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TRANSITION METAL ION BINDING BY FERRITIN AS
STUDIED BY ELECTRON PARAMAGNETIC RESONANCE SPECTROSCOPY

Duane Lee Lindner
(Ph. D. thesis)

November 1977

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ABBREVIATIONS

CPA Carboxypeptidase A
EPR Electron Paramagnetic Resonance
HEPES N-2-hydroxyethylpiperazine-N'-ethanesulfonic acid
NCF Noncrystallizable ferritin
PCMB Parachloromercuric Benzoic Acid
PIPES Piperazine-N,N'-bis(2-ethanesulfonic acid)
TRIS tris(hydroxymethyl)aminomethane
TRANSITION METAL ION BINDING BY FERRITIN AS STUDIED BY ELECTRON PARAMAGNETIC RESONANCE SPECTROSCOPY

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ABSTRACT

We have examined the binding of several transition metal ions by the iron storage protein, ferritin, as well as its iron-free derivative, apoferritin. The ions studied include ferric ion, manganous ion, cuprous and cupric ions, and vanadyl ion. The primary technique used in these investigations has been electron paramagnetic resonance spectroscopy (epr).

Ferric ion is found to bind to both ferritin and apoferritin in a site of rhombic crystal field symmetry. The ion has apparent g values \( g'_{x} = 4.28, g'_{y} = 4.24, g'_{z} = 4.33 \). Iron is bound only loosely to this site.

Cupric ion is found to bind in two different ways. One way involves a copper 'tight-binding' site which strongly and specifically binds the element. The binding is highly pH dependent with as many as two ions per protein subunit binding to both ferritin and apoferritin. The protein binds each cupric ion with three nitrogenous ligands arising from both histidine and lysine amino acid residues. This cupric ion is also seen to be reversibly reducible to cuprous ion. Copper bound in this site is shown to dramatically alter the kinetics of iron release from ferritin by reducing agents. For example, the half time for
release by ascorbate is observed to be about a factor of $10^3$ smaller for ferritin/Cu than for copper free ferritin under the same conditions. Clinical evidence that copper is important for normal iron metabolism in mammals is discussed in light of this observation.

Cupric ion in excess of that bound in the 'tight-binding' site is observed to bind in a 'solution-like' manner to the apoferritin (but not to the ferritin). Such cupric ion is free to tumble isotropically, yet its freedom of motion is somewhat limited compared to free cupric ion in aqueous solution at the same temperature. In a similar manner, manganous ion associated with apoferritin gives rise to solution-like spectra, which do, however, exhibit a line broadening of 12% relative to manganous ion under the same conditions in the absence of apoferritin. The characteristics of the epr spectra of manganous and excess cupric ions are compared with those of spectra of the same ions in the cavities of both natural and synthetic zeolites, where the ions interact with multiple cation exchange sites. It is concluded that apoferritin can concentrate metal ions in its central cavity by acting as an ion exchanger.

Vanadyl ion is also observed to associate with apoferritin in such a way as to retain a great deal of rotational freedom of motion. Again, this ion appears to be present in the apoferritin inner cavity. We observe a pH dependent spectrum resulting from the deprotonation of a water molecule coordinated to the ion. Spectral properties of this ion in association with apoferritin are discussed in light of the unusual properties of water in small cavities lined with charged groups.
I. INTRODUCTION

The past decade has witnessed a great upsurge of interest in the roles that transition metal ions play in biological systems. This interest has been spurred for several reasons. One has been the realization that a large percentage of enzymes require a metal ion for activity (over 27% according to Mildvan, 1970). Another has been the unusual catalytic and coordination properties that metal-protein systems can often display. A third has been the growing availability of these proteins which has allowed inorganic and physical chemists to study them using the techniques of these disciplines.

Metal ions can have several distinct functions in protein systems. One function is structural—the coordination of various amino acid residues of the protein by the ion can fix the tertiary structure of the polypeptide. Another is the binding or stabilization of substrates or catalytic intermediates by the metal ion. Yet another employs the oxidation-reduction properties of a metal ion for electron transport or catalysis of redox reactions of the substrate. (For reviews of metal ions in biochemistry see, for example, Mildvan, 1970 or Eichhorn, 1973.)

One of the methods of physical chemistry which has been utilized in the investigation of metalloproteins is electron paramagnetic resonance spectroscopy (epr). This technique has long been used in the study of ligand fields in which paramagnetic transition metal ions are located. The metal ions which can be so studied are characterized by nonzero electronic spin angular momentum with some restrictions (see, for example, Wertz and Bolton, 1972; Abragam and Bleaney, 1970).
If spectra are observed, one may be able to use them to characterize the metal ion binding site.

A large number of investigations of metal ions bound to proteins with epr spectroscopy have yielded a variety of different types of information about metal ion binding in a number of different systems. For example, studies of a number of iron transport compounds such as ferrichromes A and B, mycobactin P, and enterobactin have established the oxidation state of iron in these systems to be Fe(III) and revealed that the crystal field at the iron binding site has rhombic symmetry in such compounds (Oosterhuis, 1975). Studies of transferrin by epr have shown it possesses two nonequivalent metal ion binding sites (Aasa, 1972, by spectroscopy of ferric ion and Cannon and Chasteen, 1975, by spectroscopy of vanadyl ion bound in the native iron binding sites). Crystal fields in the iron-sulfur proteins have also been investigated (Blumberg and Peisach, 1974).

Numerous studies of copper containing proteins with epr spectroscopy have provided information about crystal fields at such ion binding sites and in certain cases have allowed identification of amino acid residues coordinated to cupric ions. Studies of changes in cupric binding sites in the presence of substrates in some cases, and observation of changes in oxidation state of the copper in others by epr have provided information about mechanism of enzyme action in several systems (Vanngard, 1972).

EPR studies of manganous ion in several different proteins has allowed determination of the number of binding sites as well as binding constants of the ion (Reed and Cohn, 1972; Schray and Mildvan, 1972).
Such studies have also provided information about enzyme mechanism (Maggio et al., 1975). Studies of vanadyl ion used as a probe to replace such ions as zinc and ferric ion have yielded a variety of information about ion binding sites, including crystal field symmetries and ligand identification (Chasteen, 1977). Similar information has been obtained from epr spectra of cobaltous ion (see, for example, Haffner and Coleman, 1073). Spectra of molybdenum (V) have also been obtained from a number of molybdoenzymes and have provided information about the coordination of this ion in such proteins (Bray, 1975).

We have used epr as our primary tool in the investigation of the iron storage protein, ferritin. This protein stores large amounts of iron as a ferric oxyhydroxide microcrystal which is surrounded by the polypeptide chain. The uptake and release of iron by this protein involves, respectively, catalytic oxidation and reduction of iron. We have been studying these redox reactions and have been using various metal ions as probes of metal binding sites which have been postulated to be involved in such reactions as well as in nucleation of the ferritin microcrystal. We have studied the interactions of the protein as well as the metal-free apoprotein (apoferritin) with ferric and ferrous ions as well as Cu$^{2+}$, VO$^{2+}$, and Mn$^{2+}$ which we have used as probes of metal binding sites. In the course of these investigations we have discovered a copper binding site that tightly and selectively binds both cuprous and cupric ions. The kinetics of iron mobilization are dramatically altered for ferritin with copper ion occupying this site. We have used epr of Cu$^{2+}$ bound at this copper 'tight-binding' site to characterize it.
We here present a short description of ferritin, emphasizing those features of structure and function that relate to the present study. We also review some of the characteristics of the particular metal ions as they relate to protein binding and epr studies. We then present the results of our studies with iron, manganese, copper, and vanadyl ions. Finally, we will draw on the biochemical and clinical literature as well as the present experimental results to discuss what we now know about the structure and functions of ferritin and how this relates to the iron cycle in mammals.
II. FERRITIN: A BRIEF DESCRIPTION

A. Introduction

There exist in the literature several recent excellent reviews of the protein ferritin. For example, see: Harrison and Hoy, 1973; Crichton, 1973; and Harrison et al., 1974. Also, Spiro and Saltman (1969) review some properties of iron chemistry which are important for an understanding of ferritin. Because of the existence of these reviews, we will describe here only those properties of ferritin which bear directly on the present study, and will refer the reader to the literature for further information.

The protein ferritin is widely distributed in nature as it is present in animals, plants, and even fungi (David, 1974). Most research with it has been done with ferritin isolated from animals and in particular with that isolated from equine spleen. Within a particular organism there exist isoferittins which differ depending on the type of cell (or organ) in which they are found (Crichton et al., 1973; Crichton et al., 1975). There are also differences between ferritins isolated from tumor tissue and those isolated from normal tissue (Linder et al., 1975; Munro et al., 1975).

The protein was first isolated from horse spleen by Laufberger in 1937 and its properties were reviewed by Granick (1946a) in a now classic paper. The protein was found to consist of two distinct parts (see figure 1). One is the polypeptide chain, called apoferritin, which forms a spherical shell of outer diameter about 120 Å and inner diameter about 75 Å (Harrison, 1959, 1963; Fischbach and Anderegg, 1965).
Fig 1. Ferritin in cross section. After Harrison et al., 1975, p. 274.
The other part is a ferric oxyhydroxide microcrystal located on the interior of the polypeptide shell (Mazur and Shorr, 1950; Farrant, 1954).

B. The Inorganic Core

The iron content of the inorganic microcrystalline core of ferritin is variable with a maximum content of about 4500 iron atoms. The composition is \([\text{FeOOH}]_8\text{FeOOPO}_3\text{H}_2\) \(_x\) with \(x\) between zero and about 500. The function of the phosphate is unknown, but it is known to lie on the surface of the microcrystal (Granick and Hahn, 1944). Electron microscopic studies of the core first led to speculation that it consists of several electron dense regions arranged in some regular pattern (Muir, 1960; van Bruggen et al., 1960). These results appear to be experimental artifacts, however, and higher resolution studies show the ferritin cores to be homogeneous, although irregular in shape. Some controversy still exists as to whether or not more than one microcrystal can be present in a particular ferritin molecule (Harrison and Hoy, 1970; Haydon, 1970).

Several models have been proposed for the crystal structure of the ferritin core. Two models based on an hexagonal close pack structure of the oxygen atoms with iron atoms in tetrahedral or octahedral interstitial sites (Harrison et al., 1967) or exclusively in octahedral sites (Towe and Bradley, 1967) have been suggested. A structure with all iron atoms in tetrahedral coordination sites has also been proposed (Bradly et al., 1968). Studies of electronic absorption spectra of ferritin and some of the synthetic inorganic models for the ferritin
core (Spiro and Saltman, 1969) by Webb and Gray reveal that ferritin iron is in coordination sites of octahedral symmetry and provide evidence that none of the iron is tetrahedrally coordinated (Webb and Gray, 1971; Gray, 1975).

The magnetic properties of the ferritin microcrystalline core will be discussed in chapter IV.

C. The Polypeptide Chain

Iron can be completely removed from ferritin with the use of suitable reducing and chelating agents (Granick, 1946a). It is generally accepted that the resulting apoprotein (from horse spleen) has a molecular weight of 445,000 daltons and is composed of 24 identical subunits of molecular weight 18,500 (Harrison, 1975 pp. 87-89 and references therein). However, there have been recent reports that ferritin dissociated into subunits by heating in concentrated urea solutions shows several components by isoelectric focusing (Ishitani et al., 1975). This subunit heterogeneity may be a result of partial decomposition of the protein subunits due to heating or oxidation, however (Collet-Cassart and Crichton, 1975).

Ferritin from horse spleen has an isoelectric point of 4.4 indicating that the protein is acidic. Amino acid composition of horse spleen ferritin is given in table I. Data from two different determinations are presented, but for our purposes the only important difference between the two is that of cysteine number which is found to be three per subunit in one case and two per subunit in the other. Chemical modification studies of ferritin, apoferritin, and the
Table I. Amino Acid Composition of Horse Spleen Apoferritin

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Residues per Subunit</th>
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<tr>
<td></td>
<td>A. Bryce and Crichton, 1971</td>
</tr>
<tr>
<td>Cysteine</td>
<td>2.8</td>
</tr>
<tr>
<td>Aspartic Acid + Asparagine</td>
<td>17.3</td>
</tr>
<tr>
<td>Threonine</td>
<td>5.5</td>
</tr>
<tr>
<td>Serine</td>
<td>9.0</td>
</tr>
<tr>
<td>Glutamic acid + Glutamine</td>
<td>23.9</td>
</tr>
<tr>
<td>Proline</td>
<td>2.8</td>
</tr>
<tr>
<td>Glycine</td>
<td>9.9</td>
</tr>
<tr>
<td>Alanine</td>
<td>14.0</td>
</tr>
<tr>
<td>Valine</td>
<td>6.9</td>
</tr>
<tr>
<td>Methionine</td>
<td>2.8</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>3.5</td>
</tr>
<tr>
<td>Leucine</td>
<td>25.0</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>5.0</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>7.3</td>
</tr>
<tr>
<td>Histidine</td>
<td>5.8</td>
</tr>
<tr>
<td>Lysine</td>
<td>8.7</td>
</tr>
<tr>
<td>Arginine</td>
<td>9.5</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>2.1</td>
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</table>
apoferitin subunits suggest that all of the tyrosine, one or both of the tryptophan residues and several of the basic and acidic groups are located in the subunit-subunit interface (Crichton, 1973, 1975). We will discuss the possible disposition of various metal ion binding groups later in this chapter (section F).

Recently, an x-ray study has led to the presentation of a 6Å resolution electron density map of apoferitin (Hoare et al., 1975a, 1975b). They show apoferitin to have an α-helix content of 50-60% of the polypeptide, which is in good agreement with ORD studies (Wood and Crichton, 1971). The map shows the protein to form a spherical shell penetrated by six channels along four-fold molecular symmetry axes. These channels are roughly square in cross section with a side of approximately 10Å. Boundaries between subunits are not distinguished at this resolution.

D. Uptake and Release of Iron

The mechanism of action of ferritin has been the subject of many studies. It has been known for some time that ferritin would release iron in vitro in the presence of suitable reducing agents (Granick, 1942). It is also now known that strong enough chelating agents can extract iron (as Fe^{2+}) from ferritin (Jones and Johnston, 1967). It has also been demonstrated that ferritin can be reconstituted in vitro from ferrous ion and apoferitin under oxidizing conditions (Bielig and Bayer, 1955). Niederer (1970) first demonstrated that apoferitin catalyzes the oxidative uptake of iron by apoferitin in vitro.
These initial findings resulted in numerous subsequent studies of iron movement into and out of ferritin. Among the first of such studies of iron uptake were those of Pape et al., (1968). They observed that ferritin 'reconstituted' from ferrous ion and apoferritin as mentioned above gives a protein with a total diameter and a core diameter a bit smaller than those of native ferritin. They incubated apoferritin with noncrystallizable ferritin (a ferritin fraction which does not crystallize in the normal way--some believe it to be deficient in protein subunits (Pape et al., 1968) although this point is open to dispute (Farrant, 1954; Drysdale et al., 1968)) and were able to crystallize normal ferritin from the solution. As a result of these and other experiments, they proposed a model for iron uptake in ferritin (see figure 2). In the model, apoferritin is in equilibrium with its subunits. Free subunits assemble around iron micelles which are known to form in ferric salt solutions near neutral pH in vitro (Spiro et al., 1966). Iron is then released from this ferritin by reduction and chelation. However, there is no evidence that apoferritin is in equilibrium with its subunits (Jaenicke and Bartmann, 1972) and the model is at variance with the observation that $^{59}$Fe used in tracer studies appears first in iron poor ferritin molecules and only appears in full ferritin a long time afterwards (Drysdale and Munro, 1966). Saltman and his colleagues have continued their experiments with the inorganic micelles and they have reported that addition of apoferritin subunits (prepared by dissociation at low pH) to synthetic cores gives synthetic ferritin upon rapid neutralization in vitro (Ceperneek et al., 1971).
Fig. 2. The mechanism of iron accumulation in ferritin as proposed by Pape et al., 1968. The figure is taken from that reference page 611.
Nonetheless, most workers now seem to feel that the uptake of iron in vivo proceeds via oxidation of ferrous ions and subsequent or concurrent incorporation of the resulting ferric ions in the ferritin core. As a result of many studies of oxidative iron uptake in vitro, a couple of distinct models have been advanced for the process (see figures 3 and 4). Figure 3 is the model advocated by Harrison and her co-workers (Harrison et al., 1975). The model proposes that apoferritin merely provides nucleation sites for crystal growth of the micelle. And once nucleation has occurred, further iron uptake follows a crystal growth mechanism. It assumes that the interior cavity of apoferritin is completely accessible to reagents in the protein medium (if they are small enough to pass through the channels in the polypeptide shell) and it postulates that oxidation of Fe\(^{2+}\) on uptake and reduction of Fe\(^{3+}\) on release occur on the surface of the ferric oxyhydroxide microcrystal. The model has substantial experimental backing. For example, Macara et al. (1972) found that iron uptake by apoferritin gave a sigmoidal progress curve while the curves for iron uptake by ferritins of low iron content were hyperbolic. Also, rate of iron uptake is found to be dependent on the amount of iron already present in the molecule such that a maximum rate of uptake is evidenced by ferritin fractions between 1/3 and 1/2 filled with iron. These results were interpreted as indicating a two-step 'crystal growth' mechanism. First, a slow 'nucleation' step followed by a rapid crystal growth step—with rate of iron uptake dependent on the area of the crystal surface of the growing micelle. A computer model of the proposed process gave reasonable agreement with experimental
Fig. 3. Iron uptake and release by ferritin as proposed by Harrison and her coworkers. The protein provides points of nucleation for the microcrystal, indicated here by the semicircles. After oxidation of ferrous ion at these points, further changes in the microcrystal are a result of the direct action of reagents in the protein medium with the microcrystalline surface. After Harrison et al., 1975, p. 274.
results. Numerous further studies by Harrison and her colleagues support
and refine this model (Macara et al., 1973a, 1973b, 1973c; Hoy et al.,
1974a, 1974b; Harrison et al., 1974; Harrison et al., 1975; Hoy and
Harrison, 1975). The final model proposes that oxidants and reductants
interact directly with the surface iron atoms of the ferritin core;
as do chelators such as 1,10-phenanthroline which are known to slowly
remove ferric ion from the core. Metal ion inhibitors of iron uptake
and release (such as Zn$^{2+}$) are believed to be competitive due to competition
with iron for binding sites on the micelle surface as well as competing
for the nucleation sites on the protein in the initial phases of iron
uptake. The model does have some shortcomings, however. First it
fails to provide a mechanism by which iron mobilization from the protein
can be either triggered or inhibited. Second, there is some experimental
evidence that the polypeptide plays a significant role in iron uptake -
Stefanini et al. (1976) find that apoferritin can be prepared in
either an 'oxidized' or a 'reduced' form. These two forms of the protein
take up iron in quite different ways, indicating the polypeptide has
more influence over core formation than simply providing a point of
nucleation.

There is another model, or rather a series of models, for iron
uptake and mobilization which ascribe to the protein an active role in
both of these processes. Such models were first proposed by Niederer
(1970) after a study of iron uptake and mobilization in vitro with
and without other metal ions present. He proposed that the protein
contains an 'active site' where redox reactions of iron occur during
iron incorporation and release (see figure 4). Such a model had been
Fig. 4. A particular variation of a series of possible mechanisms of iron uptake and release by ferritin as proposed by Niederer, (1970) and currently advocated by R. R. Crichton and coworkers. In this model, ferrous ion must pass through an active site where it is oxidized before being incorporated into the microcrystal. Similarly, ferric ion must pass through a reducing site (here the same as the ferrous oxidizing site) before being released to the medium. Iron in the core is in equilibrium with ions in the postulated redox sites. After Harrison et al., 1975, p. 274.
hinted at by Mazur and his coworkers (Mazur et al., 1955); Green and Mazur, 1957) who found ferric ion in the ferritin micelle to be in equilibrium with loosely bound ferrous ion—which they assumed to be bound in an iron redox site. They also noted that the loosely bound ferrous ion was not on the protein outer surface. Niederer also found evidence that the redox site was on the protein interior in a series of experiments involving chemical modification of certain amino acid residues. These studies led to a series of models for ferritin action (Macara et al., 1972) differing in the location of the postulated active site as well as the location of a possible nucleation site, e.g., the active site could either be in the protein interior or in the channels through the polypeptide shell while the nucleation site could be the same site as the redox site or a completely different site. On the other hand, there might not be a nucleation site at all, since even one ferric ion in the apoferritin interior at physiological pH would have a concentration exceeding the solubility of FeOOH, and spontaneous precipitation should therefore occur if small numbers of ferric ions are present. We have shown only one variation in figure 4. This model also has its supporting experimental evidence. Bryce and Crichton (1973) do not observe the sigmoidal progress curves for iron uptake and ascribe that observation by Macara et al. (1972) to experimental artifacts. Dognin et al. (1973) also find that iron in the ferritin core is in equilibrium with loosely bound ferric ion on the protein with the number of loose binding sites related inversely to the iron content of the protein. Boas and Troup (1971) report a broad epr line from ferritin with an apparent g value of 4 which
they attribute to ferric ion bound to some site other than those on the ferritin core. Harrison et al. (1974) however, feel that this loosely bound iron is in poorly crystalline areas of the core or in incomplete surface layers of that core and not associated with the polypeptide. Hoy and Harrison (1976) also report an experiment in vitro in which they find ferric ion to be taken up directly by rat liver ferritin. This would seem to indicate that redox activity in iron uptake and release is not necessary, and while they agree that apoferritin does exhibit ferroxidase activity, they feel these experiments prove any model requiring catalytic redox sites to be invalid.

Recent evidence suggests that the mechanism of iron mobilization from ferritin may be more complicated than either of these models suggests. Studies of iron release by various reducing agents of physiological strength have usually shown that none of them can release the iron in physiologically reasonable time scales (Bielig and Bayer, 1955; Dognin and Crichton, 1975). A recent study has, however, shown that ferritin iron is very rapidly mobilized in the presence of reduced flavins (Sirivech et al., 1974). Crichton et al. (1975a; 1975b) have extended these studies to a system containing FMN, NADH, and ferritin. They observe a lag time in which little iron is released (of 5 to 10 min) and then rapid reduction and release of iron. They ascribe the lag time to a buildup of FMNH$_2$ which they believe then binds to the ferritin and then acts as a coenzyme in the further reduction of iron with the NADH acting as an electron source as follows:

$$\text{FMN} + \text{NADH} + \text{H}^+ \longleftrightarrow \text{FMNH}_2 + \text{NAD}^+$$

$$\text{FMNH}_2 + 2\text{Fe}^{3+} \longleftrightarrow \text{FMN} + 2\text{Fe}^{2+}$$
The FMN-NADH system also quickly reduces Fe$^{3+}$ in the form FeCl$_3$ (Chrichton et al., 1975b). There have also been reports of partial purification of a protein with ferrireductase activity which was thought to be involved in ferritin iron mobilization (Osaki and Sirivech, 1971). It should also be noted here that there have been some reports that xanthine oxidase is involved in iron mobilization (Green and Mazur, 1957) but attempts to utilize that enzyme in iron release in vitro, have not resulted in any observable iron mobilization (Chrichton, 1973).

E. The Physiological Role of Ferritin

Iron in animals is treated as a very precious metal--there is no direct chemical mechanism by which it can be excreted. As a result, iron is continuously being recycled through the organism with some iron uptake from the diet to replace losses due, for example, to normal flaking off of skin or loss of blood. A simple schematic of the iron cycle in mammals is shown in figure 5. Ferritin appears to play two distinct roles in this cycle. The greater part of the ferritin seems to be simply a storage point for iron. That is, the protein provides a way in which iron can be stored in large amounts and in a water soluble form until it is needed in the biosynthesis of iron metalloproteins. In particular, ferritin in the erythroid cells stores iron for use in hemoglobin synthesis. The hemoglobin is utilized in the erythrocytes and after a lifetime of about 120 days these cells are phagocytised by cells of the reticulo-endothelial system in the spleen and elsewhere. The iron recovered during phagocytosis can be stored in ferritin at this point in the spleen or it can be transferred to transferrin for
Fig. 5. A simplified schematic of the iron cycle in mammals. After Crichton, 1973, p. 71.
distribution to other cells where it will be stored in ferritins until needed in biosynthesis (Crichton, 1973; Lynch et al., 1974).

Another distinct role has been postulated for the protein—the so-called 'mucosal block' (see figure 6) (Hahn et al., 1943; Granick; 1946a). The idea is that the ferritin found in the mucosal cells that line the intestine acts as a buffer to prevent iron overload of the organism (since there is no excretory mechanism for excess iron, mammals can be very susceptible to iron overload). It is postulated to bind most of that dietary iron absorbed by the mucosal cell and hold it for the lifetime of the cell; then when the cell is sloughed off it takes with it the iron it contains as ferritin iron and passes out of the organism in the feces. Also, while the cell lives, the iron it contains is available to the plasma transferrin. Whether or not ferritin acts as postulated in the theory of the mucosal block is still a matter of controversy, however, and there is some evidence that it merely acts as a iron storage point in the mucosa (Turnbull, 1974).

F. Possible Metal Binding Sites

From the proposed mechanisms of iron uptake and release (part D above), one can speculate about the nature of metal binding sites which ferritin may contain. If one assumes the mechanism proposed by Saltman and his coworkers, it is not clear that the protein should bind metal ions at all. The ferric oxyhydroxide core would need to bind the polypeptide subunits, but since the surface of the core is coated with phosphate groups, the interaction would probably be with
Fig. 6. The mucosal block, a proposed mechanism by which ferritin buffers iron uptake in animals, thus preventing iron overload. After Crichton, 1973, p. 72.
them; and if one postulated iron release by direct interaction of chelating and/or reducing agents with the microcrystal (after Harrison and her co-workers) then no metal binding sites need be present at all. Although we must note that the protein does require a metal ion (Cd$^{2+}$ is usually used) to crystallize--so there must be some binding sites for divalent metal ions, but these crystallization sites are probably on the protein exterior.

If, however, we postulate that iron is mobilized through some redox site then we would suppose that such a metal binding site would bind ferric ion more strongly than ferrous ion so it could readily remove iron from the micelle and upon reduction easily pass the ferrous ion to chelators in the protein medium.

Harrison's model implies that there must be metal binding sites to serve as points of nucleation for the crystal growth of the micelle. One should suppose that such sites should bind both ferrous and ferric ions with similar strength--in the former case to remove the ion from chelators and in the latter to nucleate crystal growth. Since iron reduction is postulated to go via direct reduction or chelation at the crystal surface no metal binding sites are needed for mobilization of iron.

Finally, the mechanism proposed by Niederer and advocated most extensively by Crichton involves one (or two) redox site(s) which must bind metal ions as well as a nucleation site which may or may not be the same as a redox site. We would expect the redox site involved in iron uptake to bind ferrous ion strongly and ferric ion weakly, unless it also serves as a point of nucleation, in which case both
ions should bind strongly. A redox site involved in iron mobilization could be expected to bind ferric strongly and ferrous weakly, as noted above.

Several experiments have been carried out with the purpose of illucidating the nature of any iron binding sites on ferritin and the results are often in conflict. The most direct approach to investigating the metal ion binding sites on ferritin has been utilized by Hoare et al. (1975a, 1975b)—that of x-ray crystallography. They have reported an electron density map to 6 Å resolution using Cd$^{2+}$ and UO$_2^{2+}$ as markers for divalent metal ion binding in apoferritin. Cd$^{2+}$ is found to bind in two places—one site is on a threefold molecular axis and somewhat buried, the other is on the protein surface along a molecular two-fold axis and probably represents a metal ion involved in salt bridging during crystallization (this Cd$^{2+}$ can be partially replaced with UO$_2^{2+}$). Uranyl ion binding is fairly specific for carboxyl groups, so it appears that the 'bridging' ion sites depend on carboxyls. There are also three uranyl binding sites per subunit on the protein's interior surface. These sites are over 9 Å apart. It is also noted that Cd$^{2+}$, which should behave like Fe$^{2+}$, does not bind on the protein inner surface. They also note that parachloromercuric benzoic acid (PCMB) binds on the inner surface at one site per subunit—probably at a cysteine residue. The PCMB also binds on the molecular threefold axis near the Cd$^{2+}$ binding site and there is some indication that it binds a cysteine sulfur which is usually involved in the coordination of cadmium.
There are other, less direct, ways of probing the binding of metal ions by ferritin. The 'loosely bound' iron referred to previously has been studied by chelating it (Mazur et al., 1955; Jones and Johnston, 1967; Hoy et al., 1974) and detecting the resulting complexes. A polarographic study of iron mobilization (Dognin et al., 1973) has shown ferric ion in the micelle to be in equilibrium with iron bound in a redox site on the protein. And as mentioned, Boas and Troup (1971) have observed an absorption in the epr spectrum of ferritin which they attribute to such iron (see chapter IV).

An approach to the identification of ligands involved in metal binding involves the study of effects of chemical modification of the polypeptide as well as the effects of competing metal ions on the kinetics of iron uptake and release. One of the first such studies was by Mazur et al. (1955). They found that reaction of ferritin with reagents which modify sulfhydryl groups reduced the amount of loosely bound ferrous ion. Niederer (1970) found that modification of histidine residues in the protein inhibited both iron uptake and release, but modification of sulfhydryl groups resulted in no inhibition. He also found that diazonium-H-tetrazole, a strong histidine alkylating agent, had no effect--this was attributed to the size of the reagent preventing it from penetrating to the apoferritin interior. This experiment indicates that the histidines involved in iron uptake are located at the interior of the protein. Studies of the effects of other divalent metal ions showed that Zn$^{2+}$, Ni$^{2+}$, Hg$^{2+}$, Cd$^{2+}$, Co$^{2+}$, and Mn$^{2+}$ all inhibited iron uptake while Zn$^{2+}$, Cd$^{2+}$, Mg$^{2+}$ and Fe$^{2+}$ inhibited iron release. Of course, this inhibition might be explained be postulating
that this ions all compete with Fe$^{2+}$ for binding sites on the microcrystal (Macara et al., 1973b; Harrison, 1974). In a study of proton displacement by metal ions Macara et al., (1973c) note that Zn$^{2+}$, Mn$^{2+}$, Cd$^{2+}$ and Tb$^{3+}$ each displace one H$^+$ while Cu$^{2+}$ displaces two protons at pH 5.5. Furthermore, the nature of the Mn$^{2+}$ binding was quite different from that of the other ions (see table II for their results). A study of proton displacement by Zn$^{2+}$ as a function of pH shows anomalous behavior above pH 6.3 which they believe indicates that the ion is coordinated to a ligand with a pK$_a$ in the range 6.0-7.0—implicating a histidine or a cysteine residue in the metal binding. They also find that that Zn$^{2+}$ acts as a competitive inhibitor to iron uptake at low substrate concentration which they interpret as evidence that the initial step in iron uptake is oxidation of ferrous at a site to which zinc can also bind.

Crichton and his coworkers have been attempting to ascertain which amino acid residues act as metal ligands by chemical modification studies (Bryce and Crichton, 1973; Crichton, 1975; Wetz and Crichton, 1976). They note that modification of two cysteine residues per subunit and one histidine residue per subunit results in loss of the ferroxidase activity of apoferritin. Other experiments with chemical modification are interpreted to show that one cysteine residue, one lysine residue, and four carboxyl groups are located on the protein inner surface. All five tyrosine residues, four or five lysine residues and eleven
Table II. Binding of Metal Ions to Apoferritin from Macara et al., (1973c).

<table>
<thead>
<tr>
<th>Cation</th>
<th>pH</th>
<th>H⁺ displaced</th>
<th>n₁</th>
<th>log K₁</th>
<th>n₂</th>
<th>log K₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zn²⁺</td>
<td>4.70</td>
<td>1</td>
<td>2</td>
<td>1.48</td>
<td>3</td>
<td>0.48</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>1</td>
<td>1.78</td>
<td>4</td>
<td>0.65</td>
</tr>
<tr>
<td>Zn²⁺</td>
<td>5.30</td>
<td>1</td>
<td>2</td>
<td>2.70</td>
<td>3</td>
<td>1.30</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>1</td>
<td>3.22</td>
<td>4</td>
<td>1.65</td>
</tr>
<tr>
<td>Zn²⁺</td>
<td>5.50</td>
<td>1</td>
<td>2</td>
<td>2.98</td>
<td>3</td>
<td>1.48</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1</td>
<td>3.50</td>
<td>4</td>
<td>1.84</td>
</tr>
<tr>
<td>Zn²⁺</td>
<td>6.05</td>
<td>1</td>
<td>2</td>
<td>4.7</td>
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</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1</td>
<td>5.0</td>
<td>4</td>
<td>2.74</td>
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<tr>
<td>Cd²⁺</td>
<td>5.50</td>
<td>1</td>
<td>2</td>
<td>2.88</td>
<td>3</td>
<td>1.48</td>
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<td></td>
<td>1</td>
<td>3.43</td>
<td>4</td>
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</tr>
<tr>
<td>Cu²⁺</td>
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<td>2</td>
<td>2.87</td>
<td>3</td>
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</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1</td>
<td>3.26</td>
<td>4</td>
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<tr>
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<td>0.5</td>
<td>3.30</td>
<td>2</td>
<td>1.30</td>
</tr>
<tr>
<td>Tb³⁺</td>
<td>5.55</td>
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<td>2</td>
<td>3.08</td>
<td>3</td>
<td>0.30</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1</td>
<td>4.3</td>
<td>4</td>
<td>1.62</td>
</tr>
</tbody>
</table>

n₁, n₂, K₁, K₂, are obtained from fitting of Scatchard plots, n₁ and n₂ are the number of binding sites per protein subunit of each of two types of site, K₁ and K₂ are the respective binding constants; the data does not distinguish between n₁=1 and n₁=2 in most cases, so both results are presented.
carboxyl groups are not subject to modification in either ferritin or apoferritin, and so are believed to lie in the subunit-subunit interface. Modification of one cysteine and/or three or four lysine residues per subunit does not affect apoferritin catalytic activity. Modification of seven carboxyl groups in ferritin resulted in no change in catalytic activity of the protein. However, modification of all eleven carboxyl groups in apoferritin resulted in a catalytically inactive protein. So it appears that at least one of the carboxyl groups on the protein inner surface is essential for catalytic activity.

A recent report on the interaction of $H^+$ with ferritin and apoferritin also provides information about the number and type of amino acid residues available for coordination of metal ions (Silk and Breslow, 1976). They find the cysteine sulfhydryl groups to be either oxidized or not accessible to the solvent. They also find 6.7 titratible lysines per subunit at pH 9.0—with none of them deprotonated below pH 8.5. Only two normal histidine residues per subunit are available to solvent and they titrate between pH 5.6 and 7.3. Another histidine residue is found to titrate above pH 7.3, leaving three histidines buried in the protein. A summary of their results is shown in Table III.

Other studies have also provided some indication of how metal ions interact with ferritin. Rüssel (1970) incubated both ferritin and a synthetic inorganic ferric hydroxide with various metal ions and then measured unbound metal ion concentrations by polarographic techniques. The ions examined were $Cu^{2+}, Pb^{2+}, Zn^{2+}, Cd^{2+}, Tl^{3+}$, and
Table III. Apoferritin Titration Parameters from Silk and Breslow (1976).

<table>
<thead>
<tr>
<th>Residue</th>
<th>number per subunit</th>
<th>class</th>
<th>pk$_{1 \text{int}}$</th>
<th>pk$_{2 \text{int}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-COOH</td>
<td>1</td>
<td>titratable</td>
<td>3.0</td>
<td>3.0</td>
</tr>
<tr>
<td></td>
<td>1(?)</td>
<td>(?</td>
<td></td>
<td></td>
</tr>
<tr>
<td>β,γ-COOH</td>
<td>3</td>
<td>buried (deprontonated)</td>
<td>5.56</td>
<td>5.56</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>titratable</td>
<td>3.90</td>
<td>4.07</td>
</tr>
<tr>
<td></td>
<td>21</td>
<td>titratable</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Histidine</td>
<td>3</td>
<td>buried (deprontated)</td>
<td>3.0</td>
<td>3.0</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>titratable</td>
<td></td>
<td></td>
</tr>
<tr>
<td>α-NH$_2$</td>
<td>1</td>
<td>Acetylated</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1(?)</td>
<td>titratable</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lysine</td>
<td>2</td>
<td>buried (protonated)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>titratable</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tyrosine</td>
<td>5</td>
<td>buried (protonated)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Arginine</td>
<td>1</td>
<td>buried (protonated)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>titratable</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

1 at ionic strength = 0.16
2 at ionic strength = 1.0
Ag⁺. He found that only Cu²⁺ exhibited any difference in binding between the two systems. That is, he found only Cu²⁺ interacted with the polypeptide while all the other metal ions were merely adsorbed to the ferric oxyhydroxide core. Studies of incorporation and transport of transuranic elements in rats have shown that both plutonium (Boocock et al., 1970) and americium (Stover et al., 1970) bind to ferritin; presumably because these elements form oxyhydroxide polymers similar to those of iron (Taylor, 1972) and can be incorporated into the polymer at the ferritin core.

In summary, numerous experiments have shed some light on the nature of metal ion interactions with ferritin. With the exception of cupric ion, most divalent metal ions interact only with polypeptide sites in apoferritin which are blocked by the microcrystal in ferritin. Consequently, it appears that the binding sites of interest in ferritin function are located on the protein inner surface (although x-ray work shows that some sites on the protein exterior surface do bind metal ions which are probably involved in salt bridges for crystal formation). One histidine and/or one cysteine residue have been implicated in metal ion binding by chemical modification studies, while other results indicate that the cysteine may be oxidized in the native form of the protein. Also, at least one and possibly as many as four carboxyl groups may be involved in metal ion binding, and x-ray crystallographic results show three carboxyl groups on the inner protein surface can bind uranyl ion (but not Cd(II), surprisingly). Furthermore, a number of divalent metal ions interact with the ferritin core by adsorption.

We will further consider the nature of metal ion binding by ferritin...
and apoferritin as we discuss the interaction of specific ions with these proteins in the following chapters.
III. GENERAL EXPERIMENTAL PROCEDURES

A. Protein Manipulations

The samples of equine spleen ferritin used in these studies were obtained from several different suppliers. These included Miles Laboratories, Kankakee, Illinois, 60901; Calbiochem, La Jolla, California, 92037 and Polysciences, Inc., Warrington, Pennsylvania, 18976. Although the exact procedures used by these firms in the isolation and purification of the protein are unavailable to use for proprietary reasons - the general procedures of Granick (1946a) were followed. Some of our experiments were also performed with ferritin prepared from fresh horse spleen in this laboratory by procedures which were a modification of those of Granick. In all cases the isolation of ferritin from horse spleen depends upon its unusual thermal stability. Thus heating a homogenate of horse spleen to 80°C for ten minutes denatures most extraneous, unwanted proteins causing them to precipitate. Filtration or centrifugation will remove these proteins, leaving a dark red impure solution of ferritin. There is, however, some recent evidence that ferritin is not completely unaffected by such heating (Collet-Cassart and Crichton, 1975), and it appears that a heat labile peptide bond may cleave in a percentage of the ferritin subunits (the exact percentage depending on the length of the heat treatment). This bond breakage apparently does not affect the quaternary structure of the protein or many of its other properties, so that ferritin molecules with damaged subunits may be carried through subsequent purification procedures along with undamaged protein.
Further isolation of ferritin usually involves precipitation of the protein by adding an equal volume of saturated ammonium sulfate solution to the ferritin solution in order to 'salt out' the protein (we note that such a 50% saturated ammonium sulfate solution has a pH of 5.3). The protein can then be pelleted out and redissolved in water. Recent variants of the procedure (Harrison and Hoy, 1973; Harrison et al., 1974b) require that the pH be adjusted to 4.8 or lower at this point in order to precipitate the so-called 'non-crystallizable' ferritin. The protein is then either repeatedly recrystallized with 5% cadmium sulfate (Granick, 1946a), purified by gel filtration (Harrison et al., 1974), or purified with ultracentrifugation (Crichton, 1973). Most of the protein used in our studies was twice recrystallized with cadmium sulfate, although the samples used in studies of the kinetics of iron uptake and release were cadmium free.

Apoferitin was prepared, as follows, by a modification of the procedure of Crichton (1973) in a cold room (4°C). Ferritin solutions as obtained from the several suppliers mentioned were diluted to approximately 10 mg ferritin per ml with 2% ammonium sulfate solution. This deep red ferritin solution was then placed in a dialysis bag and dialysed against a solution of mercaptoacetic acid (1% v/v) in 100 mM acetate buffer at pH 5.25 until the contents of the dialysis bag were a pale brown (this usually meant over night). If the ferritin solution used had too high a protein concentration (e.g., 100 mg/ml) the protein would precipitate upon the addition of mercaptoacetic acid; however, subsequent dialysis against buffer would redissolve it. The apoferritin solution was then dialysed exhaustively against
several changes of buffer to remove residual reducing agent. We found that the slight odor of meraptoacetic acid remains present even after dialysis against seven or eight changes of buffer (100x the apoferritin solution volume) over a period of a week. The resulting solutions of apoferritin were concentrated by burying the dialysis bag containing them in Aquacide-IIA (Calbiochem, La Jolla, California) for several hours. Apoferritin concentrations were determined by observing the absorbance of these solutions at 280nm where apoferritin has an extinction, \( E_{1\%cml} = 9.0 \) (Macara et al., 1976). We should point out that a recent study (Stefanini et al., 1976) suggests that the above preparative procedure may leave the protein in a 'reduced' state. The evidence is that 'native' apoferritin separated from ferritin preparations by ultracentrifugation and 'reduced' apoferritin prepared by a procedure similar to the one do not take up iron in the same manner unless the 'reduced' apoferritin is first treated with an oxidizing agent. Since all our apoferritin preparations were followed by several days dialysis against buffer in the presence of atmospheric oxygen, we do not feel that our apoferritin remains in this 'reduced' state.

In order to minimize interference from extraneous metal ions, polyethylene containers for solutions were used whenever possible. Any glassware employed was soaked for over three hours in 6 M HCl and then rinsed exhaustively with water. All water used was twice distilled--the second time using a Corning AG-1b automatic still which contains Vicor elements.
Buffers used were Calbiochem Ultrol® grade. All other chemicals used were reagent grade or better except for the mercaptoacetic acid which was 98% pure (Mallinckrodt) and the sodium dithionite which was Baker and Adamson technical grade (90%).

Metals were introduced to the protein in two different ways. One way involved the dialysis of a solution of apoferritin against a solution of metal ion in 100 mM acetate buffer at a pH of 5.5 or lower (to minimize hydrolysis of the metal ions). The resulting protein/ion solution was then dialysed against several changes of a buffer solution at the pH of interest. This procedure resulted in such low concentrations of unbound metal ions at higher pH that we experienced no problems with hydrolysis and precipitation of metal ions in the dialysis sack.

A different procedure involved titration of small amounts (1 - 10 µl) of concentrated metal ion solutions into a 300 - 500 µl volume of protein solution buffered at the pH of interest. As long as sufficiently small amounts of metal ion (roughly 4-5 ions per apoferritin subunit) were added to the protein solution, hydrolysis did not appear to be a problem and the final pH did not differ significantly (<10%) from that of the original protein solution.

B. Apparatus and Instruments

Electron paramagnetic resonance spectra were obtained with a Varian Associates model V4502 epr spectrometer operating at 9.0 to 9.5 GHz (X band) employing a Varian 9 inch magnet. Microwave frequencies were measured directly with a Hewlett-Packard model 5245L frequency counter with a 5255A X band plug-in unit. Magnetic fields were measured
by detecting proton magnetic resonance with a Harvey-Wells FC-50 precision gaussmeter. A Varian V4531 microwave cavity was used at temperatures above -120°C. Such temperatures were obtained by boiling liquid nitrogen in a dewar by passing current through a resistor immersed in the liquid and passing this boil-off over the sample which was contained in a dewar in the microwave cavity. Temperatures were measured with a thermocouple and a digital voltmeter. Lower temperatures were obtained with a pumped helium dewar arrangement (Pratt, 1967) and a microwave bridge substantially different from the Varian bridge was used (Jindo, 1971). These temperatures were measured by observing the partial pressure of helium in the dewar. Cavities used at low temperatures were home built. Room temperatures spectra of aqueous solutions were obtained with the sample in an aqueous flat cell (Wilmad WG-812).

EPR spectra of frozen solutions (glasses) were simulated with a modification of the program RHOM (Venable, 1965) or with the program VAESR (Chang, 1971), both of which are in Fortran. The former allows simulation of epr spectra of species in powders or glasses if such species are magnetically dilute and are bound in crystal fields of rhombic symmetry. The latter is similar, except that it will only simulate spectra of paramagnetic species in crystal fields of axial symmetry. These programs were run on the Control Data Corporation 7600 computer of the Lawrence Berkeley Laboratory. Other computations were performed with a Digital Equipment Corporation PDP 8/I computer in our laboratory.
IV. IRON BINDING

A. Introduction

In its physiological role, ferritin binds iron. Obviously, then, the properties of the protein interaction with both ferric and ferrous ions are of primary importance to an understanding of how the protein functions. Unfortunately, epr spectroscopy of ferrous ion is rather limited (Low and Weger, 1960; Orton, 1968). In strong crystal fields the ion is diamagnetic and in weak fields the species, if observable at all, requires very low temperatures due to its very short spin lattice relaxation times which cause lines to be broadened into the base line as temperatures are raised much above those of liquid helium. We have never observed any resonance which could be attributed to ferrous ion bound to ferritin, even at temperatures as low as 1.50K.

Ferric ion is a 3d^5 system which is either high spin \((S = 5/2)\) or low spin \((S = 1/2)\) depending on the strength of the surrounding crystal field. Consequently, it is always paramagnetic and due to favorable relaxation properties, an epr spectrum can usually be observed at room temperature. Furthermore, this spectrum can be very sensitive to the properties of the crystal field about the ion.

In the case of high spin ferric ion, which has a \(6S\) ground state, the Hamiltonian usually used to fit the spectrum is:

\[
\hat{H} = \beta \cdot \hat{H} \cdot \vec{g} \cdot \vec{S} + D \left[ \hat{S}_z^2 - \frac{1}{2} \right] + E(\hat{S}_x^2 - \hat{S}_y^2)
\]
where $\beta$ is the Bohr magneton, $\tilde{B}$ is the magnetic field, $g$ is the g
tensor, $D$ and $E$ are parameters which characterize the energy level
splittings in zero magnetic field, and $\hat{S}, \hat{S}_x, \hat{S}_y, \hat{S}_z$ are the usual
spin operators. Sometimes quartic terms are also included but they
are usually small compared to the quadratic terms and will be ignored
here. The exact physical origin of the terms in $D$ and $E$ is not fully
understood but they appear in the usual perturbation scheme to arise
from coupling of excited quartet electronic states to the ground
state by matrix elements of the crystal field. It can be shown that
if one chooses the correct coordinate system (see Blumberg, 1967) the
ratio $|E/D|$ must be less than 1/3; this ratio is also called $\lambda$. If
$E/D = 0$ we have the case of axial symmetry in the crystal field of
the ferric ion. If $|E/D| = 1/3$ the symmetry of the crystal field
is said to be 'completely' rhombic. For intermediate values of $\lambda$
we have cases of less than axial, but not completely rhombic symmetry.
The magnitude of $D$ relative to the Zeeman term in the Hamiltonian
governs the size of the zero field splittings; if the term in $D$ is much
larger than the Zeeman terms, the energy levels can be thought of as
three separate Kramer's doublets. The splittings of such doublets
in a magnetic field for two limiting cases of $\lambda$ is shown in figure 7.
The eigenfunctions of the Hamiltonian above are not of the form $|M_S\rangle$
where $M_S \in \{\pm 5/2, \pm 3/2, \pm 1/2\}$ but rather are linear combinations of
this basis set (Wickman et al., 1965). Hence, transitions in each
of the Kramer's doublets of figure 7 are all allowed to some extent
under the selection rule $\Delta M_S = \pm 1$, which is operative in epr transitions.
Fig. 7. First order splitting of the three doublets of the electronic ground state of ferric ion by a magnetic field. The limiting cases of $\lambda = 0$ (axial crystal field symmetry) and $\lambda = 1/3$ (rhombic crystal field symmetry) are shown. The numbers for each doublet are the apparent $g$ values ($g'$) for electron paramagnetic resonance transitions in the doublet. After Oosterhuis, 1975.
If one defines an 'effective' g value, $g'$, through the equation:

$$g' = \frac{h\nu}{\beta H}$$

where $h$ is Planck's constant, $\nu$ is the microwave frequency and $H$ is the magnitude of the magnetic field at which resonance occurs, one can the plot 'effective' g values as a function of $\lambda$ as shown in figure 8. Notice that the value $g'$ for such a system is quite distinct from any of the elements of the g tensor of the Hamiltonian, and that even though one can determine a $g'_x$, $g'_y$, and $g'_z$, these values do not comprise the diagonal elements of a tensorial quantity. In the case of high spin ferric ion under consideration here the ground state is an $S$ state, so to first order, we expect no contribution from orbital angular momentum to the Zeeman term of the Hamiltonian and consequently $g'_x = g'_y = g'_z = 2.00$, while the values of $g'_x$, $g'_y$, and $g'_z$ vary anywhere from zero to ten (Blumberg, 1967).

It is of special interest to note in figure 8 than when $\lambda = 0$, $g' = 2.0$ and $g' = 6.0$ for the lowest Kramer's doublet; meanwhile the range of the effective g values in the other doublets is from 0.0 (absorption at infinite field) to 6.0 in one and 10.0 in the other. In the spectra of glass or powder samples, absorption intensity is distributed between the fields corresponding to the maximum and minimum values of $g'$, so that for such samples, transitions in the upper two doublets are too broad to be easily observed. In such systems, the transition in the lower doublet is characteristic of high spin ferric ion in axial crystal field. Similarly, for $\lambda = 1/3$, the transitions in the upper and lower doublets are too broad to be easily observed in
Fig. 8. Effective $g$ values for transitions in each of the Kramer's doublets of high spin Fe$^{3+}$. The ordering of the doublets assumes a positive value of $D$. After Wickman et al., 1964, p. 2115.
disordered samples while the central doublet has an isotropic transition with \( g' = 4.826 \), and so an isotropic transition with this value of \( g' \) is characteristic of ferric ion in a crystal field of 'completely' rhombic symmetry. Notice that our ordering of the doublets in this discussion depends upon an assumption that \( D \) is positive. There is no reason that this should be so, if \( D \) is negative, then the order of the doublets is inverted.

B. Results and Discussion

There have been three previous reports of epr spectra of ferritin (Ishida and Tachimori, 1968; Boas and Troup, 1971; and Hanjan et al., 1972). Ishida and Tachimori investigated dried powder samples of horse spleen ferritin. They report that the spectra are different depending on the method of drying employed and they report effective \( g \) values of 6.4, 2.06, 2.0, and 0.86 in one sample and a single line at \( g' = 2.0 \) in another. They also report a spectrum of \( \gamma\text{-FeOOH} \) powder which exhibits a strong line at \( g' = 2 \) and a weaker, sharp line at \( g' = 4 \). Boas and Troup report that for dried polycrystalline ferritin from horse spleen they observe a broad resonance at \( g' = 2 \) and another, weaker broad resonance at \( g' = 4 \). They also investigated the temperature dependence of these resonances at both X band and Q band. They report that spectra of various molecular weight fractions of ferritin are similar with the intensity of the \( g' = 2 \) line dependent on iron content. Hanjan et al., report the spectrum of lyophilized human liver ferritin to consist of a single sharp line at \( g' = 1.982 \).
A spectrum of ferritin in a frozen saline solution is shown in figure 9. We observe a comparatively sharp line with $g' = 4.27$ and a much broader line at $g' = 2$ as well as an absorption at high field (near $g' = 0.67$). A spectrum of the same sample at room temperature is shown in figure 10. As can be seen, it consists of a single broad line at $g' = 2$ which is much more intense than the same line at $-105^\circ C$. If we add small amounts of Fe$^{3+}$ (as Fe(NO$_3$)$_3$) to apoferritin in 100 mM acetate buffer (pH 5.25) we observe no spectrum at room temperature but upon freezing, we observe a sharp line at $g' = 4.27$. If the solvent is diluted 1:1 with glycerine (in order to inhibit the formation of microcrystals and so assure good glass formation) we observe this line to have a width of 30 gauss at $-104^\circ C$ and to be somewhat asymmetric (see figure 11). We have also noted that some samples of ferritin may not exhibit the $g' = 4.27$ line.

Magnetic susceptibility studies of ferritin show that ferric ions in the core microcrystal are antiferromagnetically coupled (Schoffa, 1965) with a magnetic order transition at $20 \pm 3^\circ K$ which Schoffa interpreted as the Neél temperature. A Mössbauer study of ferritin also showed it to have a magnetic order transition below 30$^\circ K$ but a concurrent study of the field dependence of the magnetic susceptibility showed the Neél temperature to be about 200$^\circ K$ (Blaise et al., 1965). The transition below 30$^\circ K$ is a feature of magnetically ordered particles of small size (<100 Å diameter) and is a result of a property called superparamagnetism. In the case of the ferritin core, not all of the spin angular momentum is removed by the antiferromagnetic exchange interaction because the large surface to volume ratio of the core
Fig. 9. An EPR spectrum of unfractionated ferritin in 0.15 M NaCl solution (as obtained directly from Polysciences, Inc.) Temperature is -105°C; modulation amplitude is 6.0 G. Magnetic field sweep shown from 0 to 10000 G.
Fig. 10. An EPR spectrum of the same sample as used for figure 9, except that the temperature is 22°C. Other conditions are the same.
Fig. 11. Enlargement of the $g' = 4.27$ line of figure 9. Modulation amplitude is 6.0 G. Temperature is $-104^\circ$C. Magnetic field sweep is from 1000 to 2000 G.
results in many of the ferric spins remaining unpaired as a result of edge effects. This effect leads to a magnetization associated with the entire particle, or in this case the magnetization is associated with the entire ferritin core. At temperatures for which the anisotropy energy of magnetization is less than or equal to the thermal energy, \( kT \), a collection of such particles will have some properties similar to those of a collection of paramagnetic particles. When the temperatures is lowered there is a phase transition when the anisotropy magnetization energy is of the order of \( kT \). The temperature at which this occurs is called the blocking temperature (Kneller, 1966) and is about 200 K in ferritin. Between this blocking temperature and the Neél temperature the ferritin cores behave like a system of paramagnetic resonance. It has been shown that a system of superantiferromagnetic particles should have an effective \( g \) factor of 2, and that the line width of the resonance should vary as \( \exp(KV/kT) \) where \( K \) is the anisotropy energy per unit volume, \( V \) is the particle volume, \( k \) is Boltzman's constant, and \( T \) is absolute temperature (d'Aubigné and Winger, 1962).

As mentioned above, Boas and Troup (1971) have carried out a complete temperature dependent linewidth study of the \( g' = 2 \) line in ferritin and they find that this resonance follows the predicted dependence. Therefore, the \( g' = 2 \) resonance line of ferritin can be completely explained as a feature of the superparamagnetic nature of the ferric oxyhydroxide microcrystal.

The line at \( g' = 4.27 \) is not a result of this property, however. As noted above such a line is characteristic of high spin Fe\(^{3+} \) in a site of near rhombic symmetry (see figure 7). Boas and Troup also
observed such a transition, but they found it to be very broad. They postulated that such broadening was due to a magnetic interaction with the ferritin core. We have found that if ferritin is lyophilized it will exhibit a broad line at $g' = 4.3$, but we suspect that the broadening is a result of loss of water molecules essential to protein structure giving an inhomogeneity in these iron binding sites upon drying. The sharpness of the line that we observe in frozen glasses indicates that the ferric ion which gives rise to it is well removed from the ferric oxhydroxide core. We have also found that this resonance is not observed at $4.2^\circ K$, which is to be expected since it arises from a thermally populated doublet. The width of the line at $g' = 4.27$ as well as its effective $g$ value indicate that for this site $\lambda = 0.325$ (Wickman, 1964). From Wickman we have the following values for $g'$:

<table>
<thead>
<tr>
<th></th>
<th>lower doublet</th>
<th>middle doublet</th>
<th>upper doublet</th>
</tr>
</thead>
<tbody>
<tr>
<td>$g_x'$</td>
<td>0.90347</td>
<td>4.2839</td>
<td>0.81264</td>
</tr>
<tr>
<td>$g_y'$</td>
<td>9.6583</td>
<td>4.2393</td>
<td>0.58102</td>
</tr>
<tr>
<td>$g_z'$</td>
<td>0.63421</td>
<td>4.3317</td>
<td>9.6975</td>
</tr>
</tbody>
</table>

From this table we see that there should be an absorption edge near $g' = 9.6$—as is seen in figure 9. We also might expect to see evidence of absorption edges near $g' = 0.9, 0.6, 0.8,$ and $0.7$. However, these fall in a region where the cavity used has a false resonance and at any rate are probably too broad to be observed above $1^\circ K$ (Wickman, 1964; Spencer, 1965). Finally, the unusual line shape of figure 11 appears to be the result of a resonance with $g' = 4.3$ but a line width of roughly 200 gauss underlying the sharp resonance. It probably
represents iron in a site of rhombic crystal field symmetry which differs from the site giving rise to the sharp resonance--perhaps in coordination of water molecules.

As we can see from figure 8, the $g'$ value for the transition in the central Kramer's doublet is quite isotropic. As a result, we should expect to see this transition in solution at room temperature. The fact that we do not indicates that the ferric ion in this site is in rapid equilibrium with iron in some other magnetic environment resulting in an averaged spectrum which is too broad to be observed. This indicates that the ferric ion giving rise to this resonance is rather loosely bound to the protein. This observation is in agreement with other experiments that show that ferritin contains loosely bound iron (Mazur et al., 1954; Jones and Johnston, 1967; Dognin et al., 1973; Hoy et al., 1974). The fact that identical spectra of this 'loosely bound' ferric ion are obtained with both ferritin and apoferritin indicates that it is associated with the polypeptide and not with poorly crystalline areas or incomplete layers of the microcrystal as Harrison and coworkers have suggested (Harrison et al., 1974).
V. MANGANESE BINDING

A. Introduction

The most stable oxidation state of manganese is Mn(II), or manganous ion, which has a 3d⁵ electronic configuration. In crystal fields of either octahedral or tetragonal symmetry the manganous ion is usually high spin with a ⁶A ground electronic state. As in the case of ferric ion, which is isoelectronic with manganous ion, the relaxation properties are such that epr spectra of Mn(II) can usually be observed at high temperatures. If the zero field splitting of the Kramer's doublets is not too large, we might expect five fine structure transitions (see figure 12). Furthermore, the isotope ⁵⁵Mn, which is 100% naturally abundant, has a nuclear spin of ⁵/₂. The resulting hyperfine interaction should split each fine structure transition into six hyperfine transitions. Since the ground state of the free ion possesses no electronic orbital momentum to first order, the g values for coordinated manganous ion are very close to those of the free electron.

In aqueous solution, manganous ion is complexed to six water molecules. This complex has an average octahedral crystal field symmetry, and only has small dynamic zero-field splittings (D = 0.005 cm⁻¹, see McGarvey, 1957). As a result of these small splittings, the fine structure transitions are broadened and they overlap at X band frequencies. Consequently, the spectrum of hexaquomanganous ion consists of six well resolved lines with result from the hyperfine interaction. The fine structure transitions are not completely superimposed because of incomplete Paschen-Back effect however, and the result is a variation in line width among the six hyperfine lines.
Fig. 12. Fine structure splittings in manganous ion in a crystal field of axial symmetry. The transitions indicated by the vertical lines are all superimposed in one of the hyperfine lines in figure 13. The fact that they occur at slightly different magnetic fields results in line broadening of those hyperfine lines. This figure is for the external magnetic field applied along the axis of tetragonal distortion. Kets indicate the different $M_s$ states.
(see figure 13). If manganous ion is bound to ligands which reduce the symmetry of the crystal field, the zero field splitting increases and the mismatch in the superposition of the fine structure transitions becomes greater, resulting in an apparent increase in the linewidth of the epr spectrum. In practice, solution spectra of manganous ion coordinated to such ligands have spectra so broadened by this effect that they essentially disappear (see figure 13).

Another useful feature of the Mn(H\textsubscript{2}O\textsubscript{6})\textsuperscript{2+} spectrum is that the height of a derivative peak from minimum to maximum is a linear function of the concentration of this species in solution. As first noted by Cohn and Townsend (1954), these two facts can be used to study the binding of manganous ion to ligands, including proteins, using epr spectroscopy. Since any bound Mn\textsuperscript{2+} has essentially no spectral intensity, one need only know the total manganese concentration, measure a peak height in the Mn(H\textsubscript{2}O\textsubscript{6})\textsuperscript{2+} spectrum to get free managanese concentration, and one can calculate the binding constant for the metal ion.

B. Experimental

The interaction of manganous ion with apoferritin was studied at two different pH values. One pH used was 5.5, maintained with 100mM acetate buffer, the other was pH 6.0 maintained with 50mM PIPES--HCl buffer. Total concentrations of Mn\textsuperscript{2+} (from MnSO\textsubscript{4}) were in the range 24 \textmu\text{M} to 2400 \textmu\text{M}. Apoferritin concentrations used were 11 \textmu\text{M} (264 \textmu\text{M in subunits) and 24 \textmu\text{M (590 \textmu\text{M in subunits) at pH 5.5 and pH 6.0, respectively. All spectra were obtained with samples in the aqueous flat cell (Wilmad WS-812) with a sample volume of 400 \textmu\text{l.}
Fig. 13. Upper curve: EPR spectrum of Mn(H2O)62+ in aqueous solution at room temperature. Modulation amplitude is 6.0 G. Magnetic field sweep is from 2900 G to 3900 G. The peak indicated by the vertical line is the one used to determine concentrations of free manganous ion as explained in the text. Lower curve: EPR spectrum of the same sample as above except that excess EDTA has been added and the phase detector gain has been increased by a factor of 10.
The spectrometer was calibrated by observing spectra of standard manganous solutions and then plotting peak height of the $m_I = \frac{1}{2}$ peak (see figure 13) vs. Mn(H$_2$O)$_6^{2+}$ concentration for various standard gain settings.

Experimental samples were prepared by mixing 200 µl of a MnSO$_4$ solution of known concentration with 200 µl of apoferritin solution in the same buffer in the flat cell in order to avoid loss of sample in any transfer. Spectra of these samples were recorded and the concentration of free hexaquomanganous ion determined with the use of the calibration curves.

C. Results and Discussion

If the manganous ion binds to one type of site on the protein with an association constant $K$ and a site concentration of $n$ per subunit then:

$$K = \frac{[\text{Mn}]_b}{[E][\text{Mn}]_f}$$

where $[\text{Mn}]_f$ is the free manganous ion concentration

$[\text{Mn}]_b$ is the concentration of bound manganous ion

$[E]$ is the concentration of empty metal binding sites.

Also:

$$[E] = n[S] - [\text{Mn}]_b$$

where $[S]$ is the concentration of protein subunits.

Manipulation of these equations gives:

$$\frac{[\text{Mn}]_b}{[S][\text{Mn}]_f} = nK - \frac{[\text{Mn}]_b}{[S]}$$
or with the obvious change in notation after Scatchard et al. (1957):

\[
\frac{\bar{v}}{a} = nK - \bar{v}K
\]

So a plot of \(\frac{\bar{v}}{a}\) vs \(\bar{v}\) will give a straight line with slope \(K\) and intercept \(nK\). Our data are plotted in figures 14 and 15 as Scatchard plots. The fact that we do not obtain straight lines indicates that the above equations are not obeyed and we most likely have more than one type of binding site for manganous ion.

If we assume that manganese can bind to two independent sites on the apoferritin, we can, in a manner similar to that outlined above, obtain the equation:

\[
\bar{v} = \frac{n_1K_1a}{1+K_1a} + \frac{n_2K_2a}{1+K_2a}
\]

where the subscripts refer to binding sites one and two. A nonlinear least squares fit of this equation to the data then gives values of \(n_1, n_2, K_1, K_2\). The values of these parameters found by this procedure are:

<table>
<thead>
<tr>
<th>pH</th>
<th>(n_1)</th>
<th>(n_2)</th>
<th>(K_1)</th>
<th>(K_2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.5</td>
<td>0.21</td>
<td>8.9</td>
<td>1740</td>
<td>2.5</td>
</tr>
<tr>
<td>6.0</td>
<td>0.2</td>
<td>10</td>
<td>85000</td>
<td>160</td>
</tr>
</tbody>
</table>

Curves calculated with these parameters are plotted in the Scatchard plots of figures 14 and 15, showing the fit to four parameters to be quite good.
Fig. 14. Scatchard plot of the manganese binding data. Solid curve is calculated using the parameters shown, which were obtained from a nonlinear least squares fit of the data.
Fig. 15. As in figure 12, but for the data taken at pH 6.0. Note that the units of the abscissa should be 1/M, not 1/μ.
A closer examination of our spectra reveals that the manganous spectrum is slightly broadened in the presence of apoferritin. This broadening is independent of concentration (for the concentration range studied) and amounts to some 12% of the linewidth in manganous sulfate solutions. This broadening must account for some of the observed loss of peak height. If we make the simple assumption that the product of peak height and width is proportional to the amount of free Mn$^{2+}$, we can calculate a 'corrected' peak height. From this we can calculate a 'corrected' concentration of free manganous ion. If we then recalculate the parameters we find:

<table>
<thead>
<tr>
<th>pH</th>
<th>$n_1$</th>
<th>$n_2$</th>
<th>$K_1$</th>
<th>$K_2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.5</td>
<td>0.2</td>
<td>6</td>
<td>1500</td>
<td>2</td>
</tr>
<tr>
<td>6.0</td>
<td>0.2</td>
<td>8</td>
<td>65000</td>
<td>150</td>
</tr>
</tbody>
</table>

The most striking feature of both of these results is the low value found for $n_1$. The value of 0.2 indicates that one tight manganese binding site exists for every five apoferritin subunits. To explain this, within the assumptions made here, means we must postulate a heterogeneity in the apoferritin which allows 20% of the subunits to differ from the remainder or else we must postulate that Mn$^{2+}$ binds at a site which involves the interaction of five protein subunits. The latter postulation seems very unlikely on the face of it and the 6 Å resolution study of the protein (Hoare et al., 1975b) shows apoferritin to contain no five-fold axes. Heterogeneity of apoferritin subunits has been reported with as many as 10% of the subunits being different from the rest (Ishitani et al., 1975). Collet-Cassart and Crichton (1975) argue that such heterogeneity is a result of thermal
degradation of the protein during isolation. They report that under carefully controlled conditions less than 5% of the subunits will differ from the rest with a "considerable increase" in that percentage in less well controlled preparations. We have observed during titration studies with Cu$^{2+}$ that the first two to three cupric ions per apoferritin molecule bind anomalously and tightly (see chapter 6). This would indicate that at least 10% of the apoferritin subunits differ from the rest. Whatever the origin of this inhomogeneity in the apoferritin subunits, it probably accounts for the observed tightly bound manganous ion which results in the loss of manganous per spectral intensity.

Manganous ion binding to ferritin has also been investigated by Macara et al. (1973c) by following the displacement of protons from the protein upon addition of the metal ion. They find at pH 5.5 that $n_1 = 0.5$, $n_2 = 2$, $K_1 = 2000$, and $K_2 = 20$. They also find that each manganous ion will displace only one proton from the protein.

Comparison of the results of Macara et al., with ours would seem to indicate that the manganous-apoferritin interaction is somewhat unusual (if we neglect that portion of the ion bound to differing subunits). The ion can displace protons from apoferritin and yet remain unbound with very little distortion of its crystal field. In fact, the only effect that the ion-apoferritin interaction has on the epr spectrum is to broaden lines. Such broadening is probably a result of small distortions in the crystal field of the ion which result in increased zero field splittings (McGarvey, 1957).

Distortions of the crystal field may be a result of the presence of counter ions (such as sulfate) in the first and second coordination
spheres of the ion (Hayes and Myers, 1964). Silk and Breslow (1976) have found apoferritin, but not ferritin, to bind chloride ion and it is possible that there is a large concentration of anions in the protein interior under our experimental conditions (in this case $SO_4^{2-}$ may well be present). Such small anions could interact with manganous ion there and broaden its spectrum. Alternatively, $Mn^{2+}$ may interact with carboxylate groups on the apoferritin via an outer sphere mechanism. Such an interaction would be expected to give a 'solution-like' spectrum with some broadening as is observed. Wetz and Crichton (1976) have found the interior surface of apoferritin to be lined with 96 carboxylate groups.

In the absence of distortions in the crystal field of the manganous ion produced by counter ions, line broadening is a result of simple distortions in the inner sphere of water molecules surrounding the ion. Such distortions are a function of viscosity of the medium, with broadening increasing as viscosity increases. Such broadening of manganous epr lines has been observed in studies of epr spectra of aqueous manganese perchlorate solutions as a function of temperature (Hayes and Myers, 1964; McCain and Myers, 1968). In other words, the broadening observed here may be a result of an apparent increase in the viscosity of water in the apoferritin inner cavity.

Effects similar to those reported here have been observed with manganous ion interacting with cation exchange sites in $\gamma$-alumina and silica gel (Burlamacci et al., 1976). Manganous ion absorbed in the cavities (of ca. 10 Å diameter) of these materials has a 'solution-like' spectrum with lines broader then those of the free ion in solution.
In the case of hexaquomanganous ion interacting with the cation exchange sites of γ-alumina, the line width increases by 9%. For the interaction of silica gel, line width increases by 35%. In both cases, the manganous ion appears to remain coordinated to six water molecules and this complex interacts with multiple cation exchange sites in the cavities of the polyelectrolytes. The obvious implication is that the similar spectral effects in the apoferritin/Mn$^{2+}$ system arise for similar physical reasons, i.e., hexaquomanganous ion is in the apoferritin interior interacting with multiple cation exchange sites there. Some of these sites presumably contain H$^+$ in the absence of metal ion, and these protons are displaced and observed by Macara et al., when metal ions are added. We will discuss this model of apoferritin--metal ion interaction further in later chapters.
VI. COPPER BINDING

A. Introduction

Cupric ion has been used as a probe species in several studies of metalloenzymes. For example, it has been used to replace Zn$^{2+}$ in carboxypeptidase (Målmström and Vanngard, 1960) and in carbonic anhydrase (Lindskog and Malmstrom, 1962) and it replaces ferric ion in transferrin (Aasa et al., 1963; Aasa and Aisen, 1968). Cupric ion also has several properties which make its electron paramagnetic resonance spectroscopy particularly informative. Since Zn$^{2+}$ is known to inhibit both iron uptake and release in ferritin (Niederer, 1970); Cu$^{2+}$ seemed to have potential as a probe to the iron binding site we know apoferritin to contain (see chapter 3). With this motivation, a study of copper binding to apoferritin and ferritin was undertaken. As we show below, copper binds to a site distinct from any iron binding site and it now appears that this copper binding site could have great significance for the mobilization of iron from ferritin in vivo.

B. Properties of Copper Ions

Copper usually exists in either of two oxidation states: Cu$^+$ (cuprous) or Cu$^{2+}$ (cupric) (however, we note that there have been recent reports that the Cu$^{3+}$ ion may play important roles in biological systems (Dyrkacz et al., 1976)). The cuprous ion is a $3d^{10}$ species and hence is diamagnetic and so is epr inactive. Cuprous ion is normally colorless but can be yellow or red as a result of charge transfer absorptions (Cotton and Wilkinson, 1966). Because Cu$^+$ disproportionates in aqueous solution little is known about its interactions with amino...
acids or small polypeptides. This instability is due, in large part, to the strong solvation of cupric ion by water and is not a result of some intrinsic instability of the cuprous ion (Österberg, 1974).

Cupric ion is a $3d^9$ system and can be regarded as a single hole in the $d$ orbitals. Empirically, the most common coordination found for Cu$^{2+}$ is octahedral with a strong tetragonal distortion leaving the axial ligands well removed from the copper center and so giving essentially square planar coordination. Octahedral coordination splits the $d$ orbitals into two sets of different energy (see figure 16). The upper set ($e_g$) consists of the $d_{x^2-y^2}$ and the $d_{z^2}$ orbitals. The further tetragonal distortion splits these two and the weak interaction with the axial ligands usually leaves the $d_{z^2}$ orbital lower in energy since the electrons in that orbital are further removed from the negative charges of the ligands than are the electrons in the $d_{x^2-y^2}$ orbital (or, alternatively, the positively charged hole is attracted to the negatively charged ligands and so resides in the $d_{x^2-y^2}$ orbital). Either way, the result is that the unpaired electron spin density lies primarily in the $xy$ plane.

Because there is only one unpaired electron in Cu$^{2+}$, the ion has a net spin of 1/2 and for the case of tetragonally distorted coordination, it has an orbitally nondegenerate ground state. Since there are also no spin-orbit matrix elements connecting the $d_{z^2}$ and $d_{x^2-y^2}$ orbitals, the ion is a Kramer's ion with little orbital angular momentum and we expect to see epr transitions with $g$ values near that of the free electron. Copper occurs naturally as two isotopes, $^{63}\text{Cu}$ (69.09%) and $^{65}\text{Cu}$ (30.91%), both of which have a nuclear spin of 3/2. Since
Fig. 16. The orbitals and states of cupric ion $3d^9$. The tetragonal distortion shown is for elongation of the octahedron of ligands along one axis.
their gyromagnetic ratios are nearly identical (7.09 x 10^3 rad/G vs. 7.60 x 10^3 rad/G, respectively) they interact with the unpaired hole in a nearly identical fashion, giving rise to an almost equal hyperfine splitting of the electron resonance. The observed elements of the g tensor and the hyperfine coupling tensor (A tensor) can be used to provide qualitative information about the ions binding the cupric ion in the xy plane. Cupric ion has the further interesting property that its bonding is somewhat covalent leading to a considerable unpaired hole density on the ligands. Interaction with ligand nuclei possessing nonzero spin (especially nitrogen) can lead to further splittings (called ligand hyperfine or superhyperfine splittings) which yield further information about the identity of the ligands (vida infra).

The spectroscopy of copper in biological systems as well as in model systems has been extensively reviewed (Vännärd, 1972; Malkin and Malmström, 1970; Österberg, 1974). We will here summarize some of its most general features. Copper occurring naturally in proteins has been classified as one of three types: the type 1, or 'blue' copper; the type 2, or 'nonblue' copper; and the so-called 'epr-nondetectable' copper. This classification is based on both epr and optical spectroscopic properties. For example, the type 1 copper has unusually low values of A_1 (smaller than 100 G) and extremely large extinction coefficients for absorption in the visible in comparison to most inorganic copper complexes. Type 2 copper has A_1 values larger than 130 G (similar to that of most low molecular weight copper complexes) and normal (again relative to low molecular weight complexes) extinction
coefficients. 'EPR-nondetectable' copper centers are diamagnetic and exhibit a strong absorption band near 330 nm.

The 'blue' copper proteins have received the greatest amount of attention chiefly as a result of their intense blue color. This strong absorption is now generally believed to be due to a charge transfer to a sulfur ligand (Solomon et al., 1975). The overall coordination geometry appears to be distorted tetrahedral (Siiman et al., 1976) with one ligand being a backbone peptide nitrogen (Hare et al., 1976; Ugurbil et al., 1977). The copper center in these proteins is generally believed to participate in electron transport through outer sphere mechanisms since the blue copper center is not accessible to the medium. Also, these copper centers are not readily oxidized by molecular oxygen.

The type 2 copper center is usually found in conjunction with a type 1 center. As a result its properties have not been so thoroughly investigated as those of the type 1 center. The spectroscopy of such centers is in no way unusual, for example, galactose oxidase, which contains only a type 2 copper, has \( g_{xx} = 2.058 \), \( g_{yy} = 2.048 \), \( g_{zz} = 2.273 \), \( A_{xx} = -28.8 \) G, \( A_{yy} = -30.1 \) G, and \( A_{zz} = -176.5 \) G. \( \text{N,N'} \)-ethylenebis (trifluoroacetylacetoniminato) copper(II):

\[
\begin{array}{c}
\text{N} & \text{Cu} & \text{N} \\
\text{O} & \text{CF}_3 & \text{O} \\
\text{O} & \text{CF}_3 & \text{N}
\end{array}
\]

has been proposed as a model compound for this enzyme on the basis of its similar epr parameters (Giordano and Bereman, 1974). Type 2
copper centers occur in oxidases and in copper storage proteins. In the oxidases they have a high affinity for anions (Malkin and Malmstrom, 1970) and are accessible to the medium. These centers are thought to either participate in electron transport or to stabilize intermediates such as peroxide in redox reactions since those oxidases containing only type 2 copper reduce oxygen to hydrogen peroxide.

The mechanism by which these proteins oxidize their substrates is a matter of some debate. Early evidence from observation of epr spectra of galactose oxidase (Blumberg et al., 1965), diamine oxidase (Mondovi et al., 1967), benzyl amine oxidase (Buffoni et al., 1968), and amine oxidase (Yamada et al., 1969) seemed to indicate that the type 2 copper center in these proteins was not reduced in the presence of reducing substrate under anaerobic conditions, although addition of dithionite to the benzylamine oxidase did reduce that copper center. This was taken as evidence that the cupric ion was not involved in the electron transport. At about the same time studies on 3,4-dihydroxyphenylethylamine oxidase showed that the type 2 copper in this system was reduced by ascorbate and then reoxidized during the hydroxylation of substrate (Blumberg et al., 1965b; Friedman and Kaufman, 1966). Also, a later study of diamine oxidase (Mondavi et al., 1969) indicated that the copper in this enzyme is slowly reduced in the presence of its substrate. More recent research on the mechanism of galactose oxidase indicates that the active forms of copper in the protein are Cu(III) and Cu(I) with the Cu(II) complex existing as an inactive 'resting' form which must be oxidized to activate the enzyme (Dyrkacz et al., 1976). Although this idea has been challenged and a mechanism which
involves a cupric cysteine ligand in redox reactions has been proposed (Cleveland et al., 1975). Overall then, initial evidence seemed to indicate that type 2 copper, while essential for enzyme activity, plays a structural role. New findings indicate that this copper is actually involved in electron transport and undergoes oxidation and reduction at some point in the catalytic cycle; although the exact oxidation states through which it passes are not necessarily known.

All 'synthetic' copper proteins, i.e., proteins in which copper replaces some other native metal ion, have copper centers which may be classified as type 2.

The 'epr-nondetectable' copper could consist of two types--isolated cuprous ions or pairs of antiferromagnetically coupled cupric ions. The term is now generally taken to mean the latter type of system. These centers occur in the presence of type 1 and/or type 2 copper centers and are involved in redox reactions which involve the simultaneous transfer of two electrons.

C. Experimental

Copper was bound to ferritin and apoferritin by one of two methods. In the first, the protein was dialysed against 100 mM acetate buffer at pH 5.5 overnight. The copper (as cupric sulphate) was then added in excess to the dialysis vessel. After the copper concentration was equilized (usually overnight) buffer solution of the required pH was added and the excess copper dialysed away. This procedure prevented the precipitation of Cu(OH)₂ inside the dialysis bag even when the final pH was over 8. In the second procedure, copper (as cupric sulphate
solution) was added directly to a solution of either ferritin or apoferritin. No precipitation of copper hydroxide was noted at any pH if this addition was slow enough and in small enough quantities (vida infra). Titrations at room temperature were carried out directly into the aqueous flat cell fitted with a serum cap. The solution was withdrawn through this cap into a syringe where it was mixed and then readded to the flat cell. Amounts of copper bound to ferritin and apoferritin were determined with atomic absorption by staff of the University of California College of Chemistry analytical laboratory.

Kinetics of iron release from ferritin were investigated at 22⁰ in the presence and absence of copper bound to the protein. The ferritin used was all from one lot of cadmium free protein. A sample of ferritin/Cu was prepared by the first method described above, while a second sample of ferritin was subjected to the same dialysis procedures but without the addition of copper. EPR spectroscopy was used to ascertain that copper was present only in the normal site in the first preparation and not observable in the second. These samples were both prepared in 50 mM HEPES buffer at pH 7.5. Each solution was diluted to give an absorbance of 1.0 at 420 nm (the absorption at this wavelength is due entirely to the ferric oxyhydroxide core). At time = 0 a sample of freshly prepared solution of reductant (either ascorbic acid or sodium dithionite) was added to the cuvette by syringe. The cuvette was capped, inverted three times to mix the contents, and replaced in the spectrometer (a Cary 14). The decay of absorption was then followed as a function of time. Final reductant concentration was 43 mM. Similar experiments were carried out to investigate the effects of
other metal ions. In these cases, metal ion was added to the cuvette (which contained only buffered ferritin solution) before the reductant was added.

D. Results

1. Copper Tight Binding

Spectra of copper complexes of ferritin and apoferritin are shown in figure 17. As can be seen, the spectra of ferritin/Cu\(^{2+}\) and apoferritin/Cu\(^{2+}\) at room temperature are identical. They show the copper to be bound in a site of axial or near axial crystal field symmetry with measured epr parameters of \(g_{\|} = 2.29\), \(g_{\perp} = 2.07\) and \(A_{\|} = -145\) G. These values are characteristic of a type 2 or 'nonblue' copper center. Freezing the solution results in some overall changes in this spectrum as also seen in figure 17. The lines sharpen somewhat and we find \(g_{\|} = 2.29\), \(g_{\perp} = 2.07\) and \(A_{\|} = -161\) G. This again is a typical type 2 copper spectrum. The changes are due to a loss of motional averaging as the solution is frozen and the ferritin molecules are locked into a particular orientation with respect to the externally applied magnetic field. If we calculate the rotational correlation time of ferritin using the equation of Brownian diffusion:

\[
\tau_R = (4\pi a^3/3kT) \eta
\]

where \(a\) is the hydrodynamic radius (taken as 60 Å) and \(\eta\) is the viscosity of the medium (0.01002 poise at 20°C), we find \(\tau_R = 2 \times 10^{-7}\) seconds, or \(1/\tau_R = 4 \times 10^6\) sec\(^{-1}\). If we calculate the electron resonant frequencies at constant field we find \(\nu_{\parallel} = 9.120\) GHz and \(\nu_{\perp} = 9.096\) •
Fig. 17. Top Spectrum: EPR spectrum of ferritin/Cu$^{2+}$ in 50 mM HEPES buffer, pH 7.5; temperature is 22°; modulation amplitude is 6.0 G. Magnetic field sweep is from 2500 G to 3500 G at a frequency of 9.4308 GHz. Parameters measured are: $g_\parallel = 2.29$, $g_\perp = 2.07$, and $A_\parallel = -145$ G. Middle Spectrum: EPR spectrum of apoferritin/Cu$^{2+}$ in 50 mM HEPES buffer, pH 7.5; temperature is 22°; modulation amplitude is 6.0 G. Magnetic field sweep is from 2500 to 3500 G at a frequency of 9.4316 GHz. Parameters are the same as those of ferritin/Cu$^{2+}$, above. Bottom Spectrum: EPR spectrum of apoferritin/Cu$^{2+}$ in 50 mM HEPES buffer, pH 7.5; temperature is -105°; modulation amplitude is 6.0 G. Magnetic field sweep is from 2400 to 3400 G at a frequency of 9.1108 GHz. Parameters measured are $g_\parallel = 2.29$, $g_\perp = 2.07$, and $A_\parallel = -161$ G.
GHz giving $\Delta \omega = 151 \times 10^6 \, \text{sec}^{-1}$. This is sufficiently different from $1/T_R$ that we can expect what we observe at $20^\circ\text{C}$--a 'glass-like' spectrum with some motional averaging and broadening. In other words, the cupric ion at room temperature is tightly bound to the protein--it is not in equilibrium with any appreciable concentration of hydrated copper complex (we see no spectrum from any free copper species). We have also observed that dialysis of apoferritin/Cu$^{2+}$ against daily changes of buffer for a period of a week does not appreciably reduce copper content of the protein as determined by atomic absorption. Thus we are observing a site which binds copper very tightly.

We also observe that this metal ion binding site is very specific for copper. Experiments using Co$^{2+}$ as a probe result in the usual copper spectrum even though the copper impurity in the reagent grade cobalt sulfate used is only 0.002%. Similarly, titration studies with ferric ion (as ferric nitrate) also produce the typical apoferritin/Cu$^{2+}$ spectrum even though the copper is present in the ferric salt at only the 0.003% level. Thus in the presence of several orders of magnitude greater concentration of either divalent or trivalent metal ions, the protein will selectively bind copper in this site.

If we add imidazole to the apoferritin/Cu$^{2+}$ complex we see a change in color from light green to light blue almost immediately. There is also a change in the epr spectrum to that shown in figure 18. The additional splittings observed are a result of couplings between the spin of the unpaired copper hole and the nuclear spin of $^{14}$N atoms ($I=1$) coordinated to the metal ion. A simulation of this spectrum using the program RHOM described in chapter 3 is also shown as the
Fig. 18. Upper Curve: Simulated EPR spectrum using the program RHOM and the parameters $g_x = 2.05, g_y = 2.06, g_z = 2.26, A_x = -30 \text{ G}, A_y = -30 \text{ G}, A_z = -175 \text{ G},$ and $A_W = -15 \text{ G}$. The superhyperfine splitting is a result of calculated interaction of the unpaired hole with four equivalent nitrogens. Lower curve: Experimental EPR spectrum of apoferritin/Cu$^{2+}$/imidazole in 50% 19 mM imidazole buffer, pH 7.5; 50% glycerine. Temperature is $-125^\circ$; modulation amplitude is 3.0 G; magnetic field sweep is 2400 G to 3400 G at a frequency of 9.110 GHz.
upper curve of figure 18. The spectral parameters thus obtained are:

\[ g_x = 2.05, \quad g_y = 2.06, \quad g_z = 2.26; \quad A_x = -30 \text{ G}, \quad A_y = -30 \text{ G}, \quad A_z = -175 \text{ G}, \]

and \( A_N = -15 \text{ G}. \) Simulations using axially symmetric g tensors are not capable of reproducing the experimental spectrum. Also, one must assume that the cupric ion is bound to four nitrogenous ligands in order to duplicate the observed spectrum. Furthermore, if we titrate imidazole against the apoferritin/Cu\(^{2+}\) complex we find that after the imidazole/Cu\(^{2+}\) ratio exceeds 1, no further spectral changes are observed.

We note here that the experimental spectrum presented is of the naturally occurring mixture of copper isotopes. Spectra of apoferritin/\(^{63}\)Cu\(^{2+}\) are not significantly different.

Atomic absorption analysis of samples of apoferritin/Cu\(^{2+}\) show that the binding of copper is strongly pH dependent. At pH 5.5 we find only 2.1 cupric ions to be bound per apoferritin molecule. At pH 7.5, however, we find 47.5 ions per molecule or essentially 2 per protein subunit. We have further observed that if apoferritin is loaded with copper at pH 7.5 and the pH is then lowered to pH 5.5, by the addition of HCl solution the excess copper is released from this binding site.

It is also of interest to note that commercially supplied samples of ferritin can contain copper bound in this site. For instance, a sample obtained from Calbiochem (La Jolla, California) contained 10 copper atoms per molecule of protein, a sample from Miles Laboratories (Kankakee, Illinois) contained less than one copper per molecule of protein, and a typical sample of ferritin from Polysciences, Inc.
(Warrington, Pennsylvania) contained one copper atom per ferritin molecule.

Addition of sodium dithionite or ascorbic acid to a solution of apoferritin/Cu$^{2+}$ results in a color change from light green to reddish brown as well as a loss of the epr signal. Dialysis of this solution against buffer followed by saturation with O$_2$ will restore the original color as well as the epr signal. Clearly, the protein also binds cuprous ion (the red color, while unusual can be due to charge transfer transitions at the metal center, see Cotton and Wilkinson, 1966).

2. Kinetics of Iron Release

The results of the studies of the kinetics of iron release from ferritin with and without copper bound to it are shown in figure 19. The most apparent feature is the dramatic increase in the rate of iron release when copper is bound to the ferritin. In the case of release by ascorbate, the time for release of half the iron changes from approximately 100 hours for copper free ferritin to about 7 minutes when copper is bound to the protein. Another feature of the kinetics is that the reduction of iron by dithionite seems to be the result of an initial fast reduction followed by a slower step, while reduction of the ferritin/Cu has only one rate, as does the ferritin/Cu reduction by ascorbate. The second rate in the ferritin-dithionite system seems to be the result of buildup of ferrous ion concentration which inhibits the mobilization of more iron from the protein (see figure 20). We calculate that for an absorbance of 1.0 at 420 nm the iron concentration of the ferritin samples is about 2 mM (using $E_{1cm}^1$ = 100, Macara et al., 1972).
Fig. 19. Kinetics of iron release from ferritin. Absorbance is at 420 nm.

- ▲ iron release from copper free ferritin by 43 mM ascorbate
- + iron release from ferritin/Cu²⁺ by 43 mM ascorbate
- ○ iron release from copper free ferritin by 43 mM dithionite
- ● iron release from ferritin/Cu²⁺ by 43 mM dithionite

All in 50 mM HEPES at pH 7.5 and at 22°.
Fig. 20. Kinetics of iron release from ferritin under conditions above.
- iron release from ferritin by 47 mM dithionite
○ iron release from ferritin by 47 mM dithionite in the presence of 1.0 mM Fe²⁺
+ iron release from ferritin by 47 mM dithionite in the presence of 2 mM V²⁺
3. Copper Loose Binding

If one titrates copper against apoferritin a spectrum such as that of figure 17 is obtained for small amounts of added Cu\textsuperscript{2+} (that is, for amounts less than the total amount of copper bound at the 'tight-binding' site described. However, if additional copper is added, a spectrum such as the one in figure 21 is obtained. The arrows mark the underlying spectrum of cupric ion in the 'tight-binding' site. The additional line observed is due to cupric ion not bound to these sites. Figure 22 shows a spectrum of copper sulfate solution taken under the same conditions as figure 21, except that no apoferritin is present. The principle difference in the spectrum of the loosely bound cupric ion shown in Figure 21 and the spectrum of figure 22 is that the hyperfine structure is more clearly defined in the former. This can occur when the rotational correlation time of the copper complex is increased see Chang (1971). This could be accomplished either through coordination by small ligands or through an increase in the viscosity of the medium in which the complex rotates. We have also found that precipitation of the protein with (NH\textsubscript{4})\textsubscript{2}SO\textsubscript{4} produces no change in the spectrum of loosely bound cupric ion shown in figure 21. Since 'salting out' a protein results in protein-protein interactions that would surely affect the protein exterior, and since the spectrum we observe is significantly different from that of free Cu(H\textsubscript{2}O)\textsubscript{6}\textsuperscript{2+} under the same buffer conditions, we conclude that this solution-like copper spectrum is due to cupric ions within the apoferritin molecule. Since the x-ray crystallographic study of Hoare et al. (1975b) reveals no cavities in the molecule in which hexaquocupric ion could fit other
Fig. 21. EPR spectrum of apoferritin in the presence of excess amounts of cupric ion. Sample contains $2.3 \times 10^{-8}$ moles of apoferritin, $30 \times 10^{-8}$ moles of CuSO$_4$, and is in 100 mM acetate buffer at pH 5.25. Temperature is 20$^\circ$C. A 6.0 G modulation amplitude was used. Arrows mark the underlying spectrum of cupric ion in the copper tightbinding site. Notice that this spectrum has a 180$^\circ$ phase difference from those of figure 17 due to the tuning of the microwave bridge.
Fig. 22. Upper curve: EPR spectrum of 50 mM CuSO₄ in 50 mM acetate buffer pH 5.25. Taken under conditions of Fig. 21. Lower curve: EPR spectrum of the same sample as the upper curve, but with a concentration of ammonium sulphate of 750 mM which increases the ionic strength by a factor of ten.
than the central cavity, and since this spectrum is not observed when cupric ion is added to ferritin, we conclude that the specific location of these hexaquocupric ions is the central cavity of the apoferritin. Further addition of copper to the system results in a broadening of the hyperfine structure seen and precipitation of the protein. This latter effect is probably the result of saturation of the protein inner cavity and subsequent association of further amounts of copper with binding sites on the protein exterior where the ion can act as a salt bridge between protein molecules.

To get some idea of how much the rotational correlation time of the cupric ion complex has changed here, we compare our spectra to those of Chang (1971), which are reproduced here as figures 23 and 24 and compare them to our spectra in figures 21 and 22, respectively. The only difference in conditions in Chang's spectra is a difference of temperature and resultant change in the viscosity of the solvent, which is known. A calculation of the ratio of rotational correlation times in Chang's spectra by using the equation for Brownian diffusion above gives:

\[
\frac{\tau_R(22^\circ)}{\tau_R(-10^\circ)} = 0.33
\]

Thus by analogy, we find that we observe an apparent increase in the rotational correlation time for cupric ion associated with the interior of apoferritin, and this increase is such that the rotational correlation time of the associated cupric ion is three times that of free Cu(H_2O)_6^{2+} under the same conditions.
Fig. 23. EPR spectrum of Cu(H₂O)²⁺ at -10°. Crosses are experimental points and the solid curve is a theoretical fit to the data. From Chang, 1971, p. 61.
Fig. 24. EPR spectrum of $\text{Cu(H}_2\text{O)}_6^{2+}$ at 22°. From Chang, 1971, p. 63.
4. Copper Binding In Vivo

We have taken fresh horse spleen and isolated ferritin from it in a method similar to that of Granick (1946a) except that we have used buffer to maintain the pH at 7.0 or above. Specifically, the spleen (300 g) was homogenized with 750 ml of 100 mM Tris (pH 7.4) in a Waring blender. The homogenate was then heated to 80°C for ten minutes. The precipitate of denatured proteins was removed by filtration and centrifugation. Ferritin was then further purified from the mother liquor by centrifugation techniques (Penders et al., 1968) or by crystallization from 5% CdSO₄ (although this dropped the pH to 6.6 due to hydrolysis of the cadmium). Analysis by atomic absorption gave approximately one copper atom per ferritin molecule by the first procedure and 2.2 copper atoms per ferritin by the second. The discrepancy can be entirely accounted from by assuming that the ferritin prepared by the second method binds all of the copper present as an impurity in the cadmium sulfate used for the recrystallization.

E. Discussion

1. Properties of the Copper Tight Binding Site

There have been previous reports that the interaction of copper with ferritin is unique. In an investigation of metal ion-ferritin interactions, Rüssel (1970) examined the interactions of Cd²⁺, Zn²⁺, Pb²⁺, Cu²⁺, Tl⁺, and Ag⁺ with ferritin and synthetic ferric oxyhydroxide micelles. Of the ions studied, only cupric ion displayed an interaction with the polypeptide chain of ferritin (see chapter 2). Harrison and coworkers (Macara et al., 1973c) also investigated metal ion binding
to ferritin. They studied Zn$^{2+}$, Cd$^{2+}$, Mn$^{2+}$, Cu$^{2+}$, and Tb$^{3+}$ binding by observing the displacement of protons by these ions at pH 5.5. Cupric ion was unique in that it displaced two protons per ion bound. They interpreted their data as showing ferritin to contain two binding sites for copper per subunit (see chapter 2). This compares with our observation that copper is very selectively bound to the 'tight-binding' site, of which there are two such sites per subunit.

From computer simulation of the spectrum of figure 18, we find that the cupric ion binds in such a way as to have four nitrogenous ligands in a plane about it. Since the spectrum is split by the ligand hyperfine interaction only after the addition of imidazole, and since the addition of imidazole to solutions of apoferritin/Cu$^{2+}$ is accompanied by a color changed, we know that at least one of these ligands must be an imidazole nitrogen. However, the titration of imidazole against apoferritin/Cu$^{2+}$ shows that only one of the cupric ligands arises from the imidazole added. We therefore have three remaining nitrogenous ligands which must arise from amino acid residues. The only candidates are the imidazole nitrogens of histidine residues, ε-amino nitrogens from lysine residues, and the N-terminal nitrogen(s) of the polypeptide chain. While it has been mentioned that the binding of copper in the type 1 copper proteins has been found to involve a nitrogen from the polypeptide backbone, the epr parameters of apoferritin/Cu$^{2+}$ as well as its small extinction allow us to classify it as a type 2 copper protein, and we will assume that this unusual bonding is not to be found here. The possibility that a N-terminal nitrogen is involved in the binding rests on the outcome of the current debate on the heterogeneity of the
apoferitin. If the observed presence of two polypeptide chains per subunit is a result of thermal degradation during the heat treatment step during isolation of the protein as Collet-Cassart and Crichton (1975) have suggested then the fact that one N-terminal nitrogen is acetylated (Suran, 1966; Mainwaring and Hofmann, 1968) removes this group from consideration as a possible ligand for copper. On the other hand, if indeed the apoferitin subunit is composed of two polypeptide chains, one of which has an N-terminal nitrogen with a $pK_a = 7.5$ (Silk and Breslow, 1976) then this is a possible ligand. However, since only one such group would exist per 18,500 daltons and we see two coppers bound for that molecular weight, some assymetry in the two binding sites would be implied if such a group coordinated the cupric ion. We have never observed any evidence for nonequivalence of the copper binding sites. None-the-less, cupric epr can be somewhat insensitive to some types of changes in binding sites. For example studies of apotransferrin/Cu$^{2+}$ indicate that the two cupric ions bound by that protein are in identical sites (Aasa and Aisen, 1968) while studies of apotransferrin/Vo$^{2+}$ reveal a clear difference in the two sites (Cannon and Chasteen, 1975).

The imidazole nitrogens of histidine residues may also bind the copper ion. From the discussion in chapter two, we know that there are three titratable hisidine residues per molecular weight of 18,500. Two of these are found to titrate between pH 5.6 and pH 7.3, while one titrates at a higher pH (Silk and Breslow, 1976). Furthermore, these three histidine residues have identical reactivities with bromacetate in both ferritin and apoferitin, which is of importance since we observe
the copper to bind identically to the protein and the apoprotein. If we use a similar asymmetry argument, we could infer that the odd histidine does not bind copper. However, the insensitivity of cupric epr is again noted. Thus it is possible that one copper binding site in ferritin contains two histidine ligands while the other contains one histidine nitrogen and one N-terminal nitrogen as ligands.

Such a coordination can account for only four of an observed six nitrogenous ligands. The other nitrogens must arise from ε-amino nitrogens of lysine residues. In apoferritin, seven of these are titratable per molecular weight of 18,500 with a pK_a = 9.6 (Silk and Breslow, 1976). Of these, 3 to 4 are susceptible to chemical modification in ferritin (Wetz and Crichton, 1976). Modification of these lysine residues does not affect the catalytic activity of apoferritin and so they are not involved in the coordination of ferrous or ferric ions. Consequently, we have three or four lysine residues that could provide a nitrogen as a ligand in both ferritin and apoferritin.

In spite of the fact that the pK_a of the ε-amino nitrogen is high, it can complex cupric ion at pH < 8 (Hatamo et al., 1971). In fact, a study of complexes of Cu^{2+} with the side chains of N^α-dodecaneyl-L-lysine at the interface of an oil microemulsion gave very similar spectra to those obtained here with g_∥ = 2.26, g_⊥ = 2.07, A_∥ = -170 G, and A_N = -15 G with four coordinated ε-nitrogens. At room temperature the limited motion of these complexes resulted in an averaged spectrum which was still powder-like but with an apparent reduction in A_∥ and a loss of superhyperfine structure—exactly as we observe
with ferritin/Cu\(^{2+}\) (Smith et al., 1977). This model system also demonstrates that the \(\epsilon\)-amino group is a strong ligand for cupric ion with \(K_1 = 1.3 \times 10^6\), \(K_2 = 4.6 \times 10^5\), \(K_3 = 9.3 \times 10^4\), and \(K_4 = 8.5 \times 10^4\). It is also of interest to note that \(\epsilon\)-amino complexes of copper have been proposed as models for copper containing metalloproteins in which the copper ion undergoes oxidation and reduction because the \(\epsilon\)-amino group with its long, flexible hydrocarbon connection to the polypeptide backbone would behave as a unidentate ligand and thus allow rearrangements in the coordination sphere of the copper to accommodate the different coordination preferences of cuprous and cupric ions (Österberg, 1974).

To some extent the nature of the ligands of cupric ion can be determined by carrying out simple molecular orbital calculations employing the spin Hamiltonian parameters. The procedure is outlined by Kuska and Rogers (1971) but we will review it briefly here. We will use the results of this calculation to compare the ligands of the apoferritin/Cu\(^{2+}\) complex to those of the apoferritin/Cu\(^{2+}\) complex and to speculate on the nature of the copper ligand replaced by imidazole.

The basic set to be used consists of the real d orbitals: \(d_{xy}\), \(d_{xz}\), \(d_{yz}\), \(d_{x^2-y^2}\), and \(d_z^2\). If the spin hamiltonian
\[
\hat{H}(0) = \beta \cdot \vec{H} \cdot \vec{S} + \hat{I} \cdot \hat{A} \cdot \hat{S}
\]
is used, we find that to zero order, the ground state is a hole in the \(d_{x^2-y^2}\) orbital, if the coordinate axes are taken as being along the metal-ligand bonds. For historical reasons, the axes normally used in calculations of this type use a coordinate system rotated from the normal one by 90\(^\circ\), so that the unpaired hole is in the \(d_{xy}\) orbital to zero order; in the following we use the historical coordinate system, although, in the final expressions, all quantities
are empirical parameters and the coordinate system employed is of no importance. The spin-orbit interaction,

$$\sum_k \zeta l_k \cdot \hat{S}_k$$

where $\zeta$ is the spin-orbit interaction constant and the sum $k$ is over all electrons, is used as a perturbation on the zero order Hamiltonian above and the two wave functions of the ground state Kramer's doublet are calculated by standard first order perturbation methods. The diagonal elements of the $g$ tensor are calculated with these first order wave functions and the Zeeman Hamiltonian to give:

$$g_x = 2.0023 - 2\alpha_3$$
$$g_y = 2.0023 - 2\alpha_2$$
$$g_z = 2.0023 - 8\alpha_1$$

where:

$$\alpha_i = \zeta/E_i \quad i \in \{1,2,3\}$$

$$E_{1\alpha} = E(d_{x^2-y^2}) - E(d_{xy})$$
$$E_{2\alpha} = E(d_{yz}) - E(d_{xy})$$
$$E_{3\alpha} = E(d_{xz}) - E(d_{xy})$$

A similar calculation of the diagonal elements of the $A$ tensor to first order gives:

$$A_{xx} = P(-2\alpha_3 - \kappa + 2/7 + (3/7)\alpha_2)$$
$$A_{yy} = P(-2\alpha_2 - \kappa + 2/7 + (3/7)\alpha_3)$$
$$A_{zz} = P(-8\alpha_1 - \kappa - 4/7 - (3/7)(\alpha_2 + \alpha_3))$$
where:

\[ P = g_N g_B N (r^{-3}) \]

-89-

\( K \) is the Fermi contact hyperfine

\( g_N \) is the nuclear g factor

\( B_N \) is the nuclear magneton

\( r \) is the mean hole-metal nucleus distance

Because \( P \) is a function of the distance of the unpaired hole from

the cupric ion nucleus it is a measure of the amount of delocalization

of the valence electron density of the ion onto the ligands. Thus it

could be expected that this parameter has values which are characteris­


tic of different types of ligands.

For the apoferritin/Cu\(^{2+}\)/imidazole complex we calculate

\( P = 0.0257 \text{ cm}^{-1} \) and \( K = 0.449 \). For the apoferritin/Cu\(^{2+}\) complex we

find \( P = 0.0247 \text{ cm}^{-1} \) and \( K = 0.442 \). A note of caution is advisable

here—the values for \( g \) and \( A \) must be obtained from simulations of

the spectra in question using second order corrections. Failure to

do so in the literature has led to completely unreliable results in

many instances (see Cleveland et al., 1975 and cf. Giordano and Bereman,

1974). The point here is that the change in \( P \) is not great; about

4%, while, for example, coordination by four sulfur ligands can give

a value of \( P = 0.016 \text{ cm}^{-1} \) (Maki et al., 1964) a 38% difference from

our observed value. As another example, Cu(II)EDTA which has four

carboxyl oxygens surrounding the cupric ion has \( P = 0.0232 \text{ cm}^{-1} \) or a

10% difference from the apoferritin/Cu\(^{2+}\)/imidazole value. On the other

hand, galactose oxidase, in which the cupric ion has four nitrogenous

ligands in the xy plane is reported to have a value for \( P \) of 0.0263 \text{ cm}^{-1}
(Giordano and Bereman, 1974) or 2% above our value for a similar four nitrogen coordination.

From the above experimental evidence, we conclude that the ligands of the apoferritin/Cu$^{2+}$ complex are not very different than those of the apoferritin/Cu$^{2+}$/imidazole complex. The value of P for the former complex is consistent with coordination of the cupric ion by either an oxygen or a nitrogen ligand which is displaced by the imidazole nitrogen. It seems most likely that the fourth ligand is a molecule of water which would be easily displaced by imidazole.

In summary, the coordination of copper by both ferritin and apoferritin appears to involve a water molecule and three nitrogenous ligands derived from a mixture of histidine and lysine residues with possible involvement by one N-terminal nitrogen. Such coordination would predict the binding of the cupric ion should result in the displacement of three protons. This prediction is at variance with the observation by Harrison et al., (1973c) that such binding results in the displacement of only two protons. At the present time, we are unable to reconcile this difference.

2. The Role of Copper in Iron Release

At the time of this writing, the only system reported to release iron from ferritin on a physiological time scale is a combination of flavin mononucleotide (FMN) and nicotine adenine dinucleotide (NADH) (Sirivech et al., 1974; Dognin and Crichton, 1975; Crichton et al., 1975b). The proposed route of electron transport for iron reduction is:

\[
\begin{align*}
\text{NAD}^+ & \rightarrow \text{FMN} \rightarrow 2\text{Fe}^{3+} \rightarrow O_2 \\
\text{NADH} + H^+ & \rightarrow \text{FMNH}_2 \rightarrow 2\text{Fe}^{2+} \rightarrow ?
\end{align*}
\]
The FMN-NADH system will also rapidly reduce ferric ion in solutions of ferric chloride. One feature of the system is that low oxygen concentrations are required (<3 μM, according to Sirivech et al., 1974) to depress the direct reaction of FMNH₂ with molecular oxygen. Normal sera concentrations of oxygen in humans are in the range 50–120 μM (40–90 torr partial pressure) (Osaki et al., 1966). A study of ferritin iron release in dog liver perfusate at a controlled oxygen partial pressure of 30–40 torr showed that at this O₂ concentration, iron release from ferritin was normal (Osaki and Johnson, 1969).

With copper bound in the binding sites, we see several effects on the kinetics of iron release. Iron release occurs in physiologically reasonable times with a physiological reducing agent, namely ascorbate. Furthermore, mobilization proceeds immediately upon addition of reductant—that is it goes at oxygen partial pressures which are physiologically reasonable. Finally, reduction occurs by some novel route since ferrous ion buildup does not inhibit it. The observation that cupric ion can be reduced and the cuprous ion oxidized while bound to apoferritin indicates that the mechanism of ferric ion reduction involves the reduction of cupric ion by the reducing agent in the medium (Martell and Khan, 1973) and the subsequent reduction of ferric ion by the cuprous ion.

The implication of these studies that iron release from ferritin may be dependent on the presence of copper is especially interesting because of a substantial amount of clinical evidence that this is the case. Studies by Lee et al., (1968) found that copper deficient
swine absorbed iron from their diet into the duodenal lumen at normal rates, but that subsequent transfer of iron to the plasma was impaired. If iron supplements were injected, iron was found to accumulate in the reticuloendothelial system and the hepatic parenchmal cells. Goodman and Dallman (1969) observed similar effects in copper deficient rats and also noted that iron would accumulate in the ferritin of the erythroid cells. In other words, iron accumulated in the points where ferritin is most concentrated. A further observation was that administration of copper to the copper deficient animals resulted in the rapid release of iron from ferritin and a subsequent rise in the plasma iron concentrations. Lee attributed these results to a failure of serum ferroxidase activity (in particular, the activity of ceruloplasmin, *vida infra*) preventing oxidation of ferrous ion so that it could be incorporated into apotransferrin. The accumulation of iron in the ferritin of the erythroid cells led Goodman and Dallman to postulate that besides a defect in the plasma transport of iron, some defect in the intracellular transport of the element must also be present.

The ferroxidase activity of ceruloplasmin is well established (Osaki et al., 1966; Osaki, 1966) and it is known to contain eight copper atoms per molecular weight of 155,000 (Scheinberg, 1966). It has further been shown that the protein can catalyse the uptake of iron into apotransferrin from liver perfusate (Osaki and Johnson, 1969). However, it is not the only source of plasma ferroxidase activity. Topham and Frieden (1970) found another protein (designated ferroxidase II) which could also oxidize ferrous ion. Further studies of this protein showed that it also contained copper which was essential to
its activity (Topham et al., 1971; Topham and Sung, 1973). A study by Lee et al., (1969) found that citrate is yet another source of serum ferroxidase activity, accounting for 15% of such activity in normal patients and almost 100% in patients with Wilson's disease—a condition in which ceruoplasmin is absent and yet iron mobilization is unimpaired.

If we postulate that copper is essential for the release of iron from ferritin, we can explain the buildup of iron stores in copper deficient animals with two assumptions: (1) iron in the mucosal cells passes through ferritin and (2) there is some mechanism by which copper can be transferred to ferritin to release iron and removed from ferritin to store iron. At this time we have no evidence to support the second assumption. The first assumption deals with the question of the presence or absence of iron binding proteins besides ferritin in the mucosal cells. There are numerous reports that such other proteins do exist, although the reported properties of such proteins can conflict with each other. Blanc and Isliker (1965) report the presence of a heat stable, iron binding mucroprotein. Pearson and Reich (1969) report a similar mucroprotein and Worwood and Jacobs (1971) report a heat stable iron binding fraction, but find characterization of it to be difficult due to apparent changes in its properties on concentration from cell extracts (Worwood, 1973). A temperature labile, transferrin like protein has been reported in the mucosa by Sheehan and Frankel (1972), Pollack et al., (1972), and Pollack and Lasky (1975). Halliday et al., (1975, 1976) also report such a protein as well as three other iron binding proteins to be present. They further report that some of the
transferrin-like molecule is precipitated by antitransferrin and speculate that it may be a contaminant from serum. Hubers et al., (1971) observe that a complex of ferritin and a transferrin-like protein contains 95% of absorbed iron in the mucosal cells of rats. Finally, a study by Linder and Munro (1975) showed that unless extreme precautions are taken to exclude pancreatic enzymes from the mucosal cell preparations the ferritin in them will undergo proteolysis giving rise to several iron binding ferritin fragments. They find that if one does exclude the pancreatic enzymes, the mucosal cell is found to contain only ferritin bound iron as well as some low molecular weight iron species (mimicked by FeCl₃) which contains iron in equilibrium with the ferritin iron. Thus it appears that the assumption that iron in the mucosal cells will pass through ferritin in copper deficient animals is reasonable. As to the buildup of iron in ferritin at other points, we can only speculate that lack of copper precludes its delivery to ferritin and thus prevents iron from being released to the plasma from the reticuloendothelial cells or the proteins involved in hemoglobin synthesis in the erythroid cells. In this manner, both effects of copper deficiency on iron metabolism can be explained without the necessity of postulating two separate mechanistic defects.

There is also clinical evidence that ascorbic acid is important in the release of iron from ferritin in vivo. Wapnick et al., (1970) in a study of Bantu tribesmen in South Africa found that although these subjects often suffer from iron overload, their plasma iron levels may be normal or even depressed. At the same time ferritin stores of iron are greatly increased in the cells. Administration of ascorbic acid
resulted in a release of iron from cellular stores and an increase in plasma iron concentrations in scorbutic individuals but produced no effects in individuals with normal serum ascorbate levels. Also a study in guinea pigs (Lipschitz et al., 1971) showed ascorbate acid deprivation to increase the percentage of stored iron. Stores were located especially in the cells of the reticuloendothelial system.

3. Properties of Loosely Bound Copper

The addition of copper in excess of that bound to the copper tight-binding site results in a copper species which is associated with the protein and yet free to tumble in a 'solution-like' manner resulting in an averaging of most of the A and g tensor anisotropy. This copper is associated with sites on the apoferritin interior surface. However, addition of too much copper will saturate these sites and result in precipitation of the protein. This has also been noted by Macara et al., (1973c).

Studies of cupric ion and its interactions with zeolites give results similar to those we observe here for the interaction of excess copper with apoferritin. Zeolites are aluminosilicates which have general compositions Na$_{56}$ (Al$_{102}$)$_{56}$(Si$_{102}$)$_{136}$ (Linde type Y) and Na$_{88}$ (Al$_{102}$)$_{88}$(Si$_{102}$)$_{104}$ (Linde type X) for example. They are porous materials with roughly spherical cavities of diameters on the order of 10 Å, which can hold up to about 25 water molecules. Cations occupy specific sites in these cavities which are well defined both crystallographically and molecularly. In this case, cupric ion will displace sodium ions which normally occupy the cation exchange sites. For well hydrated zeolites, cupric ion so bound yields 'solution-like' epr
spectra (A. Nicula et al., 1965). A more complete study of the line shapes in these systems (Martini and Burlamachi, 1976) has shown that in the case of type Y zeolite, the rotational correlation time for the adsorbed cupric complex is ten times that of the ion free in aqueous solution under the same conditions. We compare that figure with our observation that excess cupric ion in the apoferritin interior has a rotational correlation time three times that of the free hexaquocupric ion. The authors of this study ascribe the effects noted to changes in the properties of water in these highly charged cavities; in particular, the hydrogen bonding properties seem to be greatly affected (see Frohnsdorff and Kington, 1958).
VII. VANADIUM BINDING

A. Introduction

Vanadium is commonly found in the oxidation states II, III, IV and V. Only vanadium(II) and vanadium(IV) are Kramer's ions, and only these species are of potential use as epr active probes of apoferritin. V(II) is not a very stable species in aqueous solution, however, so its applicability is limited to instances of strongly reducing conditions. On the other hand, V(IV) is the most stable oxidation state of the element. Molecular oxygen will oxidize V(III) to V(IV) and mild reducing agents (such as ascorbate) will reduce V(V) to V(IV). The chemistry of this oxidation state is dominated by oxovanadium or vanadyl compounds which contain the Vo2+ unit, which is extremely stable and remains intact through a variety of chemical reactions.

Chasteen and his coworkers have been the principal advocates of vanadyl as a probe of metal ion binding sites of biochemical interest. They have found it to substitute for Zn2+ in bovine insulin where zinc binding is closely paralleled by vanadyl binding (Chasteen et al., 1973). They have used the vanadyl ion as a replacement for zinc in carboxypeptidase A where they find the vanadyl substituted enzyme to retain some enzymatic activity and they also have noted that vanadyl will not reduce the CPA disulfide bond (Dekoch et al., 1974). In another study, vanadyl was bound to bovine carbonic anhydrase in place of the native Zn2+. In this case, however, no enzymatic activity was evident in the resulting metalloprotein (Fitzgerald and Chasteen, 1974). In other studies Vo2+ was found
to replace Fe$^{3+}$ in transferrin (Cannon and Chasteen, 1975; Chasteen et al., 1977; Harris, 1977). Since the inhibition of ferritin function by Zn$^{2+}$ is well known and since VO$^{2+}$ has been shown to replace both Zn$^{2+}$ and Fe$^{3+}$ in proteins, we decided to use the species as a probe of metal ion binding sites in ferritin and apoferritin.

B. Properties of Vanadyl Ion

V(IV) has one unpaired d electron and hence its electronic structure is analogous to that of Cu(II) although we are dealing now with a single unpaired electron instead of an unpaired hole in a filled shell. Figure 25 illustrates the splittings of the electronic energy levels of a d$^1$ system such as V$^{4+}$ in a crystal field of octahedral symmetry with and without a tetragonal distortion. To a very rough approximation, the vanadyl ion may be considered to be a d$^1$ system with a severe tetragonal distortion. This limit does not describe the excited states of the ion very well—and one really should use a molecular orbital description of the system—but the ground state is primarily a metal localized b$_{2g}(d_{xy})$ orbital in both the crystal field and the molecular orbital pictures so for our purposes the crystal field approximation is adequate (Ballhausen and Gray, 1962). Thus the ground state of the vanadyl ion consists of an unpaired electron in an orbital which is essentially the 3d$^0_{xy}$ orbital of the V(IV) ion, so that the unpaired spin density lies in the plane perpendicular to the V-O axis and as a result the spin states are sensitive to the ligands in this plane (see figure 25). Application of a magnetic field will split the
Fig. 25. Energy splittings of a $d^1$ electronic structure by a crystal field of octahedral symmetry with and without a tetragonal distortion. The tetragonal distortion shown is a compression.
two degenerate \( |M_s\rangle = \pm 1/2 \) states of the ion and we can observe allowed transitions between these levels by epr spectroscopy.

The strong vanadium-oxygen interaction in vanadyl results in a crystal electric field at the vanadium nucleus which has axial symmetry which the epr spectra of the species reflect. Also, the isotope \( ^{51}V \), with a nuclear spin of \( 7/2 \), is 99.8% naturally abundant and the resulting hyperfine splitting results in an eight line spectrum which is characteristic of vanadium(IV) in general and vanadyl ion in particular.

The oxidation-reduction chemistry of vanadyl is quite a bit more complicated than that of the other ions considered in this research. The oxidation of \( \text{VO}^{2+} \) to \( \text{V}^{5+} \) can be accomplished by molecular oxygen at higher values of pH; consequently, experiments with vanadyl must be carried out under nitrogen or in the presence of a mild reducing agent, or both. Too strong a reducing agent, however, will result in the reduction of \( \text{VO}^{2+} \) to \( \text{V}^{3+} \) or even \( \text{V}^{2+} \). In our experience, for example, sodium dithionite added in excess to solutions of vanadyl sulfate results in formation of \( \text{V}^{2+} \) which subsequently reacts with \( \text{VO}^{2+} \) (resulting from reoxidation of \( \text{V}^{2+} \) by molecular oxygen) to form the species \( \text{VOV}^{4+} \) which has a characteristic golden color and no epr spectrum. The \( \text{V}^{2+} \) so formed can act as an inhibitor of iron release from ferritin (see figure 20).

The aqueous solution chemistry of vanadyl can also be quite complicated since several different hydroxide complexes of vanadium can form depending on the pH of the solution. In a study of epr signal intensity of solutions of \( \text{VOSO}_4 \) as a function of pH, Francavilla and
Chasteen (1975) found that three different equilibria were needed to explain their experimental results at pH values less than 6.5. These are:

\[
\begin{align*}
&\text{VO}^{2+} + \text{H}_2\text{O} \rightleftharpoons K_{1,1} \text{VOOH}^+ + \text{H}^+ \\
&2\text{VO}^{2+} + 2\text{H}_2\text{O} \rightleftharpoons K_{2,2} (\text{VOOH})_2^{2+} + 2\text{H}^+ \\
&\text{VO}^{2+} + 2\text{OH}^- \rightleftharpoons (K_{\text{sp}})^{-1} \text{VO(OH)}_2
\end{align*}
\]

They report \(K_{1,1} = 4.4 \times 10^{-6}\) and consequently, the concentration of \(\text{VOOH}^+\), which is paramagnetic and should have an observable epr spectrum, is small for most of the pH range investigated and they found that its presence could be neglected because at those values where its presence would be important precipitation of \(\text{VO(OH)}_2\) becomes the most important process. They also found that \(K_{2,2} = 1.66 \times 10^7\) M and \(K_{\text{sp}} = 1.0 \times 10^{-22}\) M\(^3\). In their investigations, they note a gradual decrease in epr signal intensity as pH is raised from less than 2 to 5, which effect they interpret as due to the formation of the epr silent species \((\text{VOOH})_2^{2+}\). At about pH 5.0 a sharp drop in signal intensity to almost zero accompanied by formation of a precipitate is the result of formation of \(\text{VO(OH)}_2\) which actually has a polymeric structure and is epr silent. Above a pH of ca. 5 a gradual decrease in the remaining signal intensity to zero at pH ca. 6.1 is observed which they feel may be a result of the formation of some \(\text{VO(OH)}_3^-\). Addition of further base to the solution results in the very slow solution of some of the precipitated \(\text{VO(OH)}_2\) as \(\text{VO(OH)}_3^-\). Raising the pH to about 12 allows complete formation of \(\text{VO(OH)}_3^-\) which has a
characteristic epr spectrum (Iannauzzi and Rieger, 1975). Thus at physiological pH, except for the possible formation of some VO(OH)\(^{-}\) which has a very characteristic epr spectrum (and we have never observed a spectrum from this species in the pH range 5.5 - 7.5), any vanadyl not associated with a ligand should precipitate as VO(OH)\(_2\) and consequently be epr silent.

C. Experimental Procedures

Two methods were employed to introduce the vanadyl ion to apoferritin. In the first, an apoferritin solution in 100 mM acetate buffer at pH 5.5 was dialysed against 100 mM acetate buffer at the same pH but containing excess VOSO\(_4\). This dialysis was carried out under N\(_2\) and allowed to equilibrate overnight. The resulting solution was then dialysed against a buffer at the pH to be studied in order to remove unbound VO\(^{2+}\). All solutions were purged with nitrogen gas for about twenty minutes prior to use to prevent oxidation of the vanadyl. In those solutions in which the pH was greater than 6.0 ascorbic acid was added to further retard oxidation. In the second procedure nitrogen purged buffered solutions of apoferritin with added ascorbic acid were placed in the aqueous epr flat cell. Known amounts of concentrated solutions of VOSO\(_4\) were added by syringe to the flat cell. A nitrogen purged syringe was then used to withdraw the resulting solution from the flat cell. The solution was mixed in this syringe and was then readded to the flat cell.
D. Results and Discussion

A typical frozen solution apoferritin/VO$^{2+}$ spectrum along with a computer simulation is shown in figure 26. The parameters obtained from the simulation are:

\[
\begin{align*}
g_{||} &= 1.942 & A_{||} &= -184.0 \text{ G} \\
g_{\perp} &= 1.978 & A_{\perp} &= -60.0 \text{ G} \\
g_{\circ} &= 1.966 & A_{\circ} &= -101.3 \text{ G}
\end{align*}
\]

These values compare to those of VO$^{2+}$ in 100 mM acetate buffer (pH 5.5) which are found to be:

\[
\begin{align*}
g_{||} &= 1.937 & A_{||} &= -194.0 \text{ G} \\
g_{\perp} &= 1.981 & A_{\perp} &= -70.5 \text{ G} \\
g_{\circ} &= 1.966 & A_{\circ} &= -111.7 \text{ G}
\end{align*}
\]

so we are clearly observing a spectrum which is due to vanadyl interaction with the apoferritin as opposed to a VO$^{2+}$ - acetate complex.

Furthermore, attempts to bind vanadyl to ferritin by dialysis methods lead to complete loss of the vanadyl to the buffer--i.e., no ferritin/VO$^{2+}$ complex forms. If vanadyl is added by increments to ferritin, we merely observe the same vanadyl spectrum as is observed in the absence of protein. If a solution of the apoferritin/VO$^{2+}$ complex is dialysed for several days against daily buffer changes most of the vanadyl is lost to the buffer--so the vanadyl is not as tightly bound as the copper was observed to be.
Fig. 26. Upper curve: Computer simulation of vanadyl spectrum. Lower curve: Experimental spectrum of apoferritin/VO$^{2+}$ in a glass of 50% 100mM acetate buffer, pH 5.5 and 50% glycerine. Temperature is -100°C. Parameters found by simulation are: $g_\parallel = 1.942$; $g_\perp = 1.978$; $g_0 = 1.966$; $A_\parallel = -184.0$ G; $A_\perp = -60.0$ G; $A_0 = -101.3$ G. Notice that the absolute signs of the $A$ values are not directly obtained from these spectra. However, it is well known that for vanadyl these are negative.
Wüthrich (1965) first noted that for some types of ligands, the value of the isotropic hyperfine coupling constant, $A_0$, reflects the average environment about the vanadyl ion due to the four ligands in the $xy$ plane perpendicular to the vanadium-oxygen axis. This so-called 'rule of average environment' has been further investigated by Boucher et al., (1969) and applied to protein systems (Chasteen et al., 1973; DeKoch et al., 1974). This treatment provides a means of determining the nature of the axial ligands from the use of model compounds. If, for example, we choose $\text{VO(H}_2\text{O)}_5^{2+}$ ($A_0 = -116.0 \ \text{G}$) with four equatorial water oxygens and $\text{VO(porphyrin)}$ ($A_0 = -95.5 \ \text{G}$) with four equatorial nitrogens as model compounds, then using the rule of average environments we predict that a vanadyl ion complexed to two water oxygens and two nitrogens should have $A_0 = 2(-95.5) + 2(-116.0) / 4 = -105.8 \ \text{G}$, which compares very well with the experimental value of $A_0 = -105.0 \pm 1.0 \ \text{G}$ for coordination by two imidazole nitrogens and two water molecules (Chasteen et al., 1973). Other model compounds include $\text{VO(malonate)}_2$ with four equatorial sulfur ligands ($A_0 = -70.0 \ \text{G}$) (Boucher et al., 1969). The experimental value of $A_0$ for apoferritin/$\text{VO}^{2+}$ of $-101.3 \ \text{G}$ is compatible with only a limited number of coordination schemes. They are: $\text{VO(N)}_3\text{H}_2\text{O}$ with a predicted $A_0 = -100.6 \ \text{G}$; $\text{VO(H}_2\text{O)}_3(S)$ with $A_0 = -104.5 \ \text{G}$; $\text{VO(CO}^-\text{)}_4$ with $A_0 = -102.0 \ \text{G}$; $\text{VO(H}_2\text{O)}_2(S)(\text{CO}^-)$ with $A_0 = -101.0 \ \text{G}$; and $\text{VO(H}_2\text{O)}_2(S)(\text{N})$ with $A_0 = -99.4 \ \text{G}$.

If we observe the spectrum of the apoferritin/$\text{VO}^{2+}$ system at room temperature and pH 5.5 we obtain the spectrum of figure 27. The measured parameters from this spectrum (corrected for second order effects) are $g_0 = 1.966$ and $A_0 = -102.0 \ \text{G}$ so the observed vanadyl
Fig. 27. EPR spectrum of apoferritin/VO$^{2+}$ in 100 mM acetate buffer, pH = 5.5, temperature is 20°.
The most striking feature of this spectrum is that it is 'liquid-like'. We can calculate a hydrodynamic radius for this species in the following manner.

We assume the linewidth of the hyperfine lines goes as the following (Wilson and Kivelson, 1966):

\[ \Delta H = \alpha + \beta m_I + \gamma m_I^2 \]

where \( H \) is the line width, \( \alpha, \beta, \) and \( \gamma \) are empirical parameters, and \( m_I \) is the nuclear spin projection quantum number. For two hyperfine lines in the spectrum chosen such that:

\[ |m_{I,1}| = |m_{I,2}| \]

we have:

\[ \Delta(\Delta H) = \beta(m_{I,1} - m_{I,2}) \]

so we can obtain a value for \( \beta \).

From Wilson and Kivelson (1966) we find for the case of axial symmetry:

\[
\frac{\beta}{\tau_R} = (4/15)b\Delta\gamma B_0 - (8/45)(\Delta\gamma B_0)^2(A_0/\omega_0)
- (b^2A_0/\omega_0) \left\{ (1/20)I(I + 1) + (3/40) \right\}
+ u \left\{ (1/5)b\Delta\gamma B_0 - (2/15)(\Delta\gamma B_0)(A_0/\omega_0)(1 + f) \right.
- (1/20)b^2(A_0/\omega_0)uI(I + 1)(1 + 7f) \]
where:

\[ A_0 = \left( \frac{1}{3} A_\parallel + 2A_\perp \right) \]
\[ b = \left( \frac{2}{3} \right) (A_\parallel - A_\perp) \]
\[ g_0 = \left( \frac{1}{3} \right) (g_\parallel + 2g_\perp) \]
\[ \Delta g = g_\parallel - g_\perp \]
\[ \Delta \gamma = \beta \Delta g / h \]
\[ B_0 = \hbar \omega_0 / g_0 \beta \]
\[ u = (1 + \omega_0^2 R^2)^{-1} \]
\[ f = \omega_0^2 R^2 u \]

and R is the hydrodynamic radius, \( \omega_0 \) is the Larmor frequency, I is the nuclear spin (7/2 here), \( \tau_R \) is the rotational correlation time. The rotational correlation time can be expressed in terms of R by the equation for Brownian diffusion:

\[ \tau_R = \left( \frac{4 \pi R^3}{3kT} \right) \eta \]

where \( \eta \) is the viscosity. From the above equations, we can calculate the effective hydrodynamic radius of the observed vanadyl species. We find it to be 3.13\( \AA \). Thus the vanadyl appears to be tumbling freely in some sort of loose association with the apoferritin. We calculate the room temperature rotational correlation time of the vanadyl species to be \( 3.0 \times 10^{-11} \) seconds. This is in contrast to the rotational correlation of the apoferritin molecule which can be estimated to be \( 2 \times 10^{-7} \) seconds. Therefore, any coordination of the vanadyl ion by more than one amino acid residue is highly unlikely since tumbling could be expected to be severely limited in such a case. Furthermore, the chemical exchange of the vanadyl ion between
two different magnetic environments (say, for example, vanadyl bound to some site on the apoferritin and vanadyl in solution) cannot occur at a rapid enough rate at room temperature to result in an 'averaged environment' spectrum. In the present case, the fast exchange limit which would result in an epr spectrum displaying the averaged features of two different magnetic environments would require the average lifetime for the vanadyl in an environment to be much less than $4 \times 10^{-9}$ sec. Measurements of exchange of vanadyl between a pyridine and acetylacetonate ligand show that the average lifetime in one coordination is ca. $10^{-7}$ sec in this case (Walker et al., 1966). Consequently, the spectrum we observe of apoferritin/VO$^{2+}$ is not the result of chemical exchange.

Of the possible groups of ligands consistent with the 'rule of average environment' above, only those resulting in the vanadyl being primarily surrounded by water molecules are consistent with the observed freedom to tumble. This would seem to limit us to the possible configurations $\text{VO(H}_2\text{O)}_3(S)$, $\text{VO(H}_2\text{O}_2(S)(N)\text{)}$, or $\text{VO(H}_2\text{O}_2(S)(CO)}_2\text{)$. In all three cases, the presence of a sulfur ligand is essential to reduce the hyperfine splitting to the size of that observed experimentally.

We have examined complexes of vanadyl and mercaptoacetic acid ($\text{HSCH}_2\text{COOH}$) and find the complex $\text{VO(SCH}_2\text{COO)}_2^{2-}$ to have $A_\omega = -88.8$ G (we note that the rule of average environments predicts $A_\omega = -86$ G for this species). We can also observe the spectrum of $\text{VO(H}_2\text{O}_2(SCH}_2\text{COO)}$ and find $A_\omega = -101.7$ G (compared to the predicted -101.0 G). The possibility that the spectrum of vanadyl that we observe in apoferritin solutions is merely an artifact which results from quantities of
mercaptoacetic acid remaining from the preparation of the apoferritin is eliminated for two reasons. First, the binding of mercaptoacetic acid is such that at pH > 6 we see only the VO(SCH₂COO)₂⁻ species. At lower values of pH we see spectra of vanadyl with both one and two mercaptoacetate ligands as well as pentaquo vanadyl. In contrast, in solutions of apoferritin/VO²⁺ only one species is observed at these lower pH values. Second, in solutions of the apoferritin/VO²⁺ we can change the concentration of vanadyl by a factor of 10² and see only one species—if mercaptoacetate were present we should see both the mono and bis coordinated vanadyl species, especially at low concentrations of vanadyl.

The value of Δ₀ = -101.7 G is of interest because it is very close to the observed value of Δ₀ = -101.3 G for the apoferritin/VO²⁺ complex at low pH. At first glance, this would implicate a carboxyl oxygen and a sulfur as two water molecules as ligands. Such a pair of ligands from amino acid residues would establish a crystal field for the vanadyl ion which would reorient with respect to the external magnetic field with a correlation time equivalent to that of the apoferritin molecule, which is two orders of magnitude too large to explain the vanadyl spectra we observe. DeKoch et al. (1974) have observed a similar isotropic spectrum arising from a carboxypeptidase A/VO²⁺ complex. They suggest the protein is coordinated monodentate to the vanadyl ion which they believe accounts for the short rotational correlation time. This explanation is unsatisfactory, however. A monodentate ligand would establish a crystal field axis, and again, this crystal field would reorient with respect to the external magnetic field with a
correlation time equal to that of the protein. At best, one could get a partial averaging of the vanadyl spectrum as a result of rapid rotation about the ion-protein axis. For the monodentate ligand bound in the xy plane we would see apparent values of the hyperfine tensor of $A^{'}_\parallel$ and $A^{'}_\perp$ where $A^{'}_\parallel = A_\perp$ and $A^{'} = (1/2)(A_\parallel + A_\perp)$ due to rotation about the vanadyl-amino acid residue bond. The resulting spectrum is not isotropic.

The possibility exists that the vanadyl is merely associated with the apoferritin as a result of electrostatic interactions. However, if we bind vanadyl to apoferritin and then add NaCl, we see absolutely no effect on the vanadyl spectrum even at sodium to vanadyl ratios of 50 to 1. The effect is therefore not merely electrostatic attraction. Also of interest is the fact that precipitation of the apoferritin/VO$_2^+$ complex has little effect on its spectrum (see figure 28). While there is some change--indicating that the vanadyl is now in more than one environment, the spectrum remains 'liquid-like.' We must conclude that the vanadyl ion is located in the protein interior where the protein-protein interactions occurring upon salting out have minimal effect on its environment.

If we observe the apoferritin/VO$_2^+$ complex at higher pH we observe the spectrum of figure 29. Clearly, two vanadyl species are now present. Both of them must be associated with the protein since at pH 7.0 all vanadyl not so associated would precipitate as vanadyl hydroxide and give no spectrum. The values of $A_\infty$ and $g_\infty$ of the outer spectrum indicate that it is the species observed at low pH. If we vary the pH at which we observe the complex, we observe a change in the relative intensities.
Fig. 28. Upper curve: EPR spectrum of apoferritin/V02+ in 100 mM acetate buffer, pH = 5.5, temperature is 200. Lower curve: Same sample as above, but 50% saturated with (NH4)2SO4, resulting in precipitation of the protein. Temperature is 200.
Fig. 29. EPR spectrum of apoferritin/VO$^{2+}$ in 50 mM HEPES buffer, pH 7.0. Temperature is 20°C. Some ascorbic acid added. For the outer spectrum: $A_o = -101.9$ G; For the inner spectrum: $A_o = -90.9$ G; For both: $g_o = 1.966$. 
of the two spectra with the inner predominating at high pH and the outer at low pH. We therefore have a high pH and a low pH form of the same complex species. We can determine the pK_a of the vanadyl ligand which is being protonated as the pH is lowered by noting that:

\[ K_a = \frac{[B][H^+]}{[A]} \]

where \( K_a \) is the association constant

A is the low pH form
B is the high pH form

so \( \log \frac{[A]}{[B]} = pK_a - \text{pH} \) and therefore a plot of \( \log \frac{[A]}{[B]} \) vs. pH should have a slope of -1 and an intercept of \( pK_a \). The ratio \( [A]/[B] \) can be determined since it is equal to the ratio of the derivative signal peak heights for the well separated peaks (Fitzgerald and Chasteen, 1974b). Such a plot is shown in figure 30. As can be seen, we find \( pK_a = 7.15 \pm 0.1 \). Since the value of \( A_o \) for the high pH form observed is less than that of the low pH form, this ligand cannot be an imidazole nitrogen from a histidine residue or a terminal nitrogen, since deprotonation of these species would lead to coordination of the vanadyl by species which would increase, not decrease \( A_o \). The most likely explanation of our observed spectra is that increased pH results in the deprotonation of a water molecule coordinated to the vanadyl. Such a process would allow the vanadyl complex to retain its freedom of motion, while greatly decreasing \( A_o \). Such hydroxo complexes have been observed before and their epr properties as a function of pH closely parallel those of the complexes observed here (Wüthrich, 1965b; Wüthrich and Connick, 1968).
Fig. 30. Determination of pKₐ of vanadyl ligand.
Upon freezing a solution of the pH 7.0 complex, one observes that the relative concentrations of the two species changes in such a way as to favor the low pH form (figure 31). For pure water, the pK_a changes from 7.87 at 24°C to 8.34 at 0°C. It is reasonable that the pK_a of the water bound in the complex VO(H_2O)_n-apoferritin should also increase with a decrease in temperature, resulting in the observed increase in the relative concentration of the low pH form upon freezing.

The above evidence indicates that we are indeed observing an interaction of the vanadyl ion with apoferritin. The nature of this interaction is not obvious, however. We do know that the vanadyl is located in the apoferritin interior and that it is free to tumble isotropically, yet the interaction is not simply electrostatic in nature. The isotropic nature of the motion would seem to rule out even monodentate coordination by the protein as discussed above. However, we could postulate that the vanadyl is associated with a 'arm' of the protein which posses an unusual amount of motional freedom—allowing the isotropic averaging. Such an explanation does not appear likely, however, because of the small hydrodynamic radius of the vanadyl complex observed. Another possibility is that the vanadyl may be coordinated monodentate to the protein and have a great deal of fluxional lability, so that the protein ligand can vary from an axial to an equatorial coordination of the vanadyl, allowing the necessary isotropic averaging. We note, however, that vanadyl does not bind ligands axial to the V-O bound very tightly. For example, the rate of exchange of water ligands in VO(H_2O)_5^{2+} is seen to be 10^6 times larger for the axial water than for the equatorial ones.
Fig. 31. EPR spectrum of apoferritin/VO$^{2+}$ in 50 mM HEPES buffer, pH 7.0. Temperature is -120°. Some ascorbic acid added to prevent oxidation. Parameters found (corrected for second order effects) are: For the outer spectrum: $A_\parallel = -184.0$ G; $A_\perp = -60.0$ G; $A_O = -101.3$ G; For the inner spectrum: $A_\parallel = -173.3$ G; For both: $g_\parallel = 1.942$; $g_\perp = 1.978$; $g_O = 1.966$. 
(Wüthrich and Connick, 1968). A third possibility is that the vanadyl could be binding to a small anion present in the medium. Silk and Breslow (1976) have observed that Cl\(^-\) binds to apoferritin, and we know that apoferritin in its native form must have the ability to take up phosphate since this species is incorporated into the ferritin core. However, any such anions should be displaced by the massive amounts of Cl\(^-\) present when the apoferritin/VO\(^{2+}\) solution is made 1 M in NaCl (see above), and yet we observe no effect on the vanadyl spectrum in this situation.

A quite different explanation of the 'liquid-like' spectrum and the small values of \(A_0\) may lie in the unusual properties of water in small cavities lined with charged groups. As noted in chapter 6, the zeolites are such systems. Frohnsdorff and Kington (1958) have noted that the water in the cavities of Linde type A zeolite has a heat capacity 11 to 28% more than that of liquid water. They conclude from spectroscopic studies that this is a result of changes in the nature of hydrogen bonding in such cavities. If, indeed, the structure of liquid water is different in such cavities, it is very likely that the 'rule of average environments' completely breaks down. In this view, the spectra we observe at low pH are those of VO(H\(_2\)O)\(^{2+}\) with the nature of the vanadyl-water interaction affected by the electric fields in the apoferritin cavity, in which the complex is located. At high pH we see VO(H\(_2\)O)\(^4\)(OH)\(^+\) with a similar effect on complexation of water to vanadyl. This change in the properties of water could also account for the fact that such vanadyl complexes are present in solution even at high pH. This does not explain our observation.
that vanadyl is not noticeably displaced by Na⁺ in large amounts. This effect is possibly due to a difference in binding site affinities for different ions. A study of ion interactions with cation exchange sites in kaolinite (a naturally occurring aluminosilicate) has found that this material has different affinities for different metal ions, although the difference is only a factor of two (McBride, 1976).
VIII. GENERAL CONCLUSIONS

A. Copper Binding

Of the various metal ions whose interactions with ferritin and apoferritin have been investigated, both here and elsewhere, copper is unique. We have demonstrated, cupric ion binds tightly and specifically to both ferritin and apoferritin. The fact that copper bound to this site dramatically affects the rate at which iron is released from ferritin seems compelling evidence that the existence of this copper binding site is not an accidental property of the protein. The affect of copper on the release of iron from ferritin by ascorbic acid is of special interest in light of the large amount of clinical evidence that both copper and ascorbate are necessary for the proper release of iron in vivo. Unfortunately, our isolation of ferritin under conditions which should not have led to the release of any bound copper (in contrast to normal preparative procedures) failed to show significant amounts of copper to be bound to the protein. None-the-less, the fact that we have obtained samples of ferritin from biochemical suppliers containing large amounts of copper indicates to us that under some conditions, ferritin in vivo does contain copper bound at these sites. We would like to speculate that there is some mechanism by which copper can be transferred to ferritin and that this transfer functions as a 'trigger' for the rapid release of iron from ferritin stores via the sequence:
Ascorbate is known to reduce strongly coordinated Cu(II) in model systems (Martell and Khan, 1973). Furthermore, the reduction potential of copper in type 2 proteins is about 0.390 v while that of ferric ion is about 0.7 v—so the reaction should go as written. As we have demonstrated, such reductive mobilization of iron will occur under physiological conditions, i.e., under physiological reducing conditions, at physiological oxygen partial pressures, and on a physiologically reasonable time scale. In the absence of copper, iron mobilization proceeds slowly by some other mechanism. The obvious experiment to test this hypothesis is the determination of the copper content of ferritin isolated from anemic animals under conditions which will not release bound copper. Unfortunately, at this time such an experiment is beyond our capabilities.

B. Binding of Other Metal Ions

Another way in which apoferritin, but not ferritin, interacts with metal ions is exemplified by its interactions with \( \text{V}^{2+} \), \( \text{Mn}^{2+} \), and amounts of \( \text{Cu}^{2+} \) in excess of those needed to saturate the copper tight binding site. As we have shown by studies of these metal ions associated with precipitated apoferritin, the protein will concentrate such ions in its central cavity. Such ions interact with the protein in a way that their crystal fields are not substantially different from those of the free ions in aqueous solution. However some effects
are noted in the epr spectra of these ions. In particular, the manganous spectrum is broadened, indicating either increased interaction with ligands or an apparent increase in the viscosity of the medium in which the ion tumbles. Likewise, excess loosely bound cupric ion seems to be localized in a medium three times as viscous as pure water at the same temperature, causing hyperfine splittings in its epr spectrum to be better defined. Vanadyl ion displays a decrease in its $A_0$ value, perhaps indicating that it is coordinated in some very unusual way to a ligand either on the protein or free in the apoferritin interior.

Similar effects on the spectra of manganous ion and cupric ion have been observed in a number of epr studies of these ions in natural and synthetic ion exchange resins. Such resins consist of cavities lined with cation exchange sites. The effects in these resins have been attributed to changes in the physical properties of water in such cavities, and in particular to changes in the hydrogen bonding in such water.

According to Wetz and Crichton (1976) the inner surface of the apoferritin molecule contains 96 carboxylate groups, which are deprotonated at physiological pH. They further find that chemical modification of these groups produces a protein which will not take up iron.

It seems likely to us that apoferritin, via these carboxyl groups, behaves like a cation exchanger in its interactions with metal ions. From our observations, a variety of metal ions have complete access to the apoferritin cavity, as suggested by Harrison and her co-workers. In the cavity, these ions appear to remain coordinated to water molecules
much as they would be in aqueous solution. They also remain mobile
and probably interact with multiple cation exchange sites in the cavity.
The spectral effects we note for these mobile divalent metal ions
seem to be a result of the unique properties of water in the apoferritin
cavity, in analogy to water in the cavities of other ion exchangers.

In vivo, the protein probably functions in a similar manner,
concentrating ferrous ions at its interior by ion exchange. Here
the ferrous ion is oxidized, either by interaction with oxidizing
agents in the medium, or by passing an electron to some acceptor on
the protein inner surface.

Our examination of ferric ion bound to both ferritin and
apoferritin shows that this ion also binds loosely to the protein.
It is, however, definitely associated with sites on the polypeptide
and not with sites on the inorganic core. We do not understand the
significance of this type of site at the present time.
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