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THE UPTAKE, STORAGE, AND MOBILIZATION OF IRON AND ALUMINUM IN BIOLOGY

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I. Introduction

There are two major chemical problems associated with the biological use of iron, namely, the poor solubility of iron at physiological pH values and the involvement of iron in potentially harmful redox reactions. These problems have led to the evolution of a variety of elegant biochemical processes directed at minimizing the resulting complications. One of the aims of the present article is to provide a brief description of some of the key features of these processes in animals and microorganisms before moving on to consider recent work with the iron storage protein ferritin and its bacterial equivalent, bacterioferritin (bacfer).

The general review of iron metabolism will also provide a background to the subject of aluminum biochemistry. Aluminum, which is

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a toxic element and has no known beneficial role in animals, appears to be taken in and transported in animals in a fashion similar to their uptake and transport of iron. This topic is also considered in the present article, with particular emphasis on the nature of aluminum species present in blood plasma.

We have chosen to discuss a large area of bioinorganic chemistry and, clearly, we cannot be comprehensive. Therefore we have concentrated on selected topics, and have provided a detailed list of review articles that cover related areas that we do not address. Even so, there are relevant questions few authors have tackled. For example, aluminum is almost as abundant as iron in the sea yet is almost totally excluded from biological organisms, while iron is particularly sought after. What is the nature of the selection process that discriminates against aluminum?

Williams (*150*) has addressed this question in his 1981 Royal Society Bakerian Lecture on "Natural Selection of the Chemical Elements." This describes how the biological selection of elements and their chemical properties are related. Williams has also given a detailed account (*51*) of the chemical basis of the uptake of elements by biological systems, and described (*130*) the chemistry that may occur in the specific case of aluminum. Based on this analysis he suggests a major problem with acid rain is the increased biological availability of aluminum.

A major area we have not covered in detail is iron and aluminum in plants. Mineral nutrition of plants is a complex topic (*87*), with, in many cases, iron being a limiting growth factor and aluminum being harmful. Some aluminum-tolerant species are known, such as tea plants, which are actually aluminum accumulators and are given alum as a fertilizer (*31, 87*). In these plants the aluminum is stored in the leaves, a fact that has given to rise to some concern about human ingestion of aluminum (*34*), though much of this concern seems misplaced (*31, 41*). The nature of the stored aluminum is unknown. Iron uptake is based, in some plants, on siderophore-mediated mechanisms, but siderophore-independent uptake is common. A plant equivalent of ferritin, phytoferritin, acts as an iron store (*133*). Phytoferritin is not as well characterized as are its animal or bacterial equivalents, but the information that is available suggests it has a structure and iron-rich deposit similar to these (*118, 133*) (see Section IV).

II. Chemistry of Iron and Aluminum

The common oxidation states of iron are the ferrous (Fe^{2+}) and ferric (Fe^{3+}) states. These have the d^6 and d^5 electronic configurations, re-

spectively, and thus can exist in both the high-spin and low-spin states. Most of the iron species involved in the uptake, transport, and storage of iron in animals contain high-spin Fe^{3+}, though the iron may be complexed initially as Fe^{2+} (as in ferritin; see Section IV). Absorption of dietary iron across the gastrointestinal tract may also involve Fe^{2+} species (*113*), especially given that ascorbic acid influences iron absorption markedly (*102, 113*).

The Fe^{3+}/Fe^{2+} redox potential varies over a wide range depending upon the nature of the ligand. Anionic oxygen ligands, such as carboxylates and phosphates, stabilize Fe^{3+} and therefore form complexes with low redox potentials (*149*). The Fe^{3+}/Fe^{2+} reduction potential often quoted for aqueous systems is 770 mV, but this only refers to acid solutions. As the pH is raised, so that H_2O bound to iron ionizes to OH^-, the redox potential drops sharply (*56*).

Although the redox potentials of many purified components are known, the redox potentials of biological compartments are often unknown. Thus, for example, we do not know the operating redox potential(s) of the human gastrointestinal tract, and therefore the redox states of many iron complexes in the gut are also unknown.

Among the redox chemistries iron may be engaged in biologically is the formation of potentially harmful free radicals:

$$Fe^{3+} + O_2^{\cdot-} \rightarrow Fe^{2+} + O_2 \quad (1)$$

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + OH\cdot \quad (2)$$

$OH\cdot$ is particularly reactive. To minimize the damaging consequences of free-radical formation, most organisms have enzymes for removing $O_2^{\cdot-}$ (superoxide dismutase) and H_2O_2 (peroxidases and catalase). Production of $OH\cdot$ is also minimized by sequestering the iron in a form in which such chemistry is difficult to carry out (see Section III).

In aqueous solutions at low pH, simple iron salts dissociate to give the fully aquated ions. As the pH is raised, these hydrolyze and polymerize to yield insoluble oxyhydroxide precipitates (*7, 15, 48, 120*). The solubility product of $Fe(OH)_3$ is $\sim 10^{-38}$, so that at pH 5 the maximum concentration of soluble Fe^{3+} in aqueous solution, and without a competing ligand for OH^-, is 10^{-10} *M*. By contrast, the solubility product of $Fe(OH)_2$ is $\sim 10^{-15}$. Thus in aqueous and aerobic environments at $9 < pH > 3$, the most common form of iron not complexed to organic ligands is insoluble Fe^{3+} polymers (Fig. 1).

Aluminum has only one stable oxidation state, Al^{3+}, and thus does not have any biological redox chemistry comparable to that of iron. However, the ligand-binding and stereochemical preferences of Al^{3+},

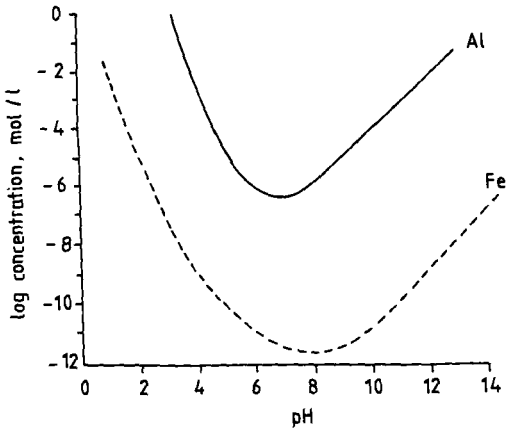

FIG. 1. Maximum solubility of aluminum and iron in water at various pH values. Adapted from the article by Ganrot (*53*), and based on data summarized by Baes and Mesmer (*7*).

TABLE I

EFFECTIVE IONIC RADII AND WATER EXCHANGE RATES FOR DIVALENT AND TRIVALENT METAL IONS[a]

Metal ion[b]	Radius (Å)[c]	k_e (sec^{-1})[d]
Fe^{3+}		
(hs)	0.65	1.6×10^2
(ls)	0.55	—
Al^{3+}	0.54	1.5×10^{-1}
Fe^{2+}		
(hs)	0.78	3×10^6
(ls)	0.61	—
Mg^{2+}	0.72	5×10^5
Ca^{2+}	1.00	$\sim 10^8$

[a] Data from Greenwood and Earnshaw (*56*), Burgess (*26*), Grant and Jordan (*55*), and Martin (*89*).

[b] hs, High spin; ls, low spin.

[c] Radii are for six-coordinate species.

[d] Values of k_e refer to the exchange rate for the process

$$M^{n+}(H_2O)_6 + H_2O^* \rightarrow M^{n+}(H_2O)_5(H_2O^*) + H_2O$$

and some aspects of its solution chemistry, closely resemble those of Fe^{3+}. Thus Al^{3+} has a preference for oxygen ligands in octahedral or tetrahedral complexes, and the solubility of Al^{3+} not complexed to organic ligands is markedly pH dependent (Fig. 1). Martin has calculated that the maximum concentrations of $[Al(H_2O)_6]^{3+}$ and $[Al(OH)_4]^-$ at pH 7.4 are $\sim 3 \times 10^{-12}$ and $\sim 8 \times 10^{-6}$ *M* (*89*). $Al(OH)_3$ is insoluble.

The similarity between Al^{3+} and Fe^{3+} chemistry does not extend to the kinetics of their ligand exchange reactions. For example, the exchange of bound H_2O for free H_2O is much faster for $[Fe(H_2O)_6]^{3+}$ than for $[Al(H_2O)_6]^{3+}$. In fact, Al^{3+} is kinetically inert compared to other biologically important metal ions (Table I). The difference between the rates of H_2O exchange for the metal ions in Table I is largely a consequence of the charge : radius ratio; the larger this ratio, the weaker the metal–H_2O interaction and thus the more labile the H_2O. Therefore, the inertness of Al^{3+} is accounted for by its relatively small size. The relevance of this to the biochemistry of Al^{3+} is described in Section III,C.

III. Aspects of Iron and Aluminum Metabolism in Animals and Bacteria

A. Introduction

The low solubility of iron is the chief hurdle to its biological uptake. Many microorganisms overcome this by secreting into the external medium small chelators, known as siderophores, that bind iron and are then reabsorbed by the organism as an iron complex. After its uptake the complex is broken down to release the iron. This breakdown may involve chemical degradation of the siderophore ligand or simple release of iron, perhaps caused by its reduction to Fe^{2+}. The uptake of the iron–siderophore complex involves its passage through at least one membrane barrier, and to facilitate this, membrane proteins form a receptor for the complex. Further details of the mechanisms of siderophore-mediated iron uptake are beyond the scope of the present article and the reviews by Griffiths (*57*), Hider (*64*), Neilands *et al.* (*103, 104*), and van der Helm *et al.* (*143*) should be consulted for additional information.

The iron uptake processes of animals appear to differ from those of microorganisms: for example, siderophores have not been identified in animals. Iron absorption from the gut is not understood in detail, though it is clear that the nature of the diet and the nutritional and iron status of the animal are important influences on iron uptake

(*113*). A key feature of uptake is that the iron must be able to cross the brush-border membrane of the duodenum to enter the mucosal cells. This means that most absorbed iron will be bound to small ligands, with the resulting complexes not carrying a high charge. Once absorbed, the iron enters the iron storage protein, ferritin, or is passed out of the mucosal cells into the blood, where it is carried by the iron transport protein, transferrin, to tissues requiring iron (*134*).

Iron transport and storage in animals are tightly regulated. Ferritin is an abundant store in tissues involved in hemoglobin breakdown (*133, 134*) (e.g., liver and spleen) and transferrin or related proteins are available in most body fluids to sequester free iron (*134*).

Iron metabolism in animals has been extensively considered elsewhere [for example, the reviews by Reddy *et al.* (*113*) and Theil and Aisen (*134*)] and so we limit ourselves to specific points concerning the structure and properties of ferritin and transferrin.

B. Iron Metabolism in Bacteria

One striking similarity between iron metabolism in animals and bacteria is that both contain ferritin (*63*). Bacterial ferritin, or bacfer, resembles ferritin in a number of respects (Section IV), but a key difference is that bacfer is also a *b*-type cytochrome (*129*), cytochrome b_1 (*126*). Thus the question arises: Is it primarily a cytochrome or primarily an iron storage protein? This question opens up a large number of avenues of research, some of which are described in Section IV, that we believe will help define further how animal ferritin functions. One important area is that of genetic control of bacfer expression.

The siderophore-mediated iron uptake process involves an extensive set of enzymes to synthesize the siderophores, and proteins to assist the uptake into the organism of the iron–siderophore complex. The biosynthesis of these proteins is controlled by an iron (**Fe**) **u**ptake **r**egulatory protein (**fur**) (*59*). The **fur** protein from *Escherichia coli* has been shown to be a 17-kDa monomer (*119*) that binds (*8*) Fe^{2+} but not Fe^{3+}. The Fe^{2+}–**fur** complex then binds to DNA at a region known as the "iron box," repressing the synthesis of the mRNA coding for the proteins necessary for siderophore-mediated iron uptake (*38*). Thus it acts as a classical negative repressor. Such a regulatory role for Fe^{2+} had been envisaged by Williams (*151*), who emphasized that because Fe^{2+} has lower binding affinities for ligands than does Fe^{3+}, it will be more dissociable, a desirable property for a reversible control signal. The fact that this low binding strength, largely a reflection of the lower

charge, permits faster exchange of ligands is indicated by the water exchange rates given in Table I.

The presence of **fur** and bacfer seems somewhat anomalous in that both interact with iron in excess of that required for synthesis of other cytochromes, and nonheme iron proteins. If the **fur** protein has a higher affinity for Fe^{2+} than does bacfer, and the siderophore route is the only way for iron to enter the cell, bacfer would presumably not get loaded with iron. Finding out whether bacfer synthesis is controlled by the **fur** protein might give further insight into this. Thus the paper by Andrews *et al.* (*4*) showing that the gene for *E. coli* bacfer is not associated with an "iron box" sequence is intriguing. Perhaps there is available an additional route to the siderophore-mediated uptake for iron to enter the cell.

If bacfer is not primarily an iron storage protein then the question arises as to what is its function. Here, the identification (*126*) of bacfer as cytochrome b_1 may be important. Cytochrome b_1 has been proposed to have a respiratory role at the level of pyruvate oxidation in *E. coli* (*37, 54*). Thus bioenergetic studies of bacfer are needed. In this connection it should be noted that bacfer has been isolated from a wide variety of bacteria, including *Pseudomonas aeruginosa* (*73, 100*), *Azotobacter vinelandii* (*25, 69, 129*), *E. coli* (*4, 156*), and *Rhodobacter sphaeroides* (*73*). In addition, the protein appears to be produced in maximal amounts only when the cell culture has entered the late exponential or stationary phase (*72*). Thus any respiratory role appears to be fairly widespread and not critical for growth, at least in the early log phase.

The idea that *in vivo* bacfer may serve as an electron store has been investigated by ^{57}Fe Mössbauer spectroscopy. Cells of *P. aeruginosa* grown to the stationary phase on ^{57}Fe-enriched media were shown to contain bacfer in which at least 90% of the iron was oxidized (*72*). Therefore, bacfer is unlikely to act simply as an electron store.

A final point on the function of bacfer is that, given it contains a high level of phosphate (Table II) when loaded with iron, one of its roles may be as a phosphate store.

C. Toxicity of Aluminum

The toxicity of aluminum is not in doubt (*53, 75*), though its relationship with certain neurological disorders, such as Alzheimer's disease, has not been firmly established (*109*). The clearest evidence that aluminum is toxic comes from patients with renal failure who have

undergone hemodialysis. When this treatment was first introduced many of the patients placed on it subsequently suffered from bone damage and neurological problems. The discovery that aluminum was the toxic agent led to the development of water treatment prior to dialysis to remove aluminum, and a resultant reduction in the incidence of the dialysis-related diseases [for further details, see the reviews by Kerr and Ward (*75*) and Wills and Savory (*154*)]. It is now generally recognized that aluminum presents a serious problem for people with kidney damage, and may constitute a health problem for a much larger fraction of the population.

The similarity (*89, 152*) between the chemical properties of Al^{3+} and Fe^{3+} explains why aluminum can be taken up and distributed in animals: it uses the iron distribution pathway. However, aluminum is unlikely to replace iron in many, if any, of its functional sites. Thus, for example, Al^{3+} has not been found in significant amounts in heme. The damaging effects of Al^{3+} appear to arise because, once mobilized within the body, it can replace Mg^{2+} and Ca^{2+} (*83, 89*), and also because insoluble aluminum-containing materials are formed. Thus aluminum toxicity is probably not directly related to iron biochemistry.

Apart from a structural role in bone, Ca^{2+}, and Mg^{2+}, have important functions as control and catalytic ions in a range of systems, for example, in the operation of kinases, in which the rapid exchange of metal ligands is vital. Al^{3+} can replace Ca^{2+} and Mg^{2+} at many of these sites, but cannot function adequately in their place, because of its slow ligand exchange reactions (Table I). In fact, aluminum is a potent inhibitor of many enzymes requiring phosphates for activity [see the reviews by Martin (*83, 89*)]. Based on this, the proposal (*18*) has been made that its interference with the phosphatidylinositol-derived second messenger system is a contributing factor in the development of Alzheimer's disease.

Insoluble aluminum species are found associated with neurofibrillary tangles, one of the main pathological signs of neurological disorders, such as Alzheimer's disease (*109*). Silicon is also associated with these tangles, consistent with the formation of aluminosilicates (*17, 40, 109*). Aluminosilicates are present in the tissue of people affected by endemic elephantiasis of the lower legs, or podoconiosis (*110, 111*). This condition is prevalent in many poor regions of Africa where the soils contain a high proportion of silica and aluminosilicates and the people often are barefooted. The aluminosilicates enter the body by penetrating the skin of the foot and then pass into the lymphatic nodes. The elephantiasis is proposed to result from the body's attempts to deal with the irritant particles, which eventually leads to the formation of

fibrous proteins enclosing the particles. It will be interesting to discover whether the fibrous proteins forming the Alzheimer neurofibrillary tangles are related to those produced in cases of podoconiosis.

The relationship between aluminum and silicon in biology has been explored by Birchall (*17, 19, 27*). He suggests the essential requirement for silicon exhibited by animals is because the silicon fulfills a protective role in complexing aluminum.

IV. Ferritin and Bacterial Ferritin

A. Structural and Functional Characteristics

Ferritin consists of 24 subunits, each with a molecular weight of ~20,000. These form a protein shell ~20 Å thick, in most cases via noncovalent packing interactions, surrounding a space of ~80 Å diameter into which the nonheme iron core is laid down. Channels through the coat, formed by the subunit interfaces, allow iron to enter and leave the core (*49, 63*). These features are discussed in the article by Harrison and her co-workers in this volume.

Many animal ferritins are not simple homopolymers; different types of subunits occur (*63, 113*). These differ in size and hence have been termed heavy and light subunits. The proportions of these types in a given ferritin oligomer are tissue dependent. A further complication is that covalently bound subunit dimers occur. In some cases, for example, sheep spleen ferritin (*95*), these constitute as much as 50% of the oligomer. The functional consequences of this structural diversity are still being investigated, but it is clear that the ease of iron uptake and release is dependent on the subunit distribution and extent of cross-linking within an oligomer.

A high-resolution X-ray diffraction structure of bacterial ferritin is not yet available but it is clear from molecular weight measurements, sedimentation velocities, and low-resolution X-ray data that bacterial ferritin consists of 24 subunits of molecular weight 18,000–21,000, depending on the source (*63*). The overall diameter of *E. coli* bacfer is 119–128 Å, consistent with this protein having the same general shape as animal ferritin.

Escherichia coli bacfer consists of a single type of subunit (*4, 156*), but *P. aeruginosa* bacfer consists of at least two types of subunits whose relative proportions vary with growth in a manner yet to be defined (*73*). *Azotobacter vinelandii* bacfer also has two types of subunits (*60*).

The X-ray structure of horse spleen ferritin reveals that each of the subunits is based on a 4-α-helical bundle type of super-secondary structure (*46*). This is also likely to be the type of structure of the bacfer subunits, given the overall similarity of the two types of ferritins (*63*). This view is supported by the secondary structure prediction analysis for *E. coli* bacfer reported by Andrews *et al.* (*5*).

1. Prosthetic Groups

Unlike animal ferritin, bacfer contains noncovalently bound heme. In the *E. coli* and *A. vinelandii* proteins as isolated, there is one heme per two subunits (*127, 129, 145*), but in the *P. aeruginosa* protein the highest heme : subunit ratio so far observed for the protein as isolated is 9 : 24 (*73*). However, additional heme has been added to the *E. coli* (*30*) and *P. aeruginosa* (*71*) bacfers to give a ratio of one heme per subunit. The reason bacfer is not isolated with its full complement of heme may be because the heme, being noncovalently bound to the protein, is lost during purification. Alternatively, there may be a biochemical explanation, such as insufficient production of heme by the organism. Whatever the explanation, it will be interesting to discover if the rate of iron deposition and release, and the nature of the iron core, are influenced by the heme content.

In the UV–visible region of its electronic spectra, the distinctive heme absorption bands of bacfer resemble strongly (*97*) those of animal cytochrome b_5 (Figs. 2 and 3). However, in the near-infrared region there are substantial differences. These differences arise because the two proteins have different types of heme axial ligands. In cytochrome b_5 the ligands are two histidine nitrogens (*91*) and in bacfer the ligands are two methionine sulfurs (*29*). Based on the secondary structure analysis of *E. coli* bacfer (*5*) and the amino acid sequence data of the *E. coli* (*4*) and *P. aeruginosa* (J. Keane, J. B. Findley, F. H. A. Kadir, and G. R. Moore, unpublished data) bacfers and the related *Nitrobacter winogradskyi* cytochrome b_{558} (*78*), the most probable heme ligands are Met 1 and Met 144 (*29*).

It is a major surprise (*98*) to find a bis(Met)heme with such a low redox potential (Table II) and a possible explanation for this is that the heme is located in a region of the protein with a relatively high local negative charge density arising from carboxylic acid groups.

The EPR and electronic spectra of the heme of bacfer are not significantly affected by the presence of the nonheme iron core (*30, 73*). Thus the redox potential difference of the heme iron in apo- and holobacfer (*145*) (Table II) is most probably caused by a long-range electrostatic effect of the core charge. This indicates that the core carries an overall

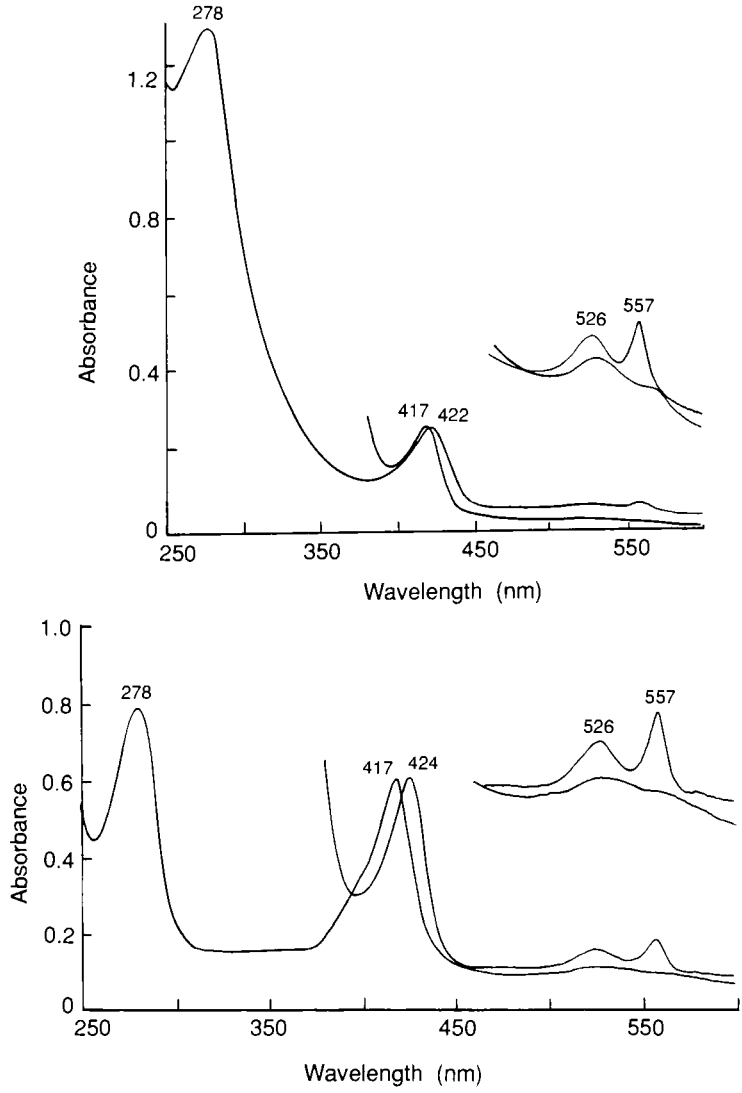

FIG. 2. Optical absorbance spectra of *Pseudomonas aeruginosa* holoferritin (top) and apoferritin (bottom) with a heme : protein subunit ratio of 9 : 24. The bands at 422, 424, 526, and 557 nm come from the dithionite-reduced protein. The nonheme iron core contributes strongly to the 278-nm absorption band. The sample conditions were 0.425 mg ml $^{-1}$ protein; 25 m*M* phosphate; pH 7.4; 20°C. The expanded α/β regions of the spectra shown as insets have an absorbance scale 3.33 times that of the main spectra.

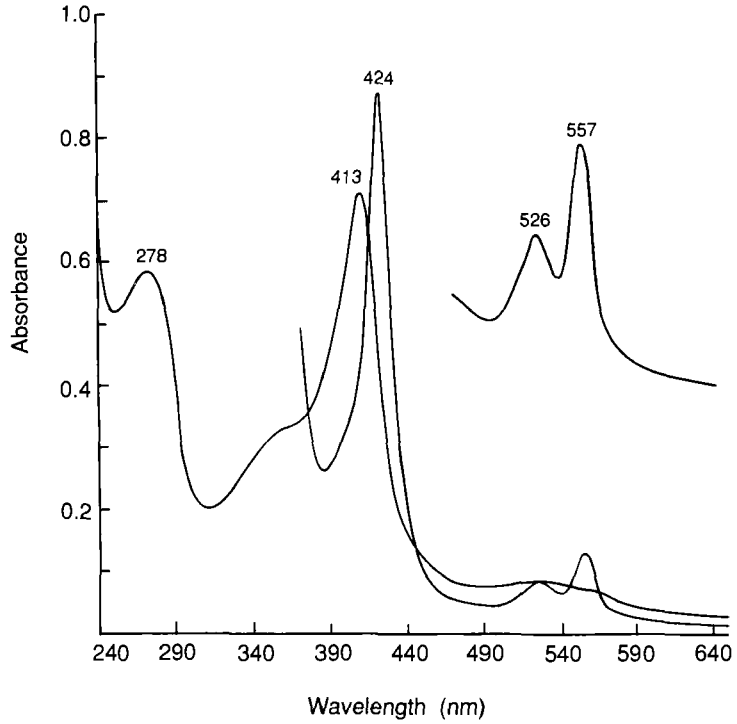

FIG. 3. The optical absorption spectrum of beef liver cytochrome b_5. The bands at 424, 526, and 557 nm come from the dithionite-reduced protein. The sample conditions were 0.375 mg ml^{-1} protein; 25 m*M* phosphate; pH 7.4; 20°C. The expanded α/β region shown as an inset has an absorbance scale 2.5 times that of the main spectra.

negative charge, because the heme redox potential is lower in its presence.

Maruyama and Listowsky (*90*) reported that ferritin from human liver, both heavy and light subunits, and horse spleen and rat liver ferritins possess fluorescence spectra in which excitation at 350 nm causes emission at 432 nm. Similar spectra (Fig. 4) have been reported for the bacterial ferritins from *P. aeruginosa* (*100*), *A. vinelandii* (*73*), and *E. coli* (*30*). Although the fluorophor has not been identified, it has been shown to have characteristics different from those of common flavins and folates (*100*). Possible candidates for the identity of the fluorophor include modified amino acids, such as derivatives of tryptophan and pyridinoline, and a redox-active group, such as pyrroloquinoline quinone or pterin. If it turns out to be one of the former groups, then the fluorescence will probably arise from oxidative dam-

TABLE II

PROPERTIES OF FERRITIN AND BACTERIAL FERRITINS

Source	Subunit types[a]	Fluorescence (445 nm)	Core				Reduction potential ($E_{m,7}$; mV)		Ref.
			Fe w/w (%)[b]	Fe : phosphate	Morphology[c]	Blocking temp.[d]	Core	Heme	
Horse	2	+	—	—	c	~40K	−190	—	*32, 49, 90, 118, 144*
Human	2	+	29	21 : 1	c	~40K	—	—	*32, 49, 90, 118, 84*
Patella laticostata	?	?	—	40 : 1	lc	~30K	—	—	*118*
Escherichia coli	1	+	—	—	a	>3K	—	—	*5, 10, 30, 118*
Azotobacter vinelandii	2	+	—	1.4 : 1	a	~20K	−445	−225 (a) −475 (h)	*60, 73, 145*
Pseudomonas aeruginosa	2	+	9	1.7 : 1	a	>3K	—	—	*73, 100, 117*

[a] Horse and human ferritins have considerable subunit diversity. The types indicated in the table are the heavy and light types. The number of subunits in *P. laticostata* ferritin has not been reported, but the number in a related chiton ferritin, that of *Clavarizon hirtosa*, has been shown to be two (*77*).

[b] These are experimentally determined values for polydisperse preparations of the holoferritins.

[c] a, Amorphous core; c, crystalline core; lc, core of limited crystallinity.

[d] This temperature is determined by ^{57}Fe Mössbauer spectroscopy. The blocking temperature, the spectral superparamagnetic transition temperature, is the temperature at which doublet and sextet signals have equal integrated intensities. The doublet arises from small particles and the sextet from larger particles.

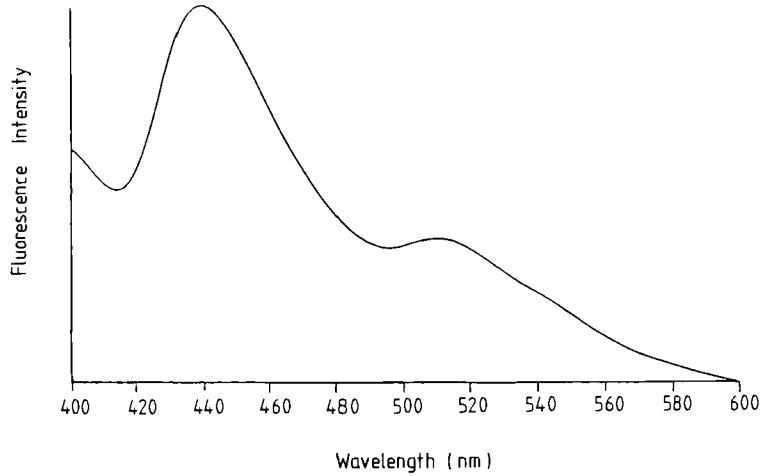

FIG. 4. Fluorescence emission spectrum of *Pseudomonas aeruginosa* holobacfer (0.5 mg ml $^{-1}$ protein; 25 m*M* phosphate and 50 m*M* NaCl; pH 7.4; 20°C) resulting from excitation at 360 nm.

age to the protein coat and probably not be of major functional significance. An indication that this may be so comes from the work of O'Connell *et al.* (*106*) with hemosiderin (Section VI). Hemosiderin has (*100, 106*) a fluorescence spectrum similar to that of Fig. 4, which O'Connell *et al.* showed could also arise from the incubation of ferritin with ascorbate and liposomes, a free-radical-generating system.

2. *The Nonheme Iron Core*

Some of the characteristics of the nonheme iron cores of ferritins and bacfers are given in Table II. As can be seen there is a wide variation in properties, though these do not seem to depend solely on overall core size. The mean core diameters measured by electron microscopy for human ferritin (*84*) and *P. aeruginosa* bacfer (*100*) were found to be 70–75 and 60–65 Å, respectively, with, in both cases, a distribution of sizes between 55 and 80 or 85 Å. The maximum core attainable for human or horse ferritin corresponds to ~4500 atoms of Fe per molecule (*49*), or ~33% of the mass of the fully loaded protein. The bacfer core contains less iron and thus is considerably less densely packed.

The core of horse and human ferritin has been identified as crystalline ferrihydrite, $5Fe_2O_3 \cdot 9H_2O$ (*49, 137*), with some adventitious phosphate bound at the surface of the core. The proportion of phosphate to iron is very low. By contrast, the cores of bacterial ferritin are

amorphous and contain a higher level of phosphate. Because the average core size of bacfer is close to that of horse ferritin and the iron content is substantially lower, it appears as though the bacfer cores, at least those of *E. coli* and *P. aeruginosa,* may consist of an iron–phosphate–oxyhydroxide species. However, the high phosphate content does not correlate simply with an amorphous core, because molluscan ferritins, such as that of *Patella laticostata,* have a low phosphate content but a core with only limited crystallinity (*118*). The properties of the core of the *A. vinelandii* bacfer are also somewhat anomalous, and may result from particles of an iron oxide resembling ferrihydrite, which are interspersed with the phosphate (*85*).

^{57}Fe Mössbauer spectroscopy has proved to be an important technique for characterizing the cores of ferritins and bacfer (*118*). This method is applicable to both isolated samples (*85, 117*) and intact biological samples, such as bacterial cells (*10, 72, 112*) and cultured animal cells (*11*). The standard Mössbauer studies of ferritins involve measuring spectra over a wide temperature range, typically 200–1.3 K. The spectra change over this temperature range as the magnetic properties of the core are affected by the variation in thermal energy. Two temperatures are particularly important: the magnetic ordering temperature and the blocking temperature. These appear to depend on the size of the magnetically ordered regions of the core, with crystalline cores being more ordered than amorphous cores, and hence requiring a greater thermal energy to disrupt the ordering. Therefore the blocking and ordering temperatures of highly crystalline cores are higher than those of amorphous cores [see St. Pierre *et al.* (*118*) for further details]. This technique has been applied to native (Table II) and reconstituted holoferritins.

In vitro a crystalline iron core can be laid down in apoferritin by the addition of an oxidant, such as O_2, to an aqueous solution of a ferrous salt and apoferritin (*32, 132, 140*). The reconstituted core of horse ferritin prepared in the absence of phosphate and with O_2 as oxidant is very similar to the native core in terms of its size and Mössbauer properties (*85*). Electron microscopy, however, reveals that it is less well ordered. Reconstitution in the presence of phosphate leads to smaller cores. Reconstituted *A. vinelandii* cores in the absence of phosphate were more ordered than were the native cores, and clearly contained ferrihydrite particles and, in some cases, crystal domains (*85*). Thus the nature of the core is not determined solely by the protein coat; the conditions of core formation are also important. This is also indicated by Mössbauer spectroscopy studies of *P. aeruginosa* cells grown under conditions different than those employed for the large-scale pu-

rification of bacfer: the spectra reveal a magnetically ordered iron species with properties more similar to those of the *A. vinelandii* bacfer than to those of the purified *P. aeruginosa* bacfer (*113*).

The ease with which the core of ferritin and bacfer can be reconstituted with Fe^{2+} and an oxidant has led to work with Fe^{3+} and other metals. Fe^{3+}, added as a citrate, oxalate, or nitrilotriacetate complex, to horse holoferritin does enter the core, but only a small amount of Fe^{3+} is taken up (*139*). No Fe^{3+} was taken up by apoferritin. This work emphasizes the requirement for core formation to occur by the oxidation of Fe^{2+}, a subject we discuss in the following section.

Given the poor uptake of Fe^{3+} by ferritin, it is not surprising that Al^{3+} is not taken up in large amounts. Both we (F. H. A. Kadir and G. R. Moore, unpublished observations) and Dedman *et al.* (*36*) have found that apoferritin and holoferritin will only take up at most ~40 Al atoms per ferritin molecule after prolonged incubation with aluminum citrate. However, in their more extensive study, Dedman *et al.* found that 120 Al atoms per ferritin molecule were bound to the iron core when the exposure to aluminum citrate occurred during its reconstitution. This is comparable to the 164 aluminum atoms bound to holoferritin reported by Sczekan and Joshi (*122*).

Joshi and his co-workers (*47, 122*) have also reported that soya ferritin containing 652 aluminum atoms per molecule can be prepared *in vitro,* and that both soya ferritin and human brain ferritin isolated from patients with Alzheimer's disease had elevated levels of aluminum compared to ferritins from other sources. However, in their study, Dedman *et al.* did not find any difference in the aluminum content of human ferritin isolated from the brains of control subjects and those with Alzheimer's disease. This topic merits further study, though the problems in analyzing small quantities of material, and minimizing contamination from extraneous aluminum, make accurate work difficult.

As we stress in the following section, Fe^{2+} does not simply exit from the core of ferritin. Watt *et al.* (*144*) have completely reduced the core of horse ferritin and shown that it has a pH-dependent redox potential corresponding to $2H^+$ taken up by the core for each Fe^{3+} reduced to Fe^{2+}. In this study, anaerobic gel-filtration columns were used to separate reduced and partially reduced ferritin from contaminating small ions, and Mössbauer spectroscopy was employed to determine the extent of core reduction. Very little of the core iron was lost during the preparation, consistent with the low yields of Prussian blue formed by the addition of $[Fe(CN)_6]^{3-}$ to reduced ferritin (*73, 146*). Partially reduced ferritin cores have Mössbauer spectra indicative (*50*) of an iron

core consisting of separate Fe^{3+} and Fe^{2+} phases, rather than a homogeneous distribution. Further spectroscopic studies of reduced ferritin cores may yield data complementary to, and extending, those obtained for the fully oxidised cores.

B. Mechanisms of Iron Uptake and Release

There is still great uncertainty surrounding the formation and breakdown of the iron core of ferritin. For relatively rapid iron uptake and release the iron needs to be in the ferrous state. Thus, because the iron in the core is mostly in the ferric state, most attention has been directed toward redox-linked iron uptake and release. However, Fe^{2+} is stable in the core for considerable periods of time (*115, 144*), $\geq$16 hr in some studies (*116*), leading to the suggestion that *in vivo* not all the iron in the core is Fe^{3+}.

The extensive series of studies on *in vitro* core formation reported by Harrison and co-workers (*32, 63, 82, 140*) and others (*133*) has led to the development of a three-step hypothesis for iron uptake. In the first step, *iron entry through the channels,* Fe^{2+} passes from the outside of the protein through the channels in the apoferritin coat to the interior cavity. The second step, *nucleation,* involves iron binding to groups on the inner surface of the protein in such a way that a small cluster of coupled Fe^{3+} ions is formed. The final step, *formation of the core,* involves the extension of a small nucleating cluster by the addition and oxidation of Fe^{2+}. This stage is characterized by an initial catalytic phase, during which the small cluster rapidly expands, followed by a reduced rate of expansion once the core has attained a particular size ($\sim$1000–1500 iron atoms per molecule).

Oxidation of the Fe^{2+}, the "ferroxidase" reaction (*24*), is a key reaction during the uptake process and it has generally been assumed that it occurs once the iron has entered the central cavity. The ferroxidase center is presumed to involve carboxylates because binding of Fe^{2+} to carboxylates favors its oxidation, by simple charge considerations. Once the nucleation cluster is formed, the cluster itself, together with exposed areas of the protein inner surface, provides the Fe^{2+} binding and oxidation sites. However, Lawson *et al.* (*79*) report a result that is not consistent with this general scheme of Fe^{2+} oxidation. These authors used X-ray crystallography and site-directed mutagenesis to locate a ferroxidase center of human heavy-chain ferritin. They found it was formed $\sim$7–10 Å away from the internal surface of ferritin within the subunit 4-α-helix bundle. Coordination of the iron to Glu 27, Glu 62, His 65, and H_2O, to yield a tetrahedral site, would result in forma-

tion of Fe^{3+}. Thus the initial oxidation of Fe^{2+} may take place in the protein coat.

Stefanini *et al.* (*128*) have also attempted to identify which carboxylates form the ferroxidase center, using horse spleen ferritin. Their study, which involved the application of various spectroscopic techniques to chemically modified ferritin, identified Asp 127 and Glu 130, groups present in the threefold channel, as the ferroxidase center. However, studies by Treffry *et al.* (*141*) of site-directed mutants of human heavy-chain ferritins indicate that the ferroxidase center is not in the threefold channels.

In summary then, at least some of the Fe^{2+} appears to be oxidized in the protein coat, though it seems unlikely that significant amounts of dimeric, or higher polymeric, clusters of iron will be produced there. The displacement of one Fe^{3+} from a site in the coat by incoming Fe^{2+} is difficult enough to envisage, let alone the displacement of an iron cluster.

This feature of the process may be clarified by the use of spectroscopic methods. EPR (*28*), EXAFS (*115*), and Mössbauer (*12, 155*) spectroscopies have been applied to the study of the early stages of iron uptake and core formation and various types of iron clusters have been identified. For example, Chasteen *et al.* (*28*) have shown by EPR that a spin-coupled Fe^{2+}–Fe^{3+} dimer is produced at low iron : protein ratios. Allied to the mutagenesis–crystallographic approach, spectroscopic examinations should aid the identification of ferroxidase and nucleation sites.

In most reconstitution experiments, O_2 has been used as the oxidant. The simplest reaction would then be as follows:

$$4Fe^{2+} + O_2 + 6H_2O \rightarrow 4FeOOH + 8H^+ \quad (3)$$

This is consistent with the observed proton–electron stoichiometry required for reducing ferritin (*144*). However, Mayer *et al.* (*93*) used $^{18}O_2$ and $H_2^{18}O$ to show that nearly all the oxygen atoms in the core were derived from solvent. This important study needs extending, not only because the result described above complicates proposals for mechanisms of core formation, but also because Mayer *et al.* observed a stoichiometry of approximately two Fe^{2+} per O_2, rather than four, and did not detect any H_2O_2 or $O_2^{\cdot-}$. In addition, their study disagreed with earlier manometric experiments showing the stoichiometry was four Fe^{2+} per O_2 (*94*).

The mechanism of iron release is less clear than that for iron uptake. Fe^{3+} chelators, such as EDTA, 1,10-phenanthroline, and desferriox-

amine B, remove only a small fraction ($\leq$10%) of the core iron during a 24-hr incubation period at pH 7, and this, together with calculations of iron turnover in cells synthesizing heme, has led Theil (*132*) and others (*32*) to dismiss Fe^{3+} release from ferritin as a physiologically significant mechanism. Even the increased release of Fe^{3+} by nonphysiological hydroxypyridinones reported by Brady *et al.* (*23*) (up to 58% of the total iron released in 24 hr) is too slow to be physiologically significant. However, dihydrolipoic acid and dihydrolipoamide do release Fe^{3+} relatively rapidly (*20, 21*); under anaerobic conditions and at pH 7, the acid removes ~40% of the core iron in 60 min. Whether this is physiologically significant remains to be established but the related aerobic release of ferritin iron catalyzed by lipoamide dehydrogenase occurs predominantly by the enzymatic generation of $O_2^{\cdot-}$, which then reduces the iron (*9*).

Faster rates of release have usually been achieved when the iron has been reduced to Fe^{2+} and when a small ligand for it is present; for example (*52*), at pH 7, thioglycollic acid removed ~5% of the core iron after a 10-min incubation, and reduced flavin mononucleotide ($FMNH_2$) removed ~25% after only a 2.5-min incubation. At lower pH values the rates are even faster; for example, after 10 min, thioglycollic acid at pH 4 released ~50% of the core iron. Other reductants, such as $O_2^{\cdot-}$ (*8, 16*) diphenols (*22*), dithionite (*52*), bipyridyls (*68*), and viologenes (*68, 135*), also release iron rapidly.

One of the major unresolved questions concerning the iron-release processes described above remains: Does the small molecule travel through the protein coat and bind inside the protein, or does the iron travel outside the protein, where it binds to the small molecule (Fig. 5)? The main problem with the proposal of core penetration of small molecules is that the channels through the coat are only ~5 Å wide (*49*), whereas some of the reagents that mobilize iron are considerably larger than this. This suggests that there should be considerable steric problems for molecules passing through the channels, consistent with the diffusional studies of May and Fish (*92*). Those workers favoring core penetration by small molecules usually point to scattering studies that reveal that molecules such as glucose and sucrose are able to pass through the protein coat (*32*), and they cite the dynamic nature of protein structures to explain why the molecules are able to do so. However, it is difficult to envisage the dynamic processes in ferritin that allow a molecule such as $FMNH_2$ to enter, and exit from, the core, as has been proposed.

The application of spectroscopic techniques to this question may clarify the situation. At present, it seems clear from the experiments of

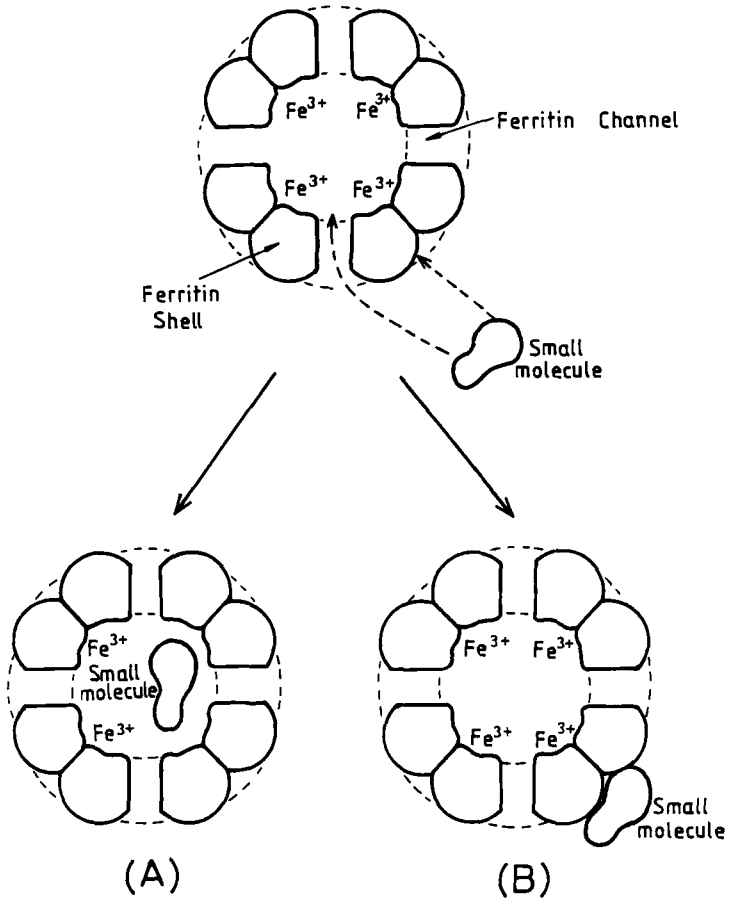

FIG. 5. Possible modes of interaction of small molecules, reductants, and/or chelators, with ferritin. (A) The small molecule penetrates to the core by passing through the channels. (B) The small molecule binds to the outer surface of the protein, either close to the mouth of a channel or, as indicated, away from the channels.

Jacobs *et al.* (*68*) that FMN cannot cross the protein coat, and the most probable explanation of the NMR data of Khodr *et al.* (*76*), concerning the interaction of ferritin with small amines, is that passage through the coat is severely limited, even for small molecules.

Another indication that small molecules cannot cross the coat is that the rates of core reduction, or iron release, for a series of redox reagents appear, with few exceptions, to depend on the redox properties of the reagent rather than its molecular dimensions (*22, 68, 70*).

Thus, in summary, our view is that iron release requires migration of the iron from the core of ferritin to the protein surface where it is bound by chelators.

The uncertainty concerning the mechanism of iron release *in vitro* is mirrored by the mechanism *in vivo*. Assuming that, *in vivo*, released iron is Fe^{2+}, many authors have attempted to identify the physiological electron donors. Because $FMNH_2$ leads to rapid *in vitro* iron release (*52, 70, 123, 142*), this has been cited as a potential physiological donor (*142*), but the amount of free $FMNH_2$ in cells is very low. Therefore, attention has begun to turn to flavoproteins (*8*) and to protein-mediated reduction (see the next section). This is one of the areas in which we believe work with bacfers can yield important results.

C. Outer-Sphere Electron Transfer and Ferritin

Electron transfer reactions can be divided into inner-sphere and outer-sphere categories (*131*). The former involve a direct interaction between the electron donor and acceptor centers via a shared ligand, whereas in the latter type of reaction the coordination spheres remain separate. Some of the redox reactions involved in forming the nonheme iron core, and in mobilizing iron from it with small reductants, may involve inner-sphere reactions, but our concern here is to consider outer-sphere reactions of ferritins.

The presence of heme in bacfer has stimulated several groups of workers to consider protein-mediated electron transfer reactions of ferritins. Moore (*97*) proposed that cytochrome b_5 might be a physiological reaction partner of mammalian ferritin based on several criteria: the optical spectral properties of the heme of bacfer resembled those of cytochrome b_5 (Figs. 2 and 3); cytochromes b_5 are widely distributed in mammalian systems in a variety of redox processes, such as cytochrome *P*-450 reduction in liver microsomes and methemoglobin reduction in red blood cells (*58, 91*); liver microsomes contain large amounts of ferritin and cytochrome b_5 (*96*); and ferritin assembly and iron uptake in rat hepatoma cells take place on the smooth endoplasmic reticulum (*80*). Since this proposal was made, however, the similarity in the heme optical spectra has been shown not to correlate with the nature of the heme ligation (*29*). Nevertheless, the reaction of mammalian ferritin with cytochrome b_5 is still of some interest and we return to this later.

Watt *et al.* (*146*) have also proposed that protein-mediated electron transfer reactions with ferritin may be physiologically important. They studied the core oxidation of reduced *A. vinelandii* bacfer and

horse spleen ferritin with the oxidized forms of cytochrome *c*, flavoproteins, ferrodoxins, and blue copper proteins and showed that in all cases the electron transfer proteins were reduced by the ferritin. However, they did not report the rates of electron transfer. Kadir *et al.* (*74*) built on this work with a range of ferrihemoproteins reacting with reduced horse ferritin (Table III). The reaction was the same as that studied by Watt *et al.* (*146*), namely, core oxidation of reduced ferritin [Eq. (4)], and the same control experiments were carried out to ensure that the reactions were the result of interprotein electron transfer and not caused by Fe^{2+} loss from ferritin.

$$\underset{\text{(core)}}{Fe^{2+}} + \underset{\text{(heme)}}{Fe^{3+}} \rightarrow \underset{\text{(core)}}{Fe^{3+}} + \underset{\text{(heme)}}{Fe^{2+}} \qquad (4)$$

The rates of ferrihemoprotein reduction with ferritin compare well with those for reduction with $[Fe(EDTA)]^-$ (Table III). $[Fe(EDTA)]^-$ was selected for comparison because it is a well-characterized reductant with some similarity to ferritin: it contains high-spin Fe^{2+} with at least four oxygen ligands, has an overall negative charge, and possesses a redox potential of ~100 mV at pH 7 (*121*). The redox potential of the core of horse ferritin is ~−190 mV (*144*) and, according to Marcus theory (*86*), the difference between these two potentials corresponds to a difference in rate of $\sim 3 \times 10^2$, assuming all other factors are the same. Thus the ferritin reductions are slower than the $[Fe(EDTA)]^-$ reductions by a factor of 10^3–10^4 when corrected for the redox potential difference.

TABLE III

RATES OF REDUCTION OF FERRIHEMOPROTEINS

Protein[a]	Charge[b]	E_m (mV)[b]	Rates (M^{-1} sec $^{-1}$) of reduction with		Ref.
			Ferritin	$[Fe(EDTA)]^-$[c]	
Cytochrome *c*	+8.4	262	1.1×10^3	2.57×10^4	*65, 98*
Cytochrome c_{551}	−2.0	260	9×10^1	4.2×10^3	*35, 98, 9*
Cytochrome b_5	−14.2	0	1.1×10^2	2.85×10^3	*91, 114*
Myoglobin	+7	50	<0.2	22	*6, 81*

[a] Protein sources are horse, *P. aeruginosa,* cow, and horse for cytochromes *c*, c_{551}, and b_5, an myoglobin, respectively; the ferritin was from horse spleen and contained ~1100 iron atoms pe molecule.

[b] At pH 7.4.

[c] At pH 7.0; EDTA, ethylenediaminetetraacetic acid.

A large difference in rate is expected for the two reductants because $[Fe(EDTA)]^-$ is smaller than the ferritin coat. Thus electron transfer takes place over a much shorter distance for $[Fe(EDTA)]^-$ than it does with ferritin. The significance of the $[Fe(EDTA)]^-$ comparison with ferritin comes in the analysis of the individual proteins. For example, reduction of metmyoglobin is slow in both cases, and reduction of ferricytochrome *c* is faster than reduction of the other cytochromes in both cases. This suggests that as far as the ferrihemoproteins are concerned, the mechanisms of electron transfer are similar for both reductants. Therefore, the reactions can be analyzed using the procedure advocated by Marcus and Sutin (*86*) [see Moore and Pettigrew (*98*) for a recent review].

In this scheme, electron transfer is proposed to take place in a noncovalently bound complex of the two proteins. The overall rate of reaction is then determined by the following parameters:

1. The redox driving energy (the difference between donor and acceptor redox potentials).
2. The work needed to form the complex.
3. The work involved in protein conformational changes accompanying electron transfer.
4. The electron transmission properties of the reactants (e.g., how readily electrons move through the organic matrix and how far they have to travel).

The comparison of cytochromes *c* and cytochrome c_{551} is particularly instructive. Here, the redox driving energies are the same, the structures of the proteins are very similar, and neither has a significant redox state conformation change (*98*). Therefore, the difference between their rates of reaction is largely due to the complex formation work terms. These are favorable for cytochrome *c*, because the reactants are oppositely charged, but unfavorable for cytochrome c_{551}. They are also unfavorable to a very great extent for the reaction with cytochrome b_5, and this, together with the reduced redox driving force for the cytochrome b_5 reaction compared to the cytochrome *c* reactions, should have produced a much lower rate of electron transfer. However, the cytochrome b_5 rate with ferritin is comparable to that of cytochrome c_{551}, reversing the $[Fe(EDTA)]^-$ trend. This indicates strongly that the cytochrome b_5–ferritin reaction is anomalous, with some feature of the system favoring electron transfer. This may be the formation of a complex, which may be only short-lived, particularly suited for electron transfer. Given the earlier proposal (*97*) concerning the

possible physiological relevance of the cytochrome b_5–ferritin reaction, this system needs to be explored further.

Interestingly, the difference in redox potential between the heme of apobacfer and the core of holobacfer is ~250 mV (*145*), which is comparable to the difference in redox potential between cytochrome b_5 and the core of ferritin of ~190 mV. It may be in both cases that the heme accepts electrons from the growing core when the incoming Fe^{2+} is oxidized to Fe^{3+}. If this is so, the marked reduction in the redox potential of the heme of bacfer once the core is partially loaded could act as a control to ensure that some of the core iron remained in the Fe^{2+} state, or even to limit the growth of the core.

Although cytochrome b_5 could be a physiological partner of ferritin, neither of the cytochromes *c* are possible physiological partners, and the myoglobin reaction is so slow, because of the intrinsic reactivity of myoglobin, that this is unlikely to be a significant reaction *in vivo*.

Work with bacfer has not progressed to the stage at which rate constants have been reported, but it is clear that a variety of hemoproteins can oxidize reduced bacfer, with rates for the bacfer heme oxidation considerably greater than those for the core oxidation (F. H. A. Kadir and G. R. Moore, unpublished observations).

The data in Table III clearly establish that electrons can travel relatively rapidly from the core of ferritin to an acceptor located outside the ferritin coat (*74, 146*). This raises the question of how the electrons travel through the coat. Two possibilities are short-range transfer via a series of electron traps, and long-range transfer (Fig. 6). In long-range transfer the electron travels through the coat without spending an appreciable period of time located on any group within the coat, but in the short-range mechanism the electrons reside on traps for a significant period of time. Possible traps for ferritin include the fluorescent

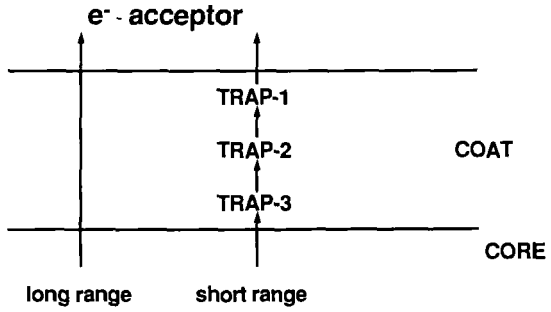

FIG. 6. Schematic representation of long-range and short-range electron transfer through the coat of ferritin.

group located in the coat and nonheme iron located in the channels (Section IV), and, of course, the heme of bacfer is an excellent electron trap.

V. Aluminum Speciation in Biology

A. Introduction

One of the outstanding chemical problems associated with aluminum biology is the characterization of its speciation, i.e., its distribution among different complexes, and their structures, stability constants, and rates of formation. As is often the case, it is the complexes formed with small ligands that present most experimental difficulties.

Martin (*89*) has provided an extensive survey of the bioinorganic chemistry of Al^{3+} that is of central importance to considerations of Al^{3+} speciation. Much of his analysis concerns the tabulation and interpretation of stability constants and we shall not duplicate this. In the following sections we describe spectroscopic studies of Al^{3+} speciation that complement calculations based on stability constants.

B. ^{27}Al NMR Spectroscopy

^{27}Al is 100% naturally abundant, has a nuclear spin quantum number (I) of 5/2, and a magnetogyric ratio of 6.9704×10^7 rad T^{-1} sec^{-1}, and therefore gives a strong NMR signal (*2*). It is one of the most receptive nuclei for NMR and is 1170 times more receptive than naturally abundant ^{13}C. Unfortunately, it has an $I > 1/2$ and thus possesses a nuclear quadrupole moment, Q, which actually has the value 0.149×10^{-28} m^2. This produces broad NMR signals in cases in which there is a significant electric field gradient at the nucleus. In practice, this means almost all cases in which the Al^{3+} ligands are not of the same type or do not form a site of high symmetry. This is illustrated by Fig. 7, which shows the ^{27}Al NMR spectra of the highly symmetric species $[Al(OH)]_4]^-$ and $[Al(H_2O)_6]^{3+}$, and spectra of less symmetric complexes of chelated Al^{3+}. The linewidth variation, which may also be influenced by chemical exchange effects, is striking.

What makes ^{27}Al NMR worth pursuing is indicated by Fig. 7: the chemical shifts of the various complexes differ markedly. The major difference between $[Al(OH)_4]^-$ and $[Al(H_2O)_6]^{3+}$ is due to the difference in coordination geometry (2)—octahedral versus tetrahedral—but even within the range associated with octahedral complexes there is a

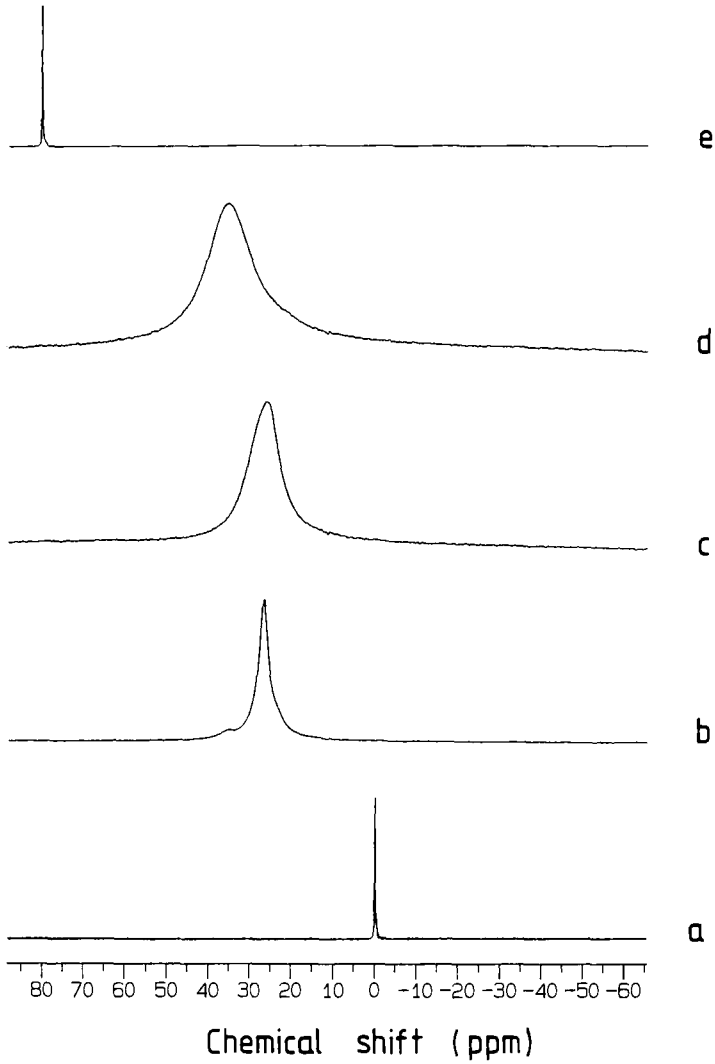

FIG. 7. The 104-MHz ^{27}Al NMR spectra at 25°C of (a) $[Al(H_2O)_6]^{3+}$, (b) [Al(1,3-propylenediaminetetraacetate)] at pH 7, (c) [Al(nitrilotriacetate)] at pH 7, (d) [Al(ethylenediaminetetraacetate)]$^-$ at pH 7, and (e) $[Al(OH)_4]^-$.

wide variation of chemical shift for apparently similar types of ligands. The origin of this variation is currently under investigation by a number of groups. Some of the possibilities are that the chemical shift varies with denticity of the ligand (*66*), charge on the ligand, or nature

of the immediate donor atoms to Al^{3+} (*2*, *44*). Currently we believe that the identity of the donor atoms is most important.

An indication of the potential of ^{27}Al NMR is given by the studies of Al^{3+}–citrate and Al^{3+}–albumin shown in Figs. 8 and 9, respectively. The ^{27}Al NMR spectra of a 1 : 1 mixture of Al^{3+}–citrate at pH 7.4 (Fig. 8) shows there are at least three signals and thus a minimum of three different Al^{3+}–citrate species (*44*).

The ^{27}Al NMR spectra of Al^{3+} added to albumin (Fig. 9) reveal that at least two Al^{3+} ions bind to one albumin molecule (*45*). From the chemical shift value we identify the Al^{3+}-binding site to be octahedral with oxygen donor atoms. Thus the most likely binding site is one consisting of carboxylates of albumin, probably at least three to give a reasonably strong binding constant. The remaining ligands to the bound Al^{3+} are probably water molecules.

The large linewidth for the ^{27}Al NMR signal of Al^{3+} bound to albumin is a consequence of the quadrupole moment of ^{27}Al. When the electric field gradient is not zero, the size of the molecule becomes important, and generally as the size increases, the line broadens. There are a number of NMR methodologies that may overcome some of the NMR broadening and we anticipate that these will be explored in the near future. If the problem of broad lines can be overcome, then ^{27}Al NMR will be a powerful method for studying Al^{3+} speciation.

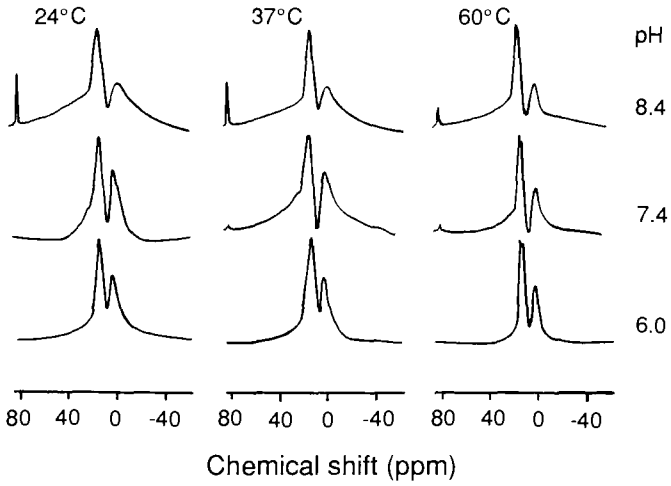

FIG. 8. The 104-MHz ^{27}Al NMR spectra of 1 : 1 mixtures of Al^{3+} and citrate. The sharp peak at ~80 ppm in some spectra comes from $[Al(OH)_4]^-$. The remaining signals are from Al^{3+}–citrate complexes; at least three peaks overlap in the region 0–35 ppm.

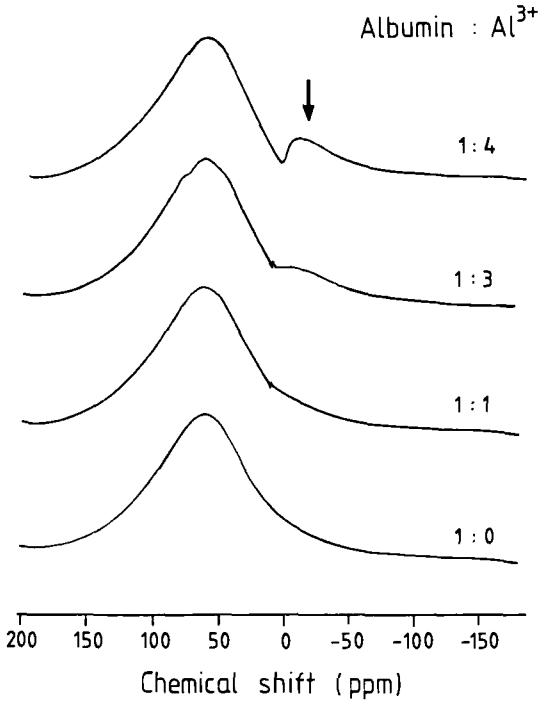

FIG. 9. The 104-MHz ^{27}Al NMR spectra of Al^{3+} binding to bovine serum albumin (130 mg ml^{-1}; 100 m*M* Tris–HCl; pH 7.4; 37°C). The molar ratios are indicated. The signal of Al^{3+}–albumin is indicated by the arrow; the broad signal between 0 and 120 ppm is a background signal from the instrument.

C. Aluminum Speciation in Blood Plasma

The uptake of Al^{3+} from the blood by tissues depends in part upon the properties of the Al^{3+}–ligand complexes that solubilize the Al^{3+}. Thus the distribution of Al^{3+} among its different ligands is valuable information for assessing the biological availability of Al^{3+}. Using calculations based on measured stability constants and chromatographic separation of the constituents of blood plasma (*13, 14, 33, 42, 53, 88, 89, 138, 154*), it has been suggested that the main carriers of Al^{3+} in normal blood are citrate, transferrin, a combination of transferrin and citrate with transferrin binding ~80% of the Al^{3+}, and a combination of transferrin and albumin. Because of the current confusion concerning the Al^{3+} carriers, Fatemi *et al.* (*43*) reexamined this topic in a spectrophotometric study of Al^{3+} binding to transferrin in the presence and absence of citrate and albumin.

The spectrophotometric technique exploits the fact that when it binds to transferrin, Al^{3+} replaces hydroxyl protons from two tyrosines, thus causing a change in the UV region of the spectrum (Fig. 10). Titration of the spectral change as a function of $[Al^{3+}]$ at constant transferrin concentration allows the binding stoichiometry and stability constant to be measured. This approach has been used to study the interaction of a large number of metals [e.g., Nd^{3+}, Sm^{3+}, Zn^{2+}, and Ga^{3+} (*61, 62, 136*)] with transferrin, including Al^{3+} (*33, 43, 136, 138*); some of these metals would otherwise be spectroscopically silent. Such

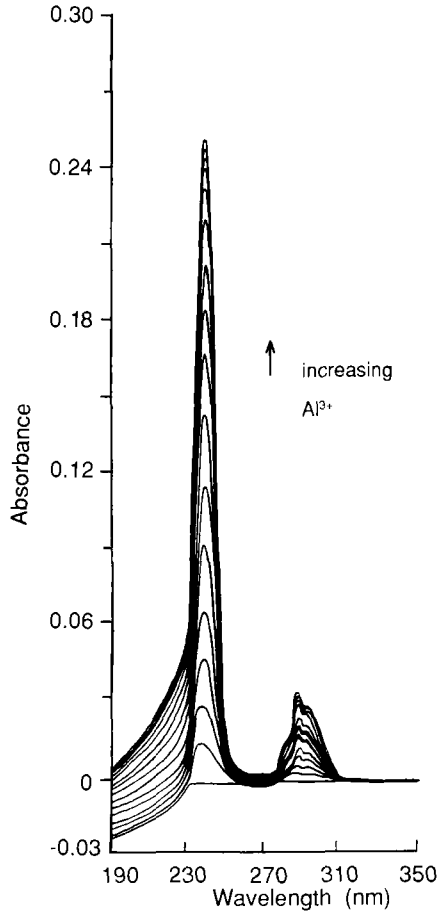

FIG. 10. Optical difference spectra for the binding of Al^{3+} to transferrin (14 μM; 25 mM HCO_3^-; 100 mM Tris–HCl; pH 7.4; 25°C). Increasing amounts of $[AlK(SO_4)_2]$ cause the observed spectral changes as a result of the formation of tyrosinate-bound Al^{3+} within transferrin.

studies have shown that the extraction of absolute binding constants is not straightforward and therefore we shall not address that topic here [but see Aisen *et al.* (*1*) and Fatemi *et al.* (*43*)].

Fatemi *et al.* (*43*) applied this approach to investigate the competition among albumin, citrate, and transferrin for aluminum. The transferrin, albumin, and citrate used in these experiments were in the same molar ratios as those occurring in normal plasma, but at 0.2 times the actual levels. The presence of albumin and citrate depressed the Al^{3+} loading of transferrin at relatively high [Al^{3+}] and at a ratio of $2Al^{3+}$: 1 transferrin; only ~50% of the Al^{3+} was bound to transferrin. Albumin at this high concentration affected Al^{3+} binding to transferrin more than did citrate. Thus these data support the view that Al^{3+} binding to albumin and citrate is only significant at relatively high concentrations of plasma Al^{3+}. They also indicate that albumin is more important than citrate as an Al^{3+}-binding agent in plasma, even though its stability constant with Al^{3+} is less than that of citrate.

In many of the chromatographic studies of plasma, a pool of Al^{3+} bound to low-molecular-weight ligands was found (*14, 42, 53*). This fraction may correspond to the component that is ultrafilterable (*53*); in plasma, in which the transferrin-binding capacity is exceeded, such as occurs with some hemodialysis patients, as much as 30–50% of the Al^{3+} is ultrafilterable. Williamson *et al.* (*153*) attempted to identify this low-molecular-weight fraction by ^{27}Al NMR spectroscopy. Using plasma from a variety of sources, including patients with Alzheimer's disease, Williamson *et al.* added Al^{3+} to a final concentration of 100 μM to saturate the transferrin; ~50 μM was needed for this. If the remaining 50 μM had formed an Al^{3+}–citrate complex this would have been detected. However, no such complex was seen. In fact, no discrete low-molecular-weight complex was identified except in plasma samples that had become aged and whose pH had risen above 7.4, usually to ≤8.0. In these samples a signal due to $[Al(OH)_4]^-$ was observed. Williamson *et al.* suggested that much of the excess Al^{3+} was bound to albumin, and thus was not detectable under the conditions of the NMR experiment because Al^{3+}–albumin has a broad signal (Fig. 9), with the low-molecular-weight fraction forming no more than 20 μM of any species. It is also possible that some of the Al^{3+} may have formed a mixed hydroxide–carbonate complex. This suggestion is based on the observation of $[Al(OH)_4]^-$ in some aged samples, the relatively high concentration of bicarbonate (25 mM) compared to Al^{3+} (100 μM) at the outset of the experiment, and the known propensity of Al^{3+} and CO_3^{2-} to complex together in solution and in minerals (*27, 108*). Such an Al^{3+} pool may be the colloidal pool suggested by Bertholf *et al.* (*14*).

Thus, in summary, transferrin and albumin appear to be the main carriers of Al^{3+} in plasma, and citrate does not seem to be a significant Al^{3+} binder.

D. Aluminum–Citrate Complexes

The nature of the complexes formed between Al^{3+} and citrate is important because even though citrate may not be a substantial binder of Al^{3+} in plasma, it can help Al^{3+} cross membranes. This may occur both for Al^{3+} transport within the body and for Al^{3+} uptake from the gut. For example, citrate in the diet leads to enhanced Al^{3+} levels in the brain and bones of rats (*124*), and to enhanced blood levels in humans taking $Al(OH)_3$–based antacids together with citrate (*125*).

A key assumption about the ability of small complexes to cross membranes passively relates to their overall charge: uncharged complexes cross membranes more easily than do highly charged complexes. Thus much effort has been directed toward determining the structures of Al^{3+}–citrate complexes and their corresponding stability constants.

Many stability constant measurements have been made and an indication of how misleading the analysis using them can be is provided by Findlow *et al.* (*46*) These authors aimed to calculate the speciation of aluminum in human and bovine milk using two sets of stability constants for Al^{3+}–citrate. Calculations with one set of constants indicated ~80% of the Al^{3+} was bound into neutral complexes, but the second set of constants, considered to be more accurate than the first, showed that less than 10% of the Al^{3+} was in the form of neutral complexes.

The major problem with such speciation calculations involving aluminum, in addition to the general criticism that they assume the system can be treated with equilibrium constants, is that because the kinetics of ligand exchange are relatively slow for Al^{3+} (Table I), measurements of stability constants are not straightforward. This is demonstrated clearly by Öhman (*107*), who found that some solutions containing Al^{3+} needed to age for 20 hr before they reached equilibrium. This is not the only problem, however. In the absence of other data, speciation analysis with potentiometric titration curves is difficult. Martin (*89*) has provided a review of this aspect of the field, noting that the interpretations of potentiometric titration results "are discouragingly discordant."

One simplification Martin (*89*) employed was to disregard species, usually polynuclear ones, found after long equilibration times. He then suggested that the following reactions were the physiologically impor-

tant ones for Al^{3+} and citrate, where C^{3-} is citrate that has lost three protons, and the inner-sphere H_2O is not shown:

$$Al^{3+} + CH^{2-} \rightleftharpoons [AlCH]^{+} \qquad \log K = 4.7 \tag{5}$$

$$[AlCH]^{+} \rightleftharpoons [AlC]^{0} + H^{+} \qquad pK_a = 2.5 \tag{6}$$

$$Al^{3+} + C^{3-} \rightleftharpoons [AlC]^{0} \qquad \log K = 8.0 \tag{7}$$

$$[AlC]^{0} \rightleftharpoons [AlCH_{-1}]^{-} + H^{+} \qquad pK_a = 3.4 \tag{8}$$

$$[AlCH_{-1}]^{-} \rightleftharpoons [(OH)AlCH_{-1}]^{2-} + H^{+} \qquad pK_a = 6.9 \tag{9}$$

$$[AlC]^{0} + C^{3-} \rightleftharpoons [AlC_2]^{3-} \qquad \log K = 5.0 \tag{10}$$

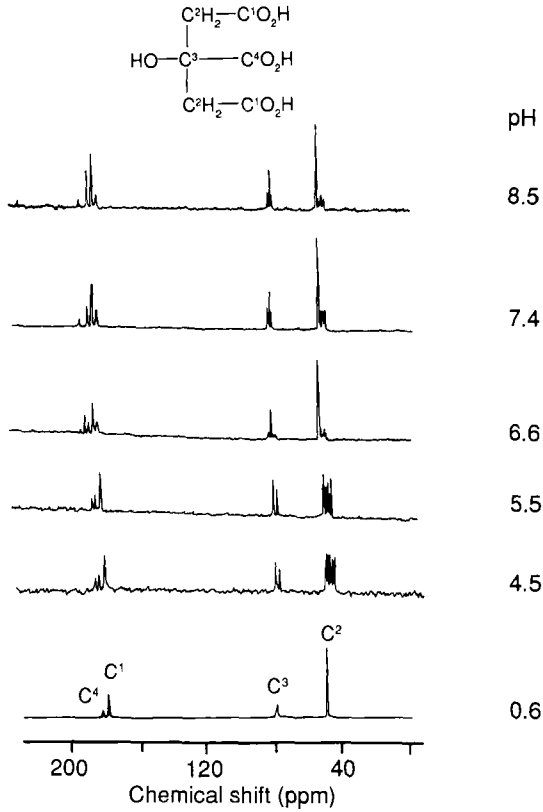

FIG. 11. The 100-MHz ^{13}C NMR spectra of 1 : 1 mixtures of Al^{3+} and citrate at various pH values. At low pH no complexation occurs, but at higher pH values complexation occurs, with multiple species being formed. The peak assignments at pH 0.6 are shown in the structure at the top.

Thus, from these equations the most likely 1 : 1 complex existing at pH 7.4 is $[(OH)AlCH_{-1}]^{2-}$ and at pH 4 is $[AlCH_{-1}]^{-}$.

Jackson (*67*) carried out a comparative study of the interaction of Al^{3+} with citrate and its analog with a hydrogen replacing the 3-hydroxy group, 3-carboxy-1,5-pentadioate, and showed clearly that the 3-hydroxy group ionized before the central carboxylic acid. Thus the structure of $[AlC]^0$ appears to be with the citrate donating three ligands (the hydroxy and terminal carboxylate groups) and three water molecules coordinating to give a six-coordinate complex (*67, 88, 101*). In $[AlCH_{-1}]^{-}$ the central carboxylate ionizes but probably remains unbound to the Al^{3+}. In $[(OH)AlCH_{-1}]^{2-}$ a coordinated water ionizes to hydroxide.

It is not immediately clear that our ^{27}Al NMR data (Fig. 8) fit into the scheme described above. The spectra at 24°C clearly arise from at least three different species not counting $[Al(OH)_4]^{-}$. The corresponding ^{13}C spectra at 27°C also provide evidence for multiple species (Fig. 11). Just considering the signal of the quaternary carbon at position 3 of citrate, there are three signals, and hence three chemical environments, at pH 7.4, in the approximate intensity ratio of 1 : 1 : 2. It may be that the NMR data are distinguishing different isomers of $[(OH)AlCH_{-1}]^{2-}$, but this cannot be the sole explanation for the multiplicity of signals (*44*). Thus further work is required to characterize the complexation of Al^{3+} by citrate.

VI. Aluminum Toxicity and Iron Overload

We end this comparative review of iron and aluminum biochemistry with a consideration of the biochemical consequences of iron overload in animals and their relationship to the effects of elevated levels of aluminum.

Iron-overload disease, or hemochromatosis, may occur as a consequence of an, as yet, undefined genetic defect, or as a secondary effect of another medical disorder, such as thalassemia. In the former condition, primary hemochromatosis, iron accumulates in various tissues because of a lack of control of iron absorption from the gut. In the latter, or secondary hemochromatosis, the accumulation of iron results from the breakdown of red blood cells and the consequent need for frequent blood transfusions, which lead to an increase in the levels of tissue iron. In both cases the predominant store for iron is hemosiderin (*147*).

The level of soluble iron in plasma is raised in cases of iron overload, with iron being bound to proteins, transferrin, and albumin, and with

low-molecular-weight complexes being formed. These latter complexes have not been determined but are presumed to include Fe^{3+} binding to citrate. Because the stability constants for Fe^{3+} and citrate are greater (*88*) than for Al^{3+} and citrate (by a factor of $\sim 10^2$–10^3), significant amounts of Fe^{3+}–citrate complexes may be formed under conditions in which only small amounts of the Al^{3+}–citrate complexes are formed (Section V,C). In addition, complexes formed between iron and CO_3^{2-}/HCO_3^- should be considered.

Electron microscopy and Mössbauer spectroscopy show (*39*) that the iron in hemosiderin is in the form of mineral phases, much like the iron core of ferritin. However, hemosiderin is insoluble in water at pH 7 and thus has not been chemically characterized to the same extent as ferritin. Nevertheless, the available evidence favors the formation of hemosiderin from the degradation and aggregation of ferritin (*3, 148*).

The extensive tissue damage associated with hemochromatosis is usually ascribed to the formation of free radicals that damage subcellular membranes, causing the organelles to become leaky (*105, 148*). However, comparison with aluminum suggests other mechanisms may also be operative. Thus iron, like aluminum (Section III), may cause damage because it displaces magnesium and calcium from key biochemical interaction sites. Also, insoluble iron deposits may stimulate the formation of free radicals, as well as produce them directly, and may activate other defense mechanisms in the body that attempt to remove or sequester particulate matter, as may happen in certain cases of aluminum overload (Section III).

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The subject of podoconiosis, discussed in Section III,C of this article, and the possible role of aluminosilicates as causative agents for the disease, were first described to us by the late Dr. E. W. Price. One of his aims in doing so was to publicize the possible

connection between this disease and Alzheimer's disease. We hope this account will help to achieve this.

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