FERRITIN: ITS PROPERTIES AND SIGNIFICANCE FOR IRON METABOLISM

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Received September 19, 1945

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Ferritin is a protein, crystallizable by cadmium sulfate, which may contain as much as 23 per cent by weight of iron. It has been isolated from bone marrow, spleen, and liver of a number of animals and has recently been detected in the gastrointestinal mucosa. The iron can be removed from ferritin without denaturing the protein. The protein thus produced is designated as apoferritin. It is homogeneous, has a molecular weight of 460,000, and crystallizes with cadmium sulfate, the space lattices of the ferritin and the apoferritin crystals being identical. The iron is present as micelles of the approximate composition $[(FeOOH)_8 \cdot (FeOPO_3H_2)]$, firmly bound to the protein. The variable size and concentration of these micelles account for the inhomogeneity of ferritin. The iron is in the ferric

state and is the only biologically occurring iron compound to possess a magnetic moment equivalent to three unpaired electrons per iron atom. Tracer studies with radioactive iron show that inorganic iron and the iron in the hemoglobin of red cells can be rapidly converted in the liver, spleen, and bone marrow into ferritin iron. Ferritin functions as a store of iron readily available for hemin synthesis, the iron being held in small micelles of large surface area on a particular protein, apoferritin, thus protecting the cell from toxic accumulations of ferric hydroxide. It is probable that ferritin may also function in the regulation of iron absorption by the gastrointestinal mucosa.

I. Introduction

With the discovery of a protein iron compound, ferritin, containing 23 per cent of iron, and the advent of radioactive iron as tracer, the study of iron metabolism has entered a new phase of development. It will be the purpose of this review to call attention not only to the few scattered facts recently arrived at but also to the newly recognized and tempting gaps which are so readily filled with conjecture and hypothesis. We shall limit ourselves primarily to a discussion of the properties of ferritin, and of its function as an iron-storage compound and as a possible regulator of iron absorption. Other aspects of iron metabolism will be discussed only as they relate to ferritin.

The total iron in a 70-kg. man averages about 4.3 g. (22). Of this iron, some 55 per cent is contained in the circulating hemoglobin, and about 10 per cent is a constituent of muscle hemoglobin and the heme catalysts. The remainder, 30–35 per cent of the body iron, may be designated as the storage-iron fraction. About two-thirds of this storage iron, or 0.8 g., is located in the liver, spleen, and red bone marrow. As pointed out by Hahn (18), the storage iron in these organs is sufficient, when converted into hemoglobin, to increase the circulating hemoglobin by about 30–50 per cent.

The storage-iron fraction, for purposes of this review, will be divided into three parts: monomolecularly dispersed iron compounds; colloidal iron compounds, namely, ferritin and non-crystallizable ferritin; and a microscopically visible granular iron material, hemosiderin.

The monomolecularly dispersed ferrous and ferric compounds, which are probably in the form of their hydroxides, may be considered to represent the metabolically active iron which may be in equilibrium with the storage-iron compound, ferritin.

The colloidal iron compounds, ferritin and non-crystallizable ferritin, appear to contain iron associated with one or possibly more proteins. The iron is in the form of a reddish brown ferric hydroxide of the approximate composition $[(FeOOH)_8 \cdot (FeOPO_3H_2)]$, the iron being uniquely characterized by magnetic measurements as possessing a constant magnetic dipole moment of 3.8 Bohr magnetons, a moment rarely observed in other iron compounds. The size of the micelles is unknown, but they are probably not uniform in size. Iron micelles with these properties we shall designate as "ferritin–iron micelles", "iron micelles" or "ferritin iron" also being used where no ambiguity arises.

When ferritin–iron micelles are attached to a specific protein, apoferritin (through an unknown chemical linkage), the complex of this protein with the

ferritin–iron micelles is designated as ferritin. Apoferritin is a homogeneous protein which may be readily crystallized with cadmium sulfate, yielding colorless crystals. When apoferritin has iron micelles attached, then red-brown crystals of ferritin may be obtained with a maximum amount of iron equivalent to as much as 23 per cent of the dry weight of the dialyzed crystalline material.

Some of the ferritin–iron micelles appear to be attached to proteins which cannot be crystallized. These inhomogeneous materials, which still possess the characteristic ferritin–iron micelles, are placed together into a group termed the "non-crystallizable ferritin" or NCF. Some of this material is undoubtedly denatured ferritin (i.e., denatured apoferritin with attached ferritin–iron micelles). It is possible that there are also apoferritin molecules with relatively large iron micelles which do not crystallize because they cannot fit spacially into the crystal lattice. Possibly still other material may represent two ferritin molecules bound together by cross links between the ferritin–iron micelles of one molecule and the ferritin–iron micelles of the other. It is conceivable that these aggregates might increase to such proportions that they would become microscopically visible and be classed with the hemosiderin granules.

The microscopically visible aggregates of iron pigment have long been familiar to histologists as "hemosiderin" granules. The iron of the granules of the horse spleen can be distinguished from ferritin iron magnetically and also chemically, since some of the granules contain a higher ratio of iron to protein than is present in ferritin.

II. History of Hemosiderin and Ferritin Iron

In the period 1870–1880 granular deposits in the liver and spleen, which gave the characteristic Prussian blue stain for iron and which are today designated as hemosiderin (38), began to be reported with frequency. These granules varied from colorless through pale yellow to deep brown in color. One of the best materials in which the iron-rich granules are easily visible is the splenic pulp of old horses where, according to Nasse (37), over 5 per cent of the dry weight of the spleen consisted of these granules. Neumann (38) and Perls (43) early recognized these granules to contain an iron oxide and iron phosphate. In other animals, iron-containing granules are not common but become apparent on the rapid breakdown of hemoglobin (55). For example, they are readily seen in the reticular cells of the bone marrow some 36 hr. after the injection of hemoglobin. Besides the reticular tissue of liver, spleen, and bone marrow, there is to a lesser degree a predilection for iron by the globus pallidus and substantia nigra of the central nervous system (48).

Succeeding investigators came to recognize that iron was present not only in granules visible under the microscope, but also in a more diffuse form. According to Schmiedeberg (47), the iron was attached to a protein and this compound, which he called Ferratin, was present in spleen, liver, and marrow. From pig liver, by boiling with water and precipitating with tartaric acid, Schmiedeberg isolated an iron protein containing 6 per cent of iron. (Today we know

that this compound was a mixture of denatured ferritin and denatured proteins.) Other investigators, including Zaleski (58), Salkowski (46), and Hammarstein, were of the opinion that the iron extracted and fractionated from liver was closely linked with nucleoproteins. Still another view was held by Burow (5), who claimed the iron to be associated with lipoids in the spleen. Of recent date but of historical vintage is the preparation of denatured ferritin, called ferrin (26).

A beginning in the understanding of the true character of the soluble iron compound was made in a notable contribution by Laufberger (25) in 1937. Laufberger isolated from horse spleen a protein to which he gave the name ferritin, in deference to the work of Schmiedeberg, who had almost isolated this compound. This protein was red-brown in color, contained over 20 per cent by dry weight of iron, and was crystallizable by means of cadmium sulfate. The isolation procedure depended on the protein being stable at 80°C.; thus other proteins could be removed by coagulation, leaving ferritin in solution. Kuhn *et al.* (24) confirmed this work in 1940 and suggested that ferritin was a nucleoprotein with the iron attached at each peptide link. Recent studies, mainly from Michaelis' laboratory, have further characterized this interesting compound. It has been found to be made up of a homogeneous protein, "apoferritin" (of molecular weight 460,000), to which are attached, in chemical linkage, small micelles of iron hydroxide–iron phosphate. The discovery of Michaelis that the iron in these micelles possesses a constant, characteristic magnetic susceptibility which may be interpreted as due to three unpaired electrons per iron atom makes it possible to distinguish ferritin iron from all other biologically occurring iron compounds of the body. Tracer studies in collaboration with Hahn at Whipple's laboratory (21) have shown that ferritin functions as an iron-storage compound of the liver, spleen, and marrow. Recently evidence has accumulated to suggest that ferritin of the gastrointestinal mucosa may play a rôle in the regulation of iron absorption (12, 20).

III. Hemosiderin

A microscopically visible granular iron pigment, hemosiderin, appears generally in the marrow, spleen, and liver after rapid hemoglobin destruction or rapid and excessive iron deposition. Of different origin may be the iron-staining granules found in certain red blood cells (17, 41). The phagocytes of the marrow, spleen, and liver have the property of engulfing damaged red cells and fragments, and decomposing them. The heme is oxidized, releasing the iron and splitting the porphyrin ring into the open-chain biliverdin, which is reduced to bilirubin and finally excreted in the bile. At the same time the globin and stroma are acted upon by proteolytic enzymes, some of the material being denatured and temporarily converted to granules which at first appear yellow-brown and give the iron stain. Within 3 or 4 days the iron stain may disappear, although some of the partially digested protein granules still have a pale yellow tinge, this color being often due to adsorbed bilirubin. (Such granules have been called hematoidin.)

Chemical studies of hemosiderin have been largely confined to granular ma-

terial obtained from the horse spleen. It is not yet known whether the granules are a result of rapid blood destruction or of an abnormally high absorption of iron or are due to a combination of both of these factors.

Cook (6) and Asher (2) have examined these granules of horse spleen and found them to consist of protein material containing brown iron hydroxide and small amounts of phosphate and calcium. By differential centrifugation in carbon tetrachloride, Asher was able to obtain granules containing as much as 35.9 per cent by weight of iron. These granules, then, may vary in composition from relatively little proteinaceous material and high iron content to relatively low iron content.

Analysis of hemosiderin granules (9) from horse spleen indicated 8.29 per cent iron, 12.9 per cent nitrogen, and 1.6 per cent phosphorus, a composition not too different from that of some ferritin samples. The apparent magnetic moment of the iron in these granules was 3.4 B.U., as contrasted with 3.8 B.U. per gram-atom of ferritin iron (32).

IV. Ferritin and Diffuse Tissue Iron

A. Isolation and preparation of crystalline ferritin

Normal horse spleen is the richest source of ferritin. By teasing a piece of horse spleen in 10 per cent cadmium sulfate solution it is possible to observe ferritin crystals forming under the microscope after a few minutes, demonstrating that ferritin is not an artifact of heating, salting out, etc.

The method of isolation (9) is a modification of the one first devised by Laufberger (25) and of that used by Kuhn, Sorensen, and Birkhofer (24). One kilogram of horse spleen is ground in a meat grinder (for smaller amounts of tissue a Waring blendor is useful), mixed with 1.5 liters of water, and heated rapidly in a water bath to 80°C. with stirring. The heavy coagulum of denatured proteins is then removed by first running the hot suspension through coarse cheese cloth, and then the filtrate onto large fluted filters. (Further liquid may be obtained by squeezing the coagulum on the cheese cloth, in a hand press.) A clear brownish solution results. To each 100 cc. of solution is added, with stirring, 35 g. of ammonium sulfate; the resulting suspension is left overnight in the ice box at 0°C. The brown precipitate containing the ferritin is then centrifuged down, and the supernatant liquid sucked off as completely as possible. (For small amounts of precipitate it is advisable to transfer the precipitate as a thick suspension to a dialyzing bag and dialyze away the ammonium sulfate before crystallizing.) The precipitate is now dissolved in a small volume of distilled water, yielding a deep red-brown solution containing ferritin (F) and non-crystallizable ferritin (NCF). For each 100 cc. of this solution is added with stirring 25 cc. of a 20 per cent solution of $CdSO_4 \cdot 8/3H_2O$. Crystallization begins within a few minutes. After 1–2 days at room temperature the crystals are centrifuged down. By differential centrifugation large heavy crystals settle to the bottom and can be separated by sucking off the supernatant mother liquor and a lighter brown sludge. The mother liquor, which is still quite red-brown in color but no longer yields crystals, will be referred to as non-crystallizable ferritin (NCF). Crystalline ferritin is only sparingly

soluble in distilled water. It is recrystallized by dissolving it in the smallest possible volume of 2 per cent by weight ammonium sulfate solution, and for each 100 cc. of the clear solution adding 25 cc. of 20 per cent cadmium sulfate solution.

B. PREPARATION OF CRYSTALLINE APOFERRITIN

The removal of the iron micelles from ferritin while maintaining the protein apoferritin undenatured may be brought about (9, 15, 16) by reducing the ferric hydroxide to the ferrous condition in a medium (pH 4.5) which is acid but not acid enough to denature the protein. In a cellophane dialyzing bag of 1 cm. diameter are placed 40 cc. of a 1–2 per cent ferritin solution. This bag is inserted into a long glass tube of 2.5 cm. diameter and 150 cc. capacity, containing acetate buffer pH 4.6, of ionic strength about 1.0. The slight acidity of this buffer is necessary to make possible the reduction of ferric to ferrous iron. Now 2.0 g. of sodium dithionite are added, together with a few glass beads, and the tube is stoppered, the inclusion of air bubbles being avoided. After 24 hr. of intermittent shaking at room temperature the brown solution has turned pale yellow. The cellophane bag is then dialyzed for 24 hr. against running distilled water. The protein solution in the bag is treated with one-fourth of its volume of 20 per cent cadmium sulfate. Pale yellow crystals form almost immediately. After several hours they are centrifuged down, the supernatant liquid is discarded, and the crystals are brought into solution with the smallest volume of 2 per cent ammonium sulfate solution.

In order to remove the last traces of iron from the protein the solution is placed in a cellophane bag, together with 300 mg. of α,α'-bipyridine. The bag is inserted into a glass tube containing the 1 M acetate buffer pH 4.6, 1 g. of sodium dithionite is added plus some glass beads, and the tube is stoppered (avoiding air bubbles) and shaken intermittently for 1 day. The cellophane bag is then removed from the glass tube and is dialyzed for 1 day against running distilled water to remove the ferrous bipyridine. Finally the apoferritin solution is crystallized with one-fourth of its volume of 20 per cent cadmium sulfate. Occasionally the crystals are not quite colorless, but pale yellow, the yellow color being due not to the presence of iron, but rather to traces of cadmium sulfide. The colorless crystals of apoferritin are identical in shape with those of the brown ferritin crystals. It is possible to obtain crystals ranging from deep brown to colorless, with an iron content of from 23 to 0 per cent, without any variation in crystal form. The ability to crystallize as a cadmium salt is evidently a property of apoferritin alone and does not depend upon nor is it interfered with by the combination with the iron micelles, even though these iron micelles can make up 38 per cent by weight of the ferritin crystal.

C. PHYSICAL PROPERTIES OF FERRITIN AND APOFERRITIN

1. Ultracentrifuge data

Careful ultracentrifuge studies of solutions of crystallized ferritin by Rothen (44) have shown that ferritin is inhomogeneous. A colorless protein fraction,

apoferritin, makes up 20–25 per cent by weight of the material; the remaining 75–80 per cent is a reddish brown fraction which sediments more rapidly, showing no sharp moving boundary. This latter fraction is made up of small iron hydroxide micelles attached to apoferritin molecules so firmly that they cannot be separated from the protein by differential ultracentrifugation. From the ultracentrifuge data Rothen suggests that the brown material consists of units which on the average contain two apoferritin molecules plus a number of small iron hydroxide micelles. Horse apoferritin has a molecular weight of 465,000; if considered as an ellipsoid, its axis ratio is 3:1. The sedimentation constants of human and horse apoferritin are identical; dog apoferritin has a sedimentation constant about 3 per cent higher. The marked stability and homogeneity of horse apoferritin and its convenient molecular weight have been made use of by Rothen in testing the precision of the ultracentrifuge technique.

2. Crystallization and crystal structure

Ferritin is an inhomogeneous substance, as shown by its variation in nitrogen, phosphorus, and iron content. Its properties of crystallization are due primarily to the protein (apoferritin) which it contains. Apoferritin and ferritin resemble the globulins in several ways. Like apoferritin, the globulins are precipitated in an amorphous state by very low concentrations of the bivalent metal ions, such as zinc, cadmium, nickel, and cobalt. At slightly higher concentrations globulins become soluble again and are not precipitable by further increasing (within certain limits) the concentrations of these ions. In contrast, apoferritin crystallizes readily at higher concentrations, especially with cadmium sulfate and less readily with zinc sulfate. An amorphous precipitate of apoferritin which is formed in a 0.5 per cent cadmium sulfate solution, dissolves in 3 per cent cadmium sulfate and soon deposits large well-formed crystals. On a dry weight basis, the amorphous precipitate is found to contain about 3.5 per cent cadmium; the large crystals contain 6–7 per cent cadmium (9).

The cadmium is considered to be present in two forms. Most of it is to be found in the relatively large pores or capillary spaces that are present in the crystal protein lattice; the remainder is attached to the individual protein molecules. Wet protein crystals appear to be made up of protein molecules with interstices or pore spaces between them that are large and filled with loosely held salt and solvent molecules. If one considers spherical protein molecules as arranged in closest hexagonal packing, the volume of space between the spherical molecules would occupy about 25 per cent of the total volume of the crystal. Often the volume of these interstices in the crystal may approach or even exceed the volume occupied by the protein molecules themselves. For example, from the data of McMeekin and Warner (31) one may calculate that lactoglobulin crystals have an interstitial volume of 53 per cent. In physicochemical studies of proteins it becomes more logical to consider the contents of the interstices as a continuum of the crystallizing mother liquor rather than as "water and salt of crystallization". This interpretation is made readily

apparent by the following experiment. When ferritin crystals were suspended in a saturated potassium chloride solution, in which the crystals are insoluble, and spun down rapidly in a centrifuge, the cadmium content of the ferritin crystals was found to decrease from 6.7 per cent to 1.7 per cent. Much of the cadmium sulfate mother liquor which had been present in the interstices of the crystals was evidently swept out by the potassium chloride solution without affecting the crystalline form (9). A portion of the cadmium, however, cannot be displaced; it appears to cling very firmly to the protein and may serve to coördinate the apoferritin molecules into a definite lattice pattern. A comparison, in this respect, with insulin is interesting. Insulin crystallizes as a zinc compound, but an extremely small concentration of zinc is sufficient to bring about crystallization. For the crystallization of ferritin a large excess of cadmium is requisite.

That the interstitial or pore space is of comparatively large magnitude follows also from a comparison of the x-ray studies of apoferritin and ferritin crystals made by Fankuchen (8), so far only with the Debye–Scherrer method, although crystals large enough for the single-crystal method are now available. The data reveal that the distances between the protein units in ferritin and in apoferritin crystals are identical. This is a surprising finding, if one considers that 38 per cent of the original ferritin can be removed as $[(FeOOH)_8 \cdot (FeOPO_3H_2)]$ and the resulting apoferritin will still crystallize with a space lattice identical with that of the original ferritin. The Debye–Scherrer lines for both ferritin and apoferritin are at the same locations but differ in intensity and correspond to a face-centered cubic cell, side 186 Å., having eight molecules per cubic cell. The fact that some of the lines obtained with ferritin are more intense indicates that the iron micelles are spaced at distances identical with the distances from one protein molecule to the next.

The only position that can be postulated satisfying x-ray and other physical and chemical data of the crystals is for the iron micelles to be arranged in the interstices between the protein molecules.

In general, ferritin and apoferritin crystals belong to the cubic system and are isotropic, whatever the species or conditions under which crystallization occurs may be (10) (figure 1). (One exception has been noted. When horse ferritin crystals are suspended in 0.6 per cent cadmium sulfate, small square plates which are optically anisotropic begin to form after several days.) In the mouse, rat, cat, and pig the crystals are octahedra; in the horse they are tetrahedra, octahedra, and most frequently twinned octahedra; in the dog they are tetrahedra; in the guinea pig they are octahedra or rectangular parallelepipeds; in the human they have rounded edges with a suggestion of octahedral form; in the pig the crystals obtained from the testicles are cubic and colorless.

3. Effect of heat

A peculiarity observed with solutions of ferritin which are 2 per cent or higher in concentration, but not with apoferritin, is the effect of heat (9). Dilute ferritin and apoferritin solutions remain clear on heating to 80°C. Above that

temperature irreversible coagulation occurs. However, in a more concentrated ferritin solution there appears at 65–70°C. a cloudiness or, according to concentration, a massive coagulation, which disappears spontaneously on cooling. Heating from 70–80°C. delays the rate of clearing at room temperature.

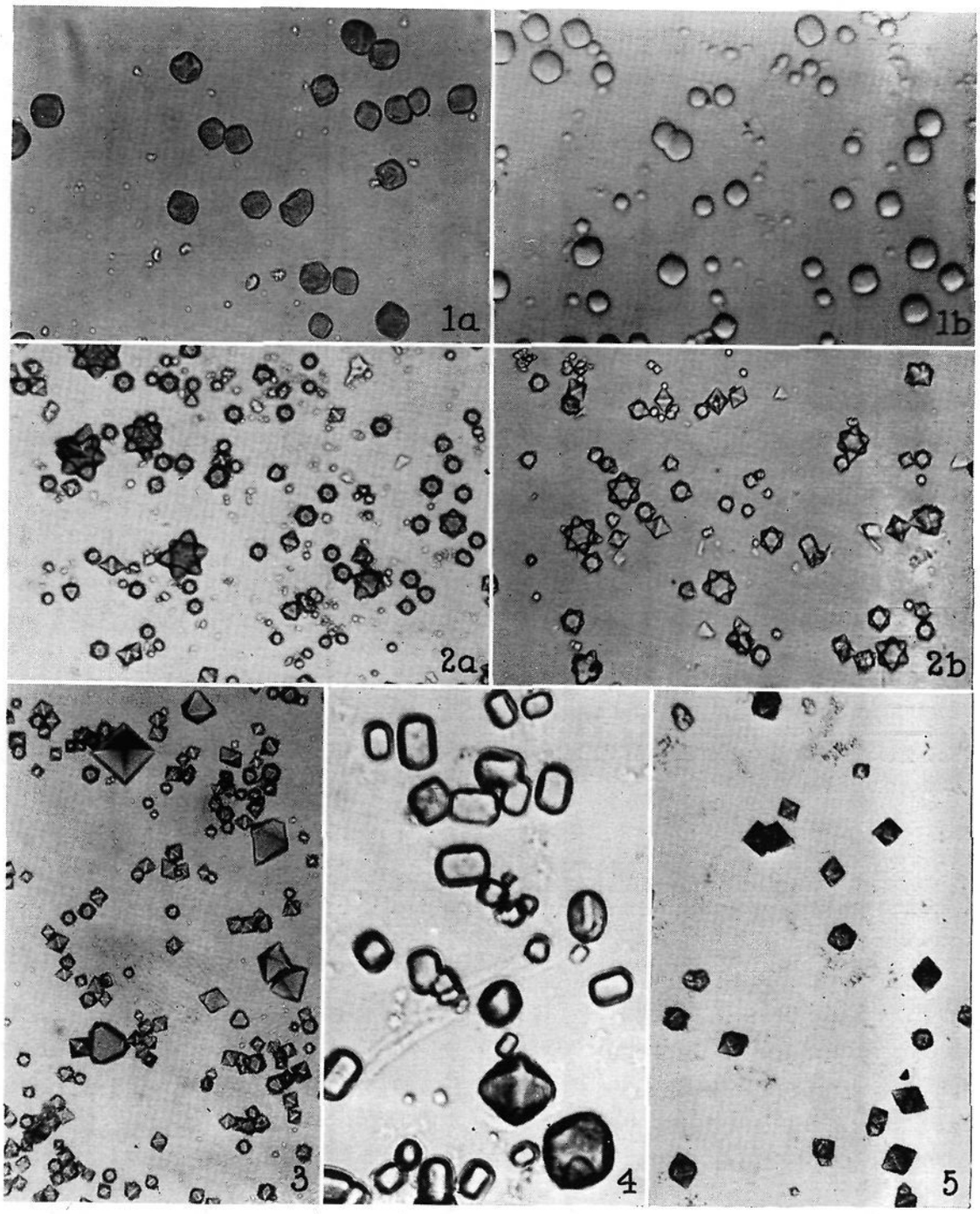


FIG. 1. 1a, human ferritin; 1b, human apoferritin; 2a, horse ferritin; 2b, horse apoferritin; 3, dog ferritin; 4, guinea pig ferritin; 5, mouse ferritin.

In concentrated ferritin solutions at temperatures below 80°C. an aggregation is observed which is spontaneously reversible at lower temperatures. Above 80°C., a denaturation of the protein molecules which is not reversible occurs, such changed molecules agglutinating to insoluble granules.

4. Absorption spectrum

The absorption of apoferritin (figure 2a) is that of a typical protein with a well-defined band at 280 mμ, indicating the presence of aromatic amino acids. The curve of ferritin (figure 2b) is due primarily to the absorption of the red-brown iron micelles and possibly to a certain amount of cadmium sulfide which may be present as an impurity. No absorption band in the region characteristic of nucleic acid has been observed.

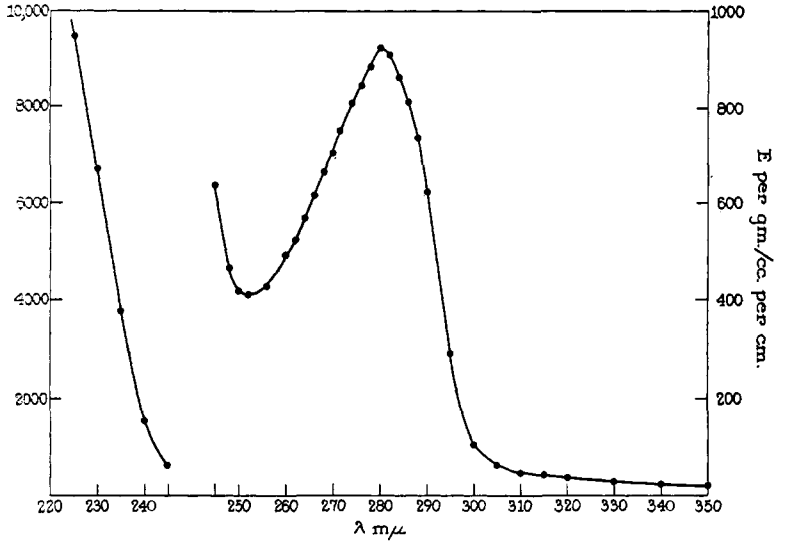


FIG. 2a. Absorption spectrum of horse apoferritin. The ordinate represents the extinction coefficient

$$E = \log_{10} \frac{I_0}{I} \cdot \frac{1}{cl}$$

where the concentration, c, is in grams of protein per cubic centimeter and the light path, l, is 1 cm. The curve at the left has units of extinction 10 times that at the right.

5. Magnetic susceptibility of ferritin iron

In 1943 Michaelis *et al.* (32, 33) discovered that ferritin iron, which is in the ferric state, could be uniquely characterized by its magnetic susceptibility. There are three possible types of ferric atoms containing, respectively, either five, three, or one unpaired electrons per iron atom. It is the three-unpaired-electron type of ferric atom which is characteristic of ferritin, and ferritin is the only biological iron compound of this type occurring naturally in the animal organism.

A ferric compound may be considered, in general, to be an octahedral complex, the iron atom being located in the center of the octahedron and bonded by six atoms or atom groups at the corners of the octahedron. A ferric ion possesses twenty-three planetary electrons, five of these electrons being present in its outermost 3*d* subshell. In the formation of ferric compounds, three cases arise, according to the treatment developed by Pauling (42). The five electrons

may remain unpaired as in $FeF_3 \cdot 3H_2O$; here all the bonds to the central iron atom are ionic or ion-dipoles. Or these five electrons may pair, leaving only one unpaired electron as in $[Fe(CN)_6]^{---}$; here all the bonds to the central iron atom are covalent. Or the intermediate case may arise infrequently in which two of the five electrons pair, leaving three unpaired electrons as in ferritin iron or as in alkaline ferrihemoglobin; here four of the bonds may be considered to be covalent and two bonds to be ionic.

In a strong magnetic field the unpaired electrons in an iron atom behave like little magnet dipoles, arranging themselves with their spins parallel to each other and tending to increase the magnetic lines of force; the temperature-dependent movement of the atoms tends to disorient this arrangement. The paramagnetic susceptibility of a substance, say per gram-atom of iron, can be determined by measuring the pull exerted by an inhomogeneous magnetic field on a cylin-

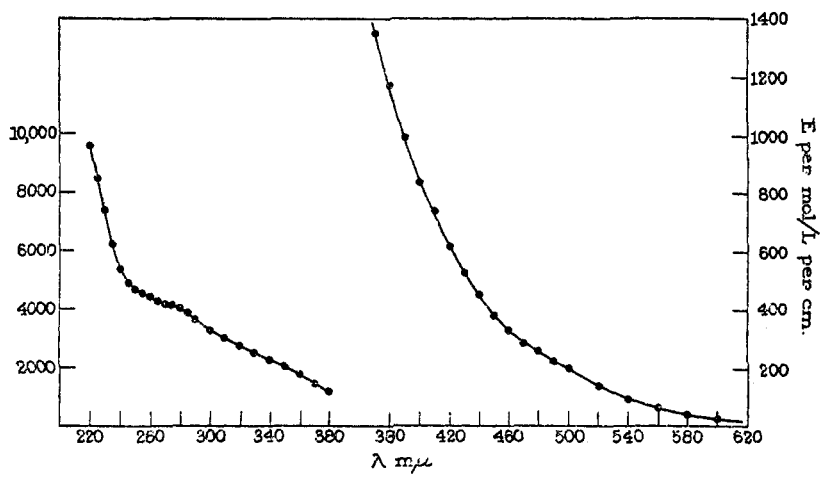


FIG. 2b. Absorption spectrum of horse ferritin. The ordinate represents the molar extinction of ferritin iron, where the concentration of iron is in moles per liter. The curve at the left has units of extinction 10 times that at the right.

der containing the iron atoms in solution. Correction of this value for the diamagnetic susceptibility of the solvent and for the effect of temperature gives the magnetic dipole moment, ϵ, in Bohr magnetons.

The Curie law expresses the temperature dependence of the paramagnetic susceptibility per gram atom, χ, at $T°$ Kelvin. The magnetic dipole moment is

$$\epsilon = 2.84 \sqrt{\chi T} \text{ Bohr magnetons} \qquad (1)$$

Small orbital contributions being neglected and ϵ being assumed due to electron spin only, then the number of Bohr magnetons, ϵ, is related to the number of unpaired electrons, n, by equation 2.

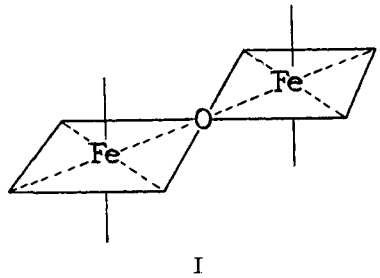
$$\epsilon = \sqrt{n(n+2)} \qquad (2)$$

The Curie law has been shown to be obeyed with sufficient accuracy by ferritin iron within the temperature range 275–301°K., so that the magnetic dipole moment can be used to determine the number of unpaired electrons in ferritin from equation 2. The magnetic dipole moment per iron atom of ferritin is 3.8 Bohr magnetons, a value which would be expected from the spins of three unpaired electrons alone, without any additional contribution from the orbital motions. Such a value occurs rarely for iron compounds, and might be attained by an appropriate mixture of types of iron atoms containing one and five unpaired electrons. However, the constancy of the magnetic dipole moment for various preparations of ferritin is a strong argument in favor of the homogeneity of ferritin iron. The value for the dipole moment remains the same even after the iron hydroxide micelles are precipitated from the protein by treatment with alkali and dialyzed.

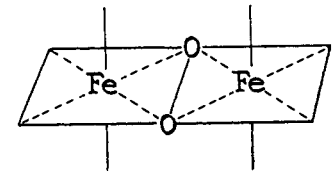
In a study of the magnetic susceptibility of various iron hydroxides, especially in the form of colloidal solutions, a large variability was found. Under specified conditions one could obtain iron hydroxides approaching either the one- or the five-unpaired-electron type per iron atom, and various gradations between them, but there was no way found to make a uniform iron hydroxide containing three unpaired electrons per iron atom. Suspensions of hemosiderin granules were found to have an apparent magnetic moment of 3.4 Bohr magnetons, a value distinctly below that for ferritin.

The relationship proposed by Michaelis between magnetic susceptibility values for iron and the bonding angles suggests the use of magnetic measurements in the structural interpretation of colloidal ferric oxides. The solubility of individual $Fe(OH)_3$ or $Fe(OH)_3 \cdot 3H_2O$ units is extremely small, roughly about 10^{-17} moles per liter. This figure indicates the instability of such a molecule and its small probability of existence as a unit. The instability is due to a great tendency for two hydroxyl groups of adjacent octahedral complexes to split out water molecules, producing Fe—O—Fe bridges and giving rise to polymers which represent the "micelles" of colloidal ferric hydroxide. Alternately, two hydroxyl groups within one molecule of ferric hydroxide may split out water, producing oxygen doubly bound to iron (i.e., Fe=O).

Two octahedral complexes may be considered to combine by sharing either a corner (I) or an edge (II) of the octahedra. The two bonds of the bridge oxygen in I (Fe—O—Fe) form an angle of approximately 180° with the oxygen,



I



II

a condition which can only arise if the bonds are ionic. In II the octahedra share an edge, the bond angles of Fe—O—Fe being compatible with the assumption that these bonds are covalent. In addition to dehydration processes, hydrogen bonding will increase the tendency toward polymerization, the hydrogen bonds forming between adjacent =O and —OH groups. From these considerations the complexity of structure and bonding of various of the colloidal iron hydroxides becomes understandable, and makes more likely the assumption that the ferric hydroxide micelles of ferritin are built up not haphazardly but according to a definite pattern which cannot as yet be reproduced in the laboratory.

D. CHEMICAL PROPERTIES

1. Analysis of ferritin

Chemical analyses of various crystalline ferritin samples (9, 25) for iron, phosphorus, and nitrogen indicate their inhomogeneity (table 1). This is compatible with the fact that ferritin consists of apoferritin molecules con-

TABLE 1

*Analyses of non-hemin iron components of horse spleen in per cent of dry weight**

PREPARATION	Fe		Cd	P	N
	Uncorrected for Cd	Corrected for Cd	Uncorrected for Cd		
	per cent	*per cent*	*per cent*	*per cent*	*per cent*
Ferritin crystallized once	22.6	23.1	1.93	1.99	12.6
Ferritin crystallized four times	19.7	20.2	2.74	1.42	11.1
Fraction precipitated with Na_2SO_4 (not crystallized)	24.5			1.45	11.0
Hemosiderin granules	8.29			1.59	12.9
"Non-crystallizable ferritin" from mother liquor	19.8	20.2	2.02	1.52	9.12

* All preparations were dried in thin layers at 80°C. for 24 hr. and then for 3 hr. at 110°C.

taining variable amounts of colloidal iron hydroxide–iron phosphate micelles. In these analyses it is necessary to correct for the rather high cadmium content present even in well-dialyzed ferritin solutions. The cadmium may amount to 1–3 per cent of the dry weight of the crystals. In several different preparations of horse spleen the iron content varied from 18 to 23 per cent and the phosphorus ranged from 1.26 to 2.0 per cent.

2. Analysis of apoferritin

The dialyzed protein apoferritin (16) is chemically quite homogeneous, various samples giving the same analytical results. The data are representative of a typical protein: nitrogen, 16.1 per cent; sulfur, 0.97 per cent; carbon, 51.3 per cent; hydrogen, 7.09 per cent; the value for cadmium is 0.73 per cent. On acid dialysis of ferritin, the micelles go into solution but the denatured protein remains in the dialysis bag. Kuhn *et al.* (22) reported the composition of this denatured protein to be: nitrogen, 16.1 per cent; sulfur, 0.88 per cent; carbon,

49.3 per cent; hydrogen, 7.14 per cent. As would be expected, these values are almost identical with those of crystalline apoferritin.

Because of the relative insolubility of apoferritin in electrolyte-free water, it has been possible to recover 84 per cent of ferritin nitrogen as apoferritin. If the nitrogen remaining in the mother liquors from the crystallizations is also included, this would make a recovery of 92 per cent of the nitrogen. This high recovery, considering losses in manipulation and denaturation, makes it unlikely that a nitrogenous compound will be found to be a constituent of the iron micelles themselves, although this possibility cannot be ruled out entirely by the data at hand.

The amino acid compositions of apoferritin as recorded by Kuhn *et al.* (24) and by Tria (53) are at marked variance with one another and will therefore not be tabulated. Archibald (1), using an arginase method for the determination of arginine, has analyzed a sample of dialyzed crystalline apoferritin and found it to contain 8.28 per cent of arginine. The figures of Kuhn *et al.* (24), recalculated in terms of apoferritin, would give about 16 per cent arginine.

3. Composition of ferritin–iron micelle

The iron micelles may be removed by denaturing ferritin with 1 *N* alkali for 10 min. at room temperature. A red-brown coagulum precipitates which is centrifuged down, washed, and dialyzed. As noted above, the magnetic susceptibility of this preparation per iron atom is identical with the magnetic susceptibility per iron atom found for ferritin. However, alkaline treatment has removed about three-fourths of the phosphorus originally attached to the iron micelles, probably replacing an $—OPO_3H_2$ group by an —OH group. Some nitrogen is also found in this preparation of alkaline-treated micelles. This is assumed to be due to the occlusion of 10 per cent by weight of the denatured protein. Assuming the nitrogen as impurity, it is concluded from the analyses that the ferritin micelles have the approximate composition $[(FeOOH)_8 \cdot (FeOPO_3H_2)]$ (14).

The evidence for phosphorus being a constituent of the iron micelles is that on treatment of ferritin with acid or alkali and subsequent dialysis there remains a denatured protein devoid of phosphorus. Likewise, treatment of ferritin with dithionite to remove the iron micelles, leaving the protein undenatured, results in an apoferritin containing no phosphorus. The claim of Kuhn *et al.* (24) that phosphorus was a constituent of a nucleic acid which formed 12.1 per cent by weight of ferritin could not be substantiated (9).

The phosphorus of the micelles does not appear to be present merely adsorbed to the surface of the micelles, since it cannot be displaced by ions like citrate, nor are appreciable amounts of radio P^*O_4 adsorbed by either ferritin or apoferritin (14). The PO_4 is not only chemically bound on the surface of the micelle but is distributed throughout the micelle. This conclusion was arrived at by studying the change in the ratio of iron to phosphorus in ferritin when treated with ammonium magnesium citrate during a several-day period. Treatment at room temperature with *N* hydrochloric acid releases the phosphate from the micelles within a few minutes, and all of the phosphorus can be accounted for as

orthophosphate. All this evidence supports the view that the iron micelle in ferritin is a basic ferric phosphate, where some of the —OH in FeOOH is replaced by $—OPO_3H_2$ groups.

There is as yet no good way of determining the size of the iron micelles, nor has the specific attachment of the micelles to the apoferritin been investigated.

E. DISTRIBUTION OF FERRITIN

1. Ferritin in species and organs

The isolation of ferritin from a number of vertebrate species was accomplished by using the identical method used for the isolation of horse spleen ferritin. In

TABLE 2

Ferritin distribution in animal species and organs as determined by isolation procedure (10)

SPECIES	ORGANS AND TISSUES EXAMINED			
	Spleen	Liver	Red bone marrow	Other organs in which ferritin was found
Horse	+++++ (0.25–0.025)*	+	+++	Testes† +
Human	++	+++ (0.23–0.006)	++	
Dog	+	++ (0.039–0.000)	0	Kidney + M
Guinea pig	+	++ (0.060–0.015)	0	
Mouse	+	+		Testes† +
Rat	+	+		
Pig	+	+		Testes† +
Rabbit	+	0	0	Testes† +
Cat	0	0		Kidney +

* The figures in parentheses represent the extremes of ferritin found in these organs, expressed in terms of the number of cubic centimeters of packed, twice-crystallized ferritin crystals per 100 g. of fresh weight of the organ. One cubic centimeter of packed crystals, dried at 110°C., weighs approximately 0.40 g.

† The crystals obtained from the testes of the horse, mouse, pig, and rabbit are either colorless or very pale yellow.

the order of decreasing ferritin content (table 2) the species are the horse, man, guinea pig, mouse, rat, rabbit, and cat (10). Kuhn *et al.* (24) also reported the isolation of ferritin from the jackal. From 100 g. fresh weight of horse spleen one may obtain as much as 0.1 g. dry weight of crystalline ferritin. No crystalline ferritin could be obtained from the liver or spleen of beef cattle, sheep, deer, various species of fowl, various species of fish, and bullfrogs. This failure to isolate crystalline ferritin from these species may in part be due to the variations in the properties of the protein.

In those species from which crystalline ferritin could be isolated it was found that it could also be made to appear in a teased preparation under the microscope. A piece of liver or spleen about 2–3 mm. thick and 5 mm. square was teased

slightly in a drop of 10 per cent cadmium sulfate solution on a slide, a cover slip was placed on the preparation, and it was placed in a moist chamber to prevent evaporation. Within half an hour, or overnight, crystals of ferritin appeared, especially in small areas where the tissue folds had entrapped cadmium sulfate. The crystals were readily distinguished from the brownish hemosiderin granules; in the human tissue preparations the crystals generally appeared as spheroids, making a distinction from hemosiderin less clear.

The organs from which crystalline ferritin could be isolated are the spleen, liver, bone marrow, and kidneys. Very pale yellow or colorless crystals were isolated in small yield from the testes of several species. There were wide individual variations in the content of ferritin from livers and spleens of the same species (10). Recently, ferritin has been detected in the mucosa of the gastrointestinal tract of the guinea pig. The significance of this finding is discussed below.

2. Antigenic studies and cross reactions

Antigenic studies and cross reactions of ferritin have been made by preparing an antibody in the rabbit against horse ferritin (10). As was to be expected, the antibody is directed against the protein apoferritin and the precipitin reaction is not hindered by the presence of the iron micelles. By using the precipitin reaction as a test for apoferritin in the different organs of the horse, apoferritin was detected in the spleen, bone marrow, liver, testicle, kidney, adrenal, pancreas, ovary, and lymph node. Its presence in the brain was uncertain. Apoferritin was absent from blood, striated muscle, stomach mucosa, and pituitary.

Apoferritin is of interest from the immunological point of view, since it represents the first clear-cut example that the pattern of a protein of one tissue is constructed to be immunologically identical with the same protein found in another tissue. There appears to be no evident relation between the distribution of apoferritin in the various tissues and the origin of these tissues from the basic embryonic germ layers. Horse apoferritin antibody cross reacts and gives a weak precipitin reaction with dog apoferritin. There is no cross reaction between horse apoferritin antibody and human apoferritin.

F. MICROCHEMICAL TESTS FOR IRON IN TISSUES

1. Methods

Iron distribution in tissues has been studied extensively by histologists, although a critical analysis of the techniques involved is often lacking. Several methods have been used which depend either upon the formation of an insoluble ferric compound in acid solution, for example, Prussian blue, or the formation of an insoluble ferrous compound in neutral or alkaline solution, for example, ferrous sulfide. They all have one defect: they require that iron be in a monomolecularly dispersed form before it can form a visually detectable insoluble compound. During the interval between dispersal and the reaction to form an insoluble compound the iron may become adsorbed preferentially to some cellular structures which normally would not contain iron, thus leading to erroneous

observations. Especially is there danger of this happening in the procedures claimed to unmask iron from organic compounds, such as hemin, by pretreatment with strong acid (39). In order to minimize this possibility, the reaction solubilizing the iron should be followed very rapidly by the reaction precipitating the iron again in a visually discernible form.

In the ferrocyanide method the inorganic iron compounds are dissolved to form ferric iron and react with ferrocyanide to form the insoluble Prussian blue. In the sulfide method it has been customary to use ammonium sulfide, the ferric iron being reduced to the soluble ferrous iron and then forming the greenish black insoluble ferrous sulfide. The use of acid or ammoniacal solutions generally requires a previous sojourn in a fixing fluid, the assumption being made that the fixing fluid has not changed the iron distribution. If no fixing fluid has been used,—as, for example, in dried blood smears,—then the acid or alkaline solution will dissolve out or coagulate proteins. Obviously, a method which can be applied to the living cell without distorting the protoplasm would be desirable. This can be achieved very simply by using a saline solution through which hydrogen sulfide has been freshly bubbled for several minutes. The hydrogen sulfide in approximately neutral solution reduces even basic ferric hydroxide to the ferrous condition and forms the very insoluble green-black ferrous sulfide (11).

2. Iron in horse spleen

Even by using this delicate hydrogen sulfide method it has not been possible to determine the localization of ferritin in particular cells (11). The fresh horse spleen tissue which is richest in ferritin after hydrogen sulfide treatment showed large black hemosiderin granules in the macrophages, tiny discrete black granules on some crenated erythrocytes, and pale grey staining erythrocytes indicating a diffuse distribution of iron in these cells. It was not possible to decide whether this diffusely staining iron in the erythrocytes represented ferritin, but it is unlikely that a cell in the process of destruction, as it is assumed these cells are in the spleen, would build a new protein. Rather it is plausible to assume ferritin to be so diffuse that no simple microscopic observation of iron distribution could demonstrate particular cells in which ferritin was present.

3. Consideration of iron in nucleoprotein

From time to time the presence of iron associated with nucleoprotein has been reported (39, 46, 58). Macallum (29), using prolonged treatment with ammonium sulfide, claimed that the chromatin became specifically darkened. Okamoto (40), studying various tissues of vertebrates and invertebrates, also used prolonged treatment with acid and ferrocyanide and reported iron to be preferentially localized in the nuclei. Saha and Guha (45) reported the isolation of nucleoproteins containing both iron and copper. The ease with which nucleic acid binds iron is attested by the common use made by histologists of iron alum as a mordant for chromosome staining. The papers suggesting that tissue extracts contain the iron bound to nucleoprotein have dealt with mixtures, and no conclusions may be drawn from them. Nor has the idea (24) that ferritin

contains nucleic acid been confirmed. As for nucleoprotein itself being rich in iron, we have examined two preparations of nucleoprotein. These were isolated by A. E. Mirsky from sheep and calf spleen nuclei, using very mild treatment with sodium chloride solutions and differential centrifugations to make the separations (34). These preparations contained 0.036 per cent and 0.007 per cent of iron, respectively, on a dry weight basis (13), i.e., negligibly small concentrations of iron. One may say categorically that no satisfactory evidence exists today which supports the view that iron is localized preferentially in nuclei or with nucleoproteins.

G. POSSIBLE FUNCTION OF FERRITIN AS A MUCOSAL REGULATOR OF IRON ABSORPTION

Recent studies have brought to light a number of interesting facts which govern the process of iron absorption. The iron is apparently absorbed in the ferrous state (23, 27, 28, 35, 52, 56), various portions of the gastrointestinal tract being capable of absorbing it (3, 7, 20).

In 1937 Widdowson and McCance (30, 57) presented evidence that iron once absorbed or injected parenterally remained in the body, no significant amounts being excreted. Evidence on the clinical side by Welch *et al.* (56) also supported this idea. Using radioactive iron as a tracer, Hahn, Bale, Lawrence, and Whipple (19), in a fundamental experiment, came to the conclusion that the rate of absorption of iron was probably governed by some process in the gastrointestinal mucosa. Further studies from Whipple's (4, 20) laboratory firmly established the fact that the total iron content of the body was not controlled by unlimited absorption and excretion of excess as are the common elements, but rather by limiting the intake of iron.

Two experiments clearly revealed the regulatory process of the mucosa (20). A dog, when suddenly made anemic and simultaneously fed radio iron, absorbed no more iron at this time than did a normal dog. The body stores were gradually depleted, and within 7 days iron absorption had increased to 10–20 times the normal rate. In another experiment a chronically anemic dog was fed a 10-mg. dose of iron and after 1–6 hr. was fed a 10-mg. dose of radio iron. The radio iron was found to be absorbed at a much lower rate than was the first dose. These experiments indicated that a "mucosal block" or saturation, with respect to iron, could be built up within a few hours and be maintained for several hours to several days, appreciably diminishing iron absorption. The lag in iron absorption, Hahn *et al.* suggested, might be due to a "mucosal acceptor" having the properties of apoferritin which could accept the iron and which when saturated would prevent any further uptake of iron. We should like to modify this hypothesis by considering the mucosal cells to be "physiologically saturated" with respect to ferrous ions rather than with respect to ferritin.

According to this hypothesis the regulation of iron absorption may occur in the following way: Iron entering the mucosal cells would be converted to ferritin iron. This ferritin iron would be in equilibrium with small amounts of ferrous ions in the cells, and the ferrous ions would be in equilibrium with the plasma Fe^{+++} iron of the blood stream (figure 3). The creation of an anemic condition

would result in draining the storage iron primarily from the marrow, spleen, and liver. Only when this major source had been depleted and the plasma iron level had been lowered would iron from the mucosa begin to move out into the blood stream. The "physiological saturation" of the mucosal cells with respect to ferrous ions would, however, be maintained until part or all of the ferritin iron had been converted to ferrous iron. Only then would the ferrous iron fall below its "saturation" value in the mucosal cells, and when this occurred radioactive iron would begin to be absorbed.

Recently (12) evidence was found which appears to support the hypothesis of the regulatory action of ferritin in iron absorption. When about 0.2 g. of the intestinal mucosa of the guinea pig was scraped off onto a slide, mixed with 10 per cent cadmium sulfate, and allowed to concentrate by slow evaporation overnight in a moist chamber, crystals of ferritin were found. By counting the number of crystals per slide it was possible to determine in a semiquantitative way the distribution of ferritin in 2–5 cm. segments along the intestinal tract.

In rapidly growing guinea pigs of 400 to 600 g. barely detectable amounts of ferritin (1–10 tiny crystals per slide) were observed and here only in the mucosa

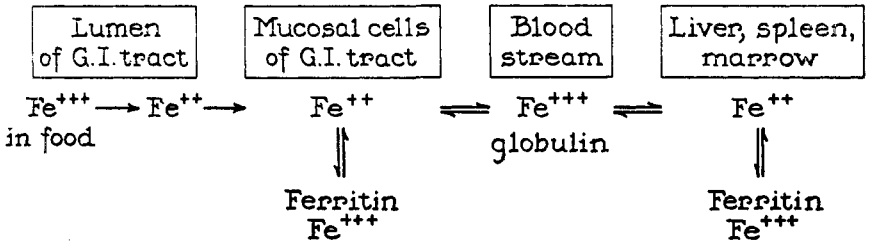


FIG. 3. Scheme representing the rôle of ferritin in the absorption and storage of iron.

of the duodenal region. When 20 mg. of iron was fed in the form of ferrous ammonium sulfate, an increase in ferritin was observed within 4 hr. after feeding (50–100 crystals per slide for the duodenal region), and the maximum amount of ferritin was reached some 8 hr. after feeding (100–500 crystals per slide for the duodenal region). Twenty hours after iron feeding, ferritin was occasionally detected in the cardiac region of the stomach; it was generally present in the pyloric region (10–50 crystals per slide); it was highest in the duodenal region (100–500 crystals per slide), i.e., in a 5-cm. segment below the pyloric sphincter; it diminished along the jejunum (50–100 crystals per slide) and ileum (10–50 crystals per slide); it was occasionally detected in the cecum; it was low in the upper portion of the large intestine (0–10 crystals per slide); and it was occasionally detectable in the lower portion of the large intestine. Some 5 to 7 days after iron was fed, the ferritin content had diminished considerably and was almost down to the concentration present in the mucosa of the control guinea pigs.

Of all the organs and tissues of the guinea pig which were examined, it was found that per unit weight the liver and duodenal mucosa were highest in ferritin. This relatively high content of ferritin in the mucosa, the properties of ferritin

as an iron-storage compound, the rapid increase of ferritin in the mucosa in response to iron feeding, the diminution of ferritin in the mucosa several days afterwards, paralleling the phenomena of the "mucosal block",—all of these factors are in keeping with the hypothesis of ferritin functioning in the regulation of iron absorption by the mucosa.

H. IRON TRANSPORT IN THE BLOOD STREAM

Once the ferrous iron has passed through the mucosa into the blood stream (36), the oxygen tension is sufficient to autoxidize immediately any ferrous iron to the ferric state. The ferric iron, probably in the form of ferric hydroxide, then attaches avidly to the serum proteins (49, 50). It should be emphasized at this point that ferritin itself is not transported through the blood, since even the very sensitive precipitin reaction has not revealed any ferritin in blood (10).

The concentration of ferric iron in plasma averages about 100–200 mg. per 100 cc. of plasma (54). Little is known of how this relatively steady state is maintained. The following picture is suggested: Ferritin in the cells may be considered to be in equilibrium with a small amount of ferrous iron in the cells, the ferrous iron being capable of transport to the blood stream. One of the important factors governing the level of ferrous iron in the cells is the rate of autoxidation of ferrous to ferric ions and the rate of reduction of ferritin iron to the ferrous condition. The level or steady state of the serum iron would then be governed, in part at least, by what one may loosely term the "redox level" of the storage tissues.

As tracer studies indicate (14), large amounts of iron in the ferric state can be taken out of circulation within a few hours by the liver, some going directly to the bone marrow. Here also we are ignorant of how iron is transported from the blood stream into the liver cells, for example. It is interesting to note that the tissues which take out iron most avidly from the blood stream are those which contain numerous phagocytes of the macrophage type. The possibility suggests itself that it is these phagocytes which might ingest the serum protein iron complexes, reducing the iron, and passing it on to adjacent cells where conversion to ferritin might occur.

I. FUNCTION OF FERRITIN AS AN IRON-STORAGE COMPOUND

The chemical and physical properties presented above make it apparent that ferritin would be an ideal substance for storage both of iron and of protein. Ferric ions are rather toxic to protoplasm, tending to coagulate proteins and enzymes. In ferritin the iron is stored in the form of colloidal micelles or aggregates of very insoluble basic iron phosphate attached to a relatively large non-diffusible protein molecule, apoferritin. When iron is required for heme synthesis it is made rapidly available. A reasonable hypothesis is that the iron of the micelles is made available by reducing the iron to the relatively soluble ferrous form. At the same time demands for globin synthesis would seem to be met in part by breaking down the apoferritin molecule itself (10).

1. Ferritin in anemic and in normal horses

One of the observations to support the idea that ferritin is an iron-storage compound arose accidentally from a study of horse spleens obtained from the Antitoxin Laboratories at Otisville, N. Y. (10). Horses which had been bled frequently for serum were found to have spleens from which relatively little ferritin could be obtained, in contrast to normal horse spleens which had a high ferritin content. Not only was ferritin iron and total iron low in bled-horse spleens, but the protein apoferritin was also low. This was interpreted as showing that in anemic animals there was a drain not only of iron but also of apoferritin and probably of other proteins to meet the demands of rapid blood formation.

2. Conversion of tagged hemoglobin iron to ferritin iron

A more direct approach to the problem of the function of ferritin was made by Hahn, Granick, Bale, and Michaelis (21), using radioactive iron as tracer. In

TABLE 3

Conversion of tagged red cell hemoglobin iron to ferritin iron (21)

	DISTRIBUTION OF LABELED IRON		PER CENT OF IRON IN THE TISSUES WHICH IS RADIOACTIVE
	Total circulating activity after transfusion taken as 100 per cent	Activity lost from circulation taken as 100 per cent	
	per cent	*per cent*	
Injected tagged blood (110 cc.)	100		
Circulating blood after transfusion	100		19.8
Circulating blood before death	15.6		7.9
Liver (335 g.)	46.1	55	21.4
F + NCF			25.3
Ferritin			25.5
Spleen (89 g.)	17.1	20	12.5
F + NCF			12.0

order to determine whether hemoglobin iron could be converted into ferritin iron, a donor dog was prepared. The donor dog was first made anemic by bleeding. Then the animal was injected with a radioactive iron solution of ferric ammonium citrate, at the same time that it was fed liver. A rapid synthesis of red cells occurred which contained the radio iron as a constituent of the heme in hemoglobin. Of this donor dog's blood 110 cc. was injected intravenously into a recipient dog (table 3). On the following day the recipient dog received a subcutaneous injection of acetylphenylhydrazine to increase the destruction of the red cells. After 6 days the dog was sacrificed. Of the radioactive iron lost from the circulation 55 per cent was found in the liver and 20 per cent in the spleen. About half of the total iron of the liver was isolated in the iron-rich fraction (F + NCF); one out of every four atoms of this iron was radioactive. Crystalline ferritin prepared from this fraction also contained the same propor-

tion of radioactive iron to total iron as did the ferritin-rich fraction. This experiment therefore demonstrates the conversion of heme iron of the red blood cells into iron which can be isolated as a constituent of crystalline ferritin.

3. Conversion of tagged inorganic iron to ferritin iron

In another experiment (21) it was demonstrated that radio ferric ammonium citrate could be injected intravenously into a dog and be converted to ferritin iron of a magnetic susceptibility characteristic of normal ferritin. Depending on the method of preparation, ferric ammonium citrate can be obtained with various magnetic susceptibilities. Radio ferric ammonium citrate prepared in a slight excess of ammonium citrate (14) has a magnetic moment of 5.69 B.U. When this compound is mixed *in vitro* with dog plasma, little change magnetically can be detected in half an hour.

Some 13 days after intravenous injection of radio ferric ammonium citrate the dog was viviperfused. Over 80 per cent of the radioactive iron was found to be present in the liver, only a trace being present in the spleen. The iron-rich fraction of the liver (F + NCF), making up about 25 per cent of the radioactive iron injected, was subjected to magnetic measurements. The magnetic moment was 3.78 B.U. per gram-atom of iron, which is the same value as that found for all previous samples of ferritin. The iron-rich fraction was further treated to isolate crystalline ferritin; this also had a radioactivity equivalent to that of the (F + NCF) fraction, i.e., about 75 per cent of the iron was radioactive iron. It was thus shown that inorganic ferric iron of a magnetic susceptibility representing approximately five unpaired electrons per iron atom could be converted into a basic iron phosphate of magnetic susceptibility representing three unpaired electrons per iron atom and could be isolated as a crystalline compound, ferritin. Here again the storage function of ferritin was clearly evident.

4. Rate of conversion to ferritin iron

In order to find out how rapidly the conversion of inorganic to ferritin iron occurred, three dogs were sacrificed 1, 2, and 5 hr. after intravenous injection with radioactive ferric ammonium citrate (14). One hour after injection 40 per cent of the radioactive iron was localized in the liver, and 2 hr. after injection 61 per cent was found in the liver. The iron-rich fraction (F + NCF) had the red-brown color and paramagnetic susceptibility characteristic of ferritin. Derangement of ferritin synthesis was suggested by the results obtained with the third dog, sacrificed after 5 hr. The liver of this dog had the ability to take up radioactive iron, since 40 per cent of the radioactive iron injected was found in the liver. However, the fraction (F + NCF) which was isolated did not possess the normal red-brown color of ferritin but was rather of a pale brownish yellow color. Its magnetic moment was 4.7 B.U. instead of the normal 3.8 B.U.; nor could ferritin crystals be isolated from this fraction. The experiment seems to indicate that in this particular case, although the liver could take up iron from circulation, it could not convert it into ferritin iron.

J. SOME FACTORS IN FERRITIN FORMATION

For ferritin formation not only should the cell be able to produce apoferritin but it must have a mechanism for accumulating iron and possibly another for attaching iron to apoferritin. These conclusions are suggested by the observation that apoferritin, but little or no ferritin, is present in testicles (10). It may be that the mechanism accumulating iron or forming micelles is not present, or is present to a very slight extent in testicular tissue. Also, the liver of the abnormal dog mentioned above could take out much iron readily from the blood stream but was unable to convert it to normal ferritin, perhaps because the protein, apoferritin, was lacking. In connection with the quantity of apoferritin present, it should be mentioned that apoferritin may serve as a protein reserve, probably along with other proteins, to be used for the synthesis of new erythrocytes when there is a demand for it or during starvation.

Recently (12) a marked increase of ferritin in the gastrointestinal mucosa was observed in response to iron feeding. When ferrous ammonium sulfate was fed to guinea pigs, the ferritin especially of the duodenal mucosa was greatly increased. No apoferritin crystals could be detected before iron feeding, indicating that apoferritin was not originally present in any appreciable concentration. These facts suggest that the feeding of iron has in some way brought about an increase in the concentration of a particular protein which combines with iron to form a storage compound of iron in the mucosa.

Ferritin formation appears to be a result of a biological mechanism. *In vitro* experiments negate the possibility that ferritin or apoferritin accumulates iron merely by a kind of crystal growth in the presence of either ferrous or ferric iron. Guinea pig liver mash, when mixed and incubated with apoferritin and radio iron, appeared to bring about the conversion of inorganic iron to ferritin iron, indicating that some specific biological mechanisms were functioning (14). The extent of this conversion was, however, very low.

An "apparent" synthesis of ferritin occurs if one mixes apoferritin with non-crystallizable ferritin and adds cadmium sulfate (16). The crystals resulting are brown, contain iron, and are indistinguishable from ferritin. We believe that this does not represent an addition of iron micelles to apoferritin molecules but rather that the apoferritin lattice is sufficiently porous to permit certain ferritin molecules in the non-crystallizable ferritin solution (9) to fit into it.

K. SUMMARY OF IRON METABOLISM IN RELATION TO FERRITIN

Figure 3 is an attempt to summarize the facts and hypotheses of iron absorption, transport, storage, and function. Iron enters the gastrointestinal tract in the ferric state with the food. It is converted to the ferrous state with the aid of acidity, —SH groups, ascorbic acid and possibly other reducing agents of the food. Absorption of ferrous iron occurs mainly into the mucosal cells of the duodenum and jejunum, and appears to be unidirectional. In response to iron feeding the protein apoferritin increases in concentration in the mucosal cells so that more ferritin accumulates in these cells. The mucosal cells regulate iron absorption, it is postulated, by maintaining within the cells a level of ferrous

iron, governed in part by the redox level of these cells. The ferrous iron of the mucosal cells is further postulated to be in equilibrium with the ferritin in the mucosal cells and with the plasma iron of the blood stream. From the mucosa the ferrous iron moves into the blood stream, where it is at once autoxidized to ferric hydroxide, which is then adsorbed to the serum proteins and is transported as a ferric hydroxide–protein complex. According to these hypotheses a lowering of the plasma iron would result in more rapid movement of iron out of the mucosal cells, depleting the stores of ferritin iron and finally lowering the concentration of ferrous iron in the mucosal cells below the "physiological saturation" level. At this time, then, increased absorption of iron from the gastrointestinal tract would be observed.

The plasma protein iron is in equilibrium and exchanges rapidly with the ferrous iron and ferritin of the liver, spleen, and bone marrow. Here also the ferritin is in equilibrium with ferrous iron, only ferrous iron being suggested as capable of transfer from the cells to the blood stream. In the marrow not only is ferrous iron converted to ferritin, but ferrous iron is also incorporated into protoporphyrin to form hemin, and the hemin is attached to globin to form hemoglobin. Damaged red cells are destroyed by the phagocytes of the liver, spleen, and marrow. The iron released from hemoglobin breakdown, unlike the porphyrin moiety, is carefully conserved by the body to be re-used.

We should like to take this opportunity to thank Dr. Leonor Michaelis for his suggestions and advice in these studies.

V. References

(1) Archibald, R. M.: Unpublished work.
(2) Asher, Th.: Z. physiol. Chem. **220**, 97 (1933).
(3) Austoni, M., and Greenberg, D.: J. Biol. Chem. **134**, 27 (1940).
(4) Balfour, W., Hahn, P., Bale, W., Pommerenki, W., and Whipple, G.: J. Exptl. Med. **76**, 15 (1942).
(5) Burow, Robert: Biochem. Z. **25**, 165 (1910).
(6) Cook, S.: J. Biol. Chem. **82**, 595 (1929).
(7) Copp, H., and Greenberg, D.: Federation Proc. **4**, 86 (1945).
(8) Fankuchen, I.: J. Biol. Chem. **150**, 57 (1943).
(9) Granick, S.: J. Biol. Chem. **146**, 451 (1942).
(10) Granick, S.: J. Biol. Chem. **149**, 157 (1943).
(11) Granick, S.: Proc. Soc. Exptl. Biol. Med. **53**, 255 (1943).
(12) Granick, S.: Science **103**, 107 (1946).
(13) Granick, S.: Unpublished work.
(14) Granick, S., and Hahn, P.: J. Biol. Chem. **155**, 661 (1944).
(15) Granick, S., and Michaelis, L.: Science **95**, 439 (1942).
(16) Granick, S., and Michaelis, L.: J. Biol. Chem. **147**, 91 (1943).
(17) Grüneberg, H.: Nature **148**, 114 (1941).
(18) Hahn, P.: Medicine **16**, 249 (1937).
(19) Hahn, P., Bale, W., Lawrence, E., and Whipple, G. H.: J. Exptl. Med. **69**, 739 (1939).
(20) Hahn, P., Bale, W., Ross, J., Balfour, W., and Whipple, G. H.: J. Exptl. Med. **78**, 169 (1943).
(21) Hahn, P., Granick, S., Bale, W., and Michaelis, L.: J. Biol. Chem. **150**, 407 (1943).

(22) HEATH, C.: *Symposium on Blood*, pp. 41–51. Wisconsin Press, Madison, Wisconsin (1939).
(23) HENDERSON, V., AND LUCAS, G.: Am. J. Digestive Diseases Nutrition **11**, 244 (1944).
(24) KUHN, R., SORENSEN, N., AND BIRKHOFER, L.: Ber. **73B**, 823 (1940).
(25) LAUFBERGER, V.: Bull. soc. chim. biol. **19**, 1575 (1937); Compt. rend. du XV Congress des Physiologie à Leningrad, p. 232 (1935).
(26) LIBET, B., AND ELLIOTT, K.: J. Biol. Chem. **152**, 613 (1944).
(27) LINTZEL, W.: Ergeb. Physiol. **31**, 844 (1931).
(28) LINTZEL, W.: Biochem. Z. **263**, 173 (1933).
(29) MACALLUM, A. B.: Proc. Roy. Soc. (London) **B50**, 277 (1891).
(30) MCCANCE, R., AND WIDDOWSON, E.: Lancet **2**, 680 (1937).
(31) MCMEEKIN, I., AND WARNER, R.: J. Am. Chem. Soc. **64**, 2393 (1942).
(32) MICHAELIS, L.: Chapter on Magnetochemistry in *Physical Methods of Organic Chemistry*, Vol. II, in press. Interscience Publishers, New York.
(33) MICHAELIS, L., CORYELL, C., AND GRANICK, S.: J. Biol. Chem. **148**, 463 (1943).
(34) MIRSKY, A. E.: Advances in Enzymol. **3**, 1 (1943).
(35) MOORE, C., DUBACH, R., MINNICH, V., AND ROBERTS, K.: J. Clin. Investigation **23**, 755 (1944).
(36) MÜLLER, FRANZ: Arch. path. Anat. (Virchow's) **164**, 436 (1901).
(37) NASSE, H.: Sitzung ges. Nat., Marberg (1873), No. 2.
(38) NEUMANN, E.: Arch. path. Anat. (Virchow's) **111**, 25 (1888).
(39) NICHOLSON, F.: J. Comp. Neurol. **36**, 37 (1923).
(40) OKAMOTO, KOZO: Acta Schol. Med. Univ. Imp. Kioto **20**, 413–560 (1937).
(41) PAPPENHEIMER, A., THOMPSON, W., PARKER, D., AND SMITH, K.: Proc. Soc. Exptl. Biol. Med. **56**, 145 (1944).
(42) PAULING, L.: *The Nature of the Chemical Bond.* Cornell University Press, Ithaca, New York (1939).
(43) PERLS, M.: Arch. path. Anat. (Virchow's) **39**, 42 (1867).
(44) ROTHEN, A.: J. Biol. Chem. **152**, 679 (1944).
(45) SAHA, K., AND GUHA, B.: Nature **148**, 595 (1941).
(46) SALKOWSKI, E.: Z. physiol. Chem. **59**, 19 (1909).
(47) SCHMIEDEBERG, O.: Arch. exptl. Path. Pharmakol. **33**, 101 (1894).
(48) SPATZ, H.: Z. ges. Neurol. Psychiat. **77**, 261 (1922).
(49) STARKENSTEIN, E.: Klin. Wochschr. **7**, 218 (1929).
(50) STARKENSTEIN, E.: Arch. exptl. Path. Pharmakol. **172**, 75 (1933).
(51) STARKENSTEIN, E.: *Handbuch der experimentellen Pharmakologie*, Vol. 3, p. 682. J. Springer, Berlin (1934).
(52) TOMPSETT, S.: Biochem. J. **34**, 961 (1940).
(53) TRIA, E.: Chem. Abstracts **37**, 898 (1943); Atti accad. Italia, Rend. classe sci. fis., mat. nat. [7] **1**, 579–89 (1940).
(54) VAHLQUIST, B.: Acta Paediat. **28**, Supplement V (1941).
(55) WEHRLE, H.: Arch. Path. **25**, 514 (1938).
(56) WELCH, C., WAKEFIELD, E., AND ADAMS, M.: Arch. Internal Med. **58**, 1095 (1936).
(57) WIDDOWSON, E., AND MCCANCE, R.: Biochem. J. **31**, 2029 (1937).
(58) ZALESKI, ST.: Z. physiol. Chem. **10**, 453 (1886).