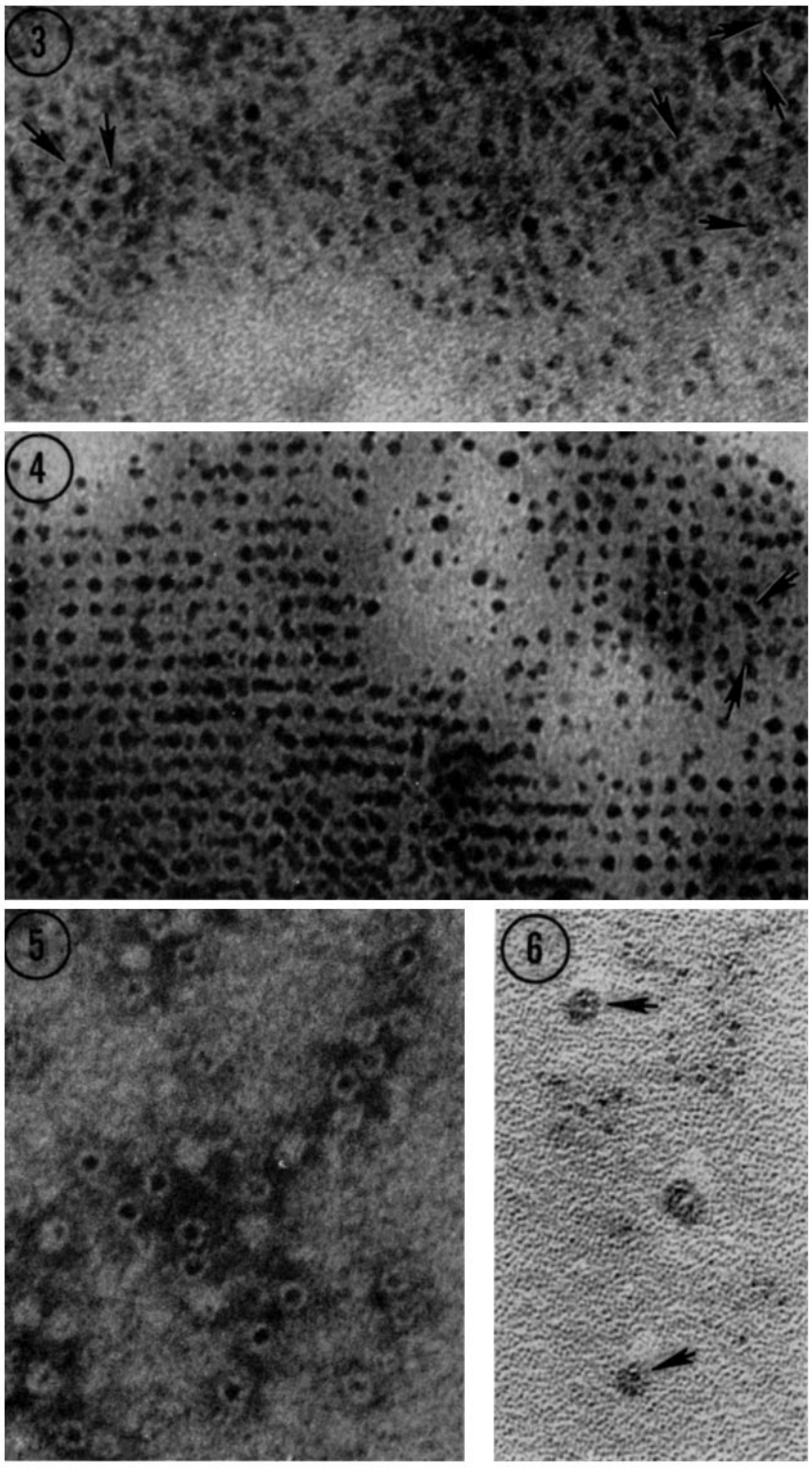
PLATE 48

FIG. 3. Part of another hemosiderin granule from the liver of Case 2. (FeOOH) micelles of ferritin molecules can be seen; they appear as characteristic profiles (mostly tetrads, triads, or dyads of particles with diameters of 30 A). See arrows. × 320,000.

FIG. 4. Part of hemosiderin granule from liver of Case 3 (secondary hemosiderosis). This granule is largely composed of ferritin. Note regions of close packing with cubic symmetry. In such regions the distances between the centers of adjacent particles are about 105 A. The particles appear to have subunits. Tetrads, triads, and dyads can be seen (arrows). The tetrads have diameters of about 60 A; the subunits again measure close to 30 A in diameter. There are also scattered smaller particles (~ 20 A). × 320,000.

FIG. 5. Phosphotungstate spray preparation of apoferritin molecules (see text for details). The apoferritin was extracted from hemosiderin granules obtained from the liver of Case 2. Pale circular profiles, 105 A in diameter, are outlined by the background of phosphotungstate. Within many of the pale halos there are dense, somewhat irregular cores, about 60 A in diameter. As shown previously (41), the cores represent phosphotungstate that has penetrated the interior of the particles. The profiles and cores have the size, configuration, and shape of apoferritin molecules. Note that some of the circular profiles (halos) do not have dense cores. Presumably these molecules have not been filled with phosphotungstate. × 375,000.

FIG. 6. Images of ferritin molecules extracted from hemosiderin granules (Case 2), and doubly shadowed with chromium. The arrows indicate typical circular profiles of entire, shadowed ferritin molecules (105 A in diameter). Within the shadowed profiles there are central arrays of dense particles: (FeOOH) micelles that measure about 60 A in diameter, and are composed of smaller particles (× 230,000).

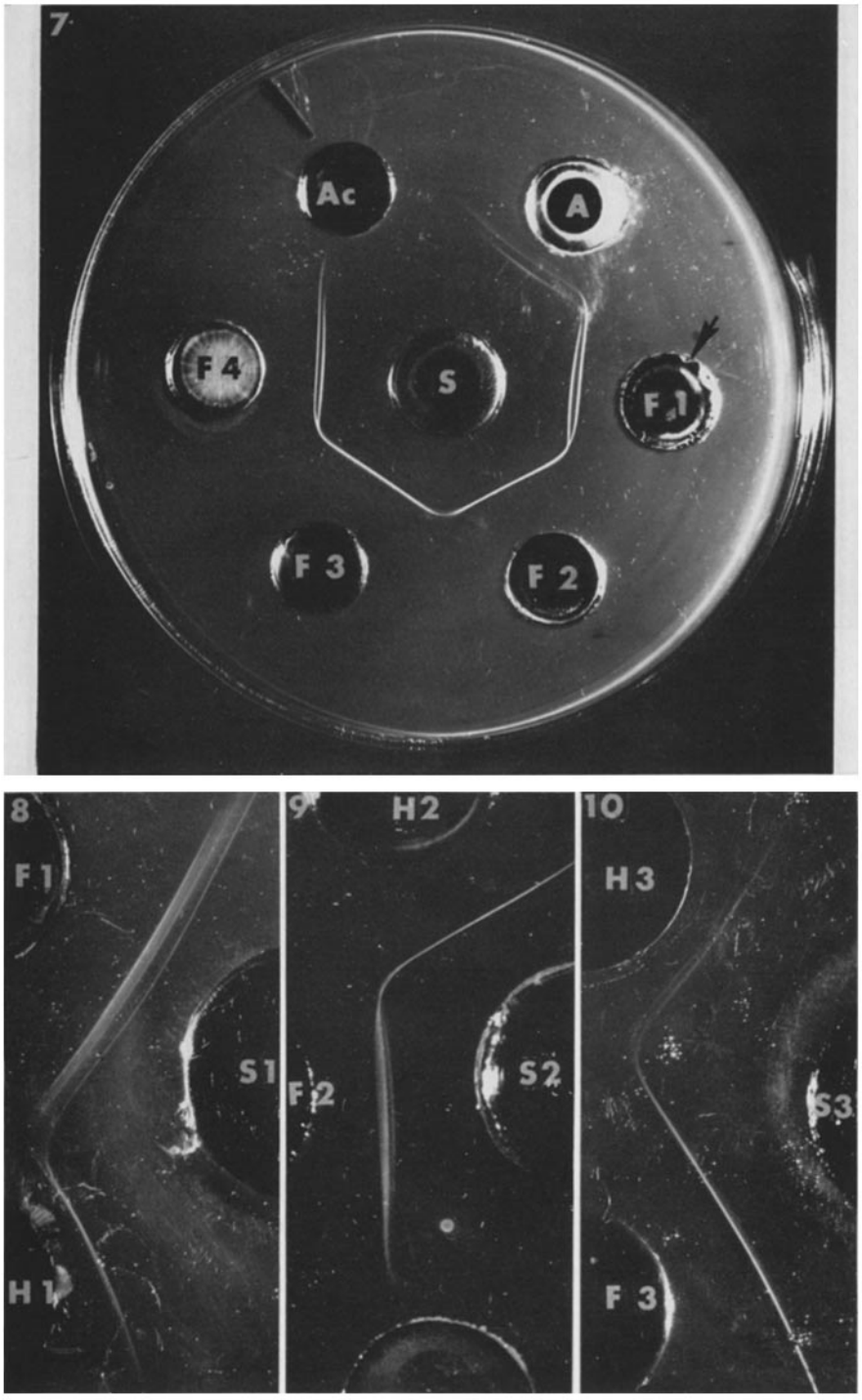


(Richter: Nature of storage iron)

PLATE 49

FIG. 7. Picture of agar-gel diffusion plate showing precipitation zones produced by the reaction of rabbit antiserum with ferritin and apoferritin, respectively. *S:* antiserum. *F 1, F 2, F 3:* ferritin isolated from tissues of Cases 1, 2, and 3 respectively (see Materials and Methods). *F 4:* ferritin used to produce the antiserum. *A:* apoferritin from Case 1. *Ac:* apoferritin that was heated at 95°C. for 10 minutes and presumably denatured. The two precipitation zones at *F 1* and *F 4* fuse with the zones at *F 2* and *F 3*, respectively. However, the concentrations of ferritin in *F 2* and *F 3* were greater than those of ferritin in *F 1* and *F 4*; hence different diffusion gradients were produced, resulting in incomplete separation of zones at *F 2* and *F 3*. Note that there are three zones at *A*. The two that are closest to *A* fuse with the zone that is closest to *F 1*. The inner zone at *A* continues into the inner zone at *F 1*. The zones at *Ac* were too faint to be seen in this picture.

FIGS. 8 to 10. Results of precipitin tests in agar-gel. *F 1, F 2, F 3:* suspensions of ferritin crystals from Cases 1, 2, and 3, respectively. *S 1, S 2, S 3:* three rabbit antisera resulting from injections of *F 1, F 2, F 3*. *H 1, H 2, H 3:* suspensions of hemosiderin granules from Case 1, 2, 3. In all three figures material diffusing from ferritin wells and from the neighboring hemosiderin wells has given rise to precipitation zones that are completely joined to each other. In each instance, at least one of the zones produced had a light tan color, confirming the presence of ferritin (see text). Although separation of the different zones is incomplete, the presence of identical antigens in ferritin and hemosiderin is indicated.



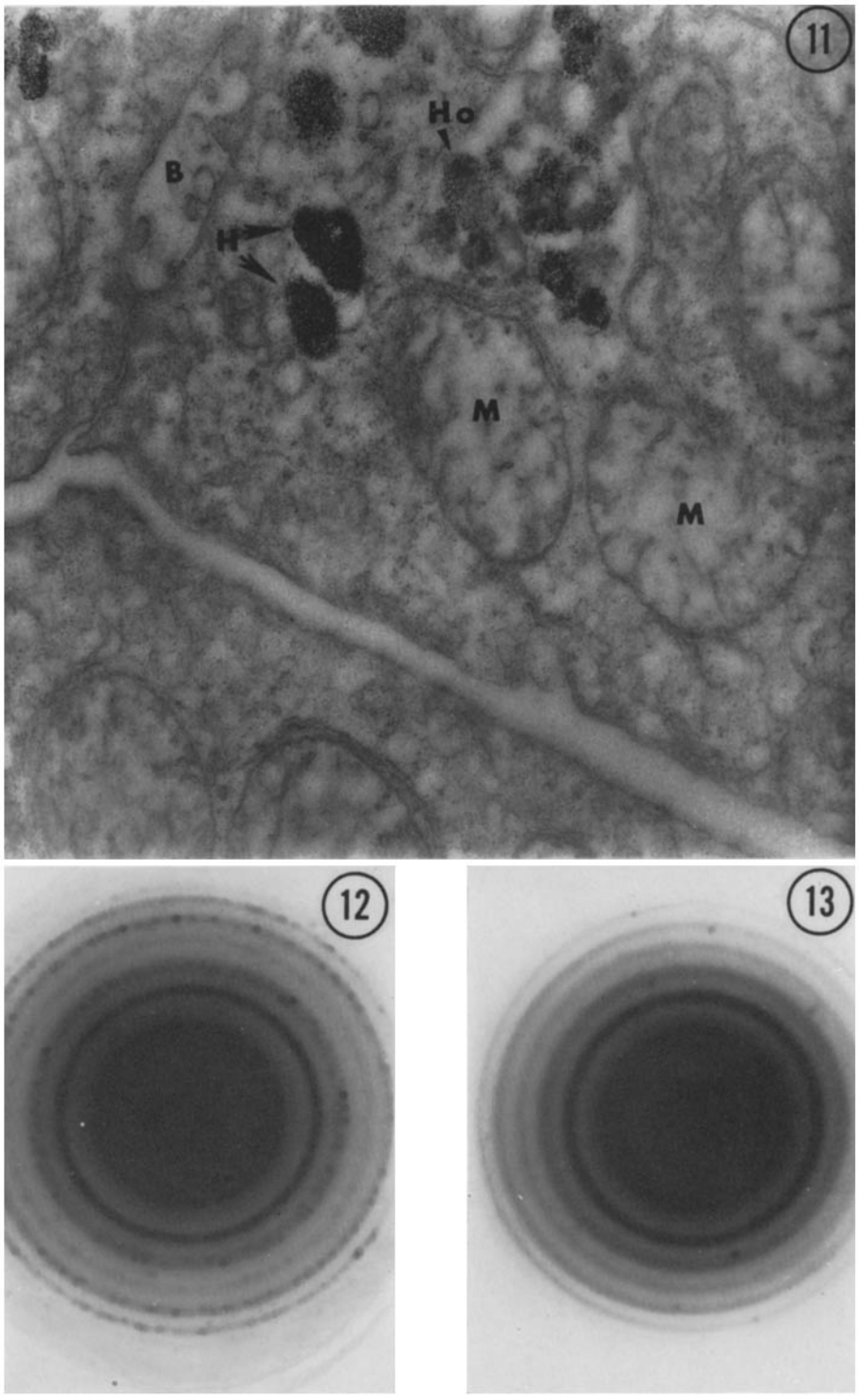
(Richter: Nature of storage iron)

PLATE 50

FIG. 11. Hemosiderin in liver cell of biopsy specimen obtained from Case 1 (thalassemia major). Parts of three liver cells are shown. *H:* hemosiderin granules containing closely packed particles, 60 A in diameter; several of the granules are enclosed by a membrane. *Ho*, hemosiderin granules that contain smudgy osmiophilic material as well as dense particles. *M*, mitochondria. *B*, bile canaliculus. Note the innumerable dense particles (60 A in diameter) in the cytoplasmic matrix, in the bile canaliculus and in a few cytoplasmic vesicles. × 50,000.

FIG. 12. Electron microdiffraction powder pattern obtained from particles of partly hydrated alpha ferric oxide (α-$Fe_2O_3 \cdot xH_2O$) prepared in the laboratory. The d_{hkl} spacings are given in Table II.

Fig. 13. Electron microdiffraction powder pattern obtained from dried hemosiderin granules from Case 2 (idiopathic hemochromatosis). The granules were attached to a formvar-coated specimen grid. The d_{hkl} spacing of this pattern are identical with those obtained from the reference sample of partly hydrated alpha ferric oxide as shown in Table II and Fig. 12.

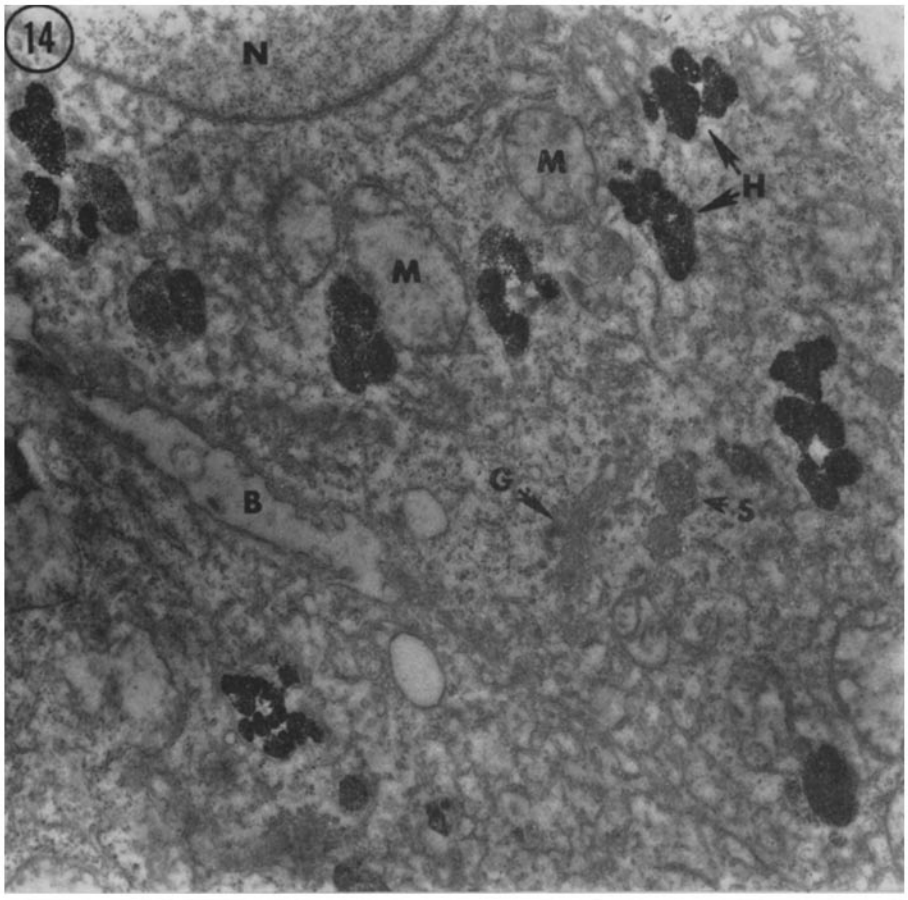


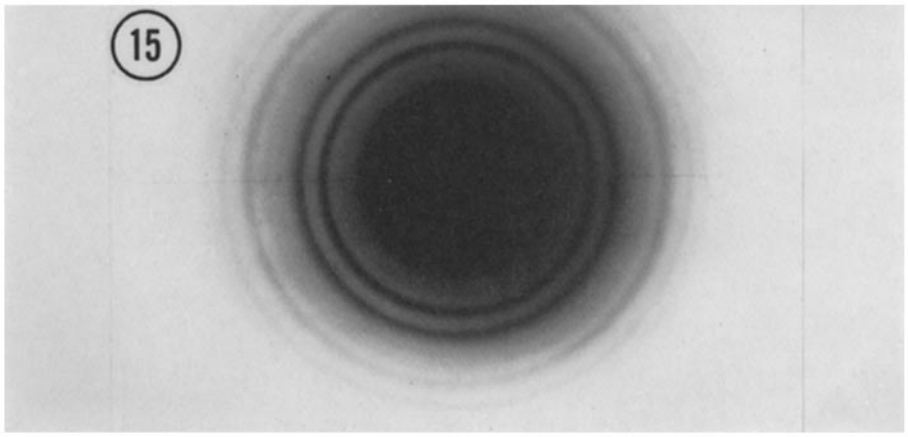
(Richter: Nature of storage iron)

PLATE 51

FIG. 14. Hemosiderin in cytoplasm of two adjacent liver cells from Case 3 (unclassified anemia with transfusional hemosiderosis). Compare with Fig. 11. *H*, hemosiderin granules. *S*, siderosome. *M*, mitochondria. *G*, Golgi apparatus. *B*, bile canaliculus. *N*, nucleus. × 33,000.

FIG. 15. Typical electron microdiffraction pattern obtained from hemosiderin granules in field shown in Fig. 14. The d_{hkl} spacings are given in Table II and are in agreement with the spacings obtained from the reference material (α-$Fe_2O_3 \cdot xH_2O$).





(Richter: Nature of storage iron)