

## Summary

When we began our neutron diffraction work in early 1974, only four metal hydride complexes had been investigated by this technique.<sup>7,8,49,50</sup> Four years later,

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 (57) A. J. Schultz, J. M. Williams, R. B. Calvert, J. R. Shapley, and G. D. Stucky, *Inorg. Chem.*, **18**, 319 (1979).  
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 (59) B. F. G. Johnson, J. Lewis, D. Pippard, P. R. Raithby, G. M. Sheldrick, and K. D. Rouse, *J. Chem. Soc., Dalton Trans.*, in press.  
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 (61) P. L. Johnson, S. A. Cohen, T. J. Marks, and J. M. Williams, *J. Am. Chem. Soc.*, **100**, 2709 (1978).  
 (62) J. M. Williams, R. K. Brown, A. J. Schultz, G. D. Stucky, and S. D. Ittel, *J. Am. Chem. Soc.*, **100**, 7407 (1978).  
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through the efforts of several different groups, the situation has changed dramatically: there are now over two dozen metal hydride complexes whose structures have been accurately analyzed by single-crystal neutron diffraction, as summarized in Table I. It seems clear that neutron diffraction will continue to be extensively used to analyze such compounds. At the rate new results are appearing, it may not take long before most of the major types of metal-hydrogen linkages are explored.

We wish to thank our many collaborators in this project for their efforts: L. C. Andrews, W. E. Carroll, H. B. Chin, D. W. Hart, Å. Kvik, R. A. Love, R. K. McMullan, J. P. Olsen, D. L. Tipton, G. J. B. Williams and R. D. Wilson. We also acknowledge the generosity of P. Chini, H. D. Kaesz, J. Müller, G. W. Parshall, and F. N. Tebbe for the samples of  $[(Ph_3P)_2N]^+ [HCo_6(CO)_{15}]^-$ ,  $HfFeCo_3(CO)_9(P(OMe)_3)_3$ ,  $H_3Ni_4Cp_4$ , and  $H_3TaCp_3$  used in this work, and valuable discussions with R. Hoffmann. We thank E. R. Bernstein, L. F. Dahl, T. J. Marks, S. A. Mason, J. L. Petersen, A. J. Schultz, J. A. Shapley, G. M. Sheldrick, and J. M. Williams, who have communicated their results to us prior to publication. Technical assistance in operating the neutron diffractometers was provided by J. Henriques. Research at Brookhaven National Laboratory was performed under contract with the U.S. Department of Energy and supported by its Office of Basic Energy Sciences. Research at the University of Southern California was supported by National Science Foundation Grants CHE-74-01541 and CHE-77-00360 and the Petroleum Research Fund, administered by the American Chemical Society.

# Coordination Chemistry and Microbial Iron Transport

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Iron is an essential element for all living things.<sup>1-3</sup> This is a consequence of the extremely large number of enzymes and proteins in which iron plays an essential part. The ubiquitous role of metal ions in biochemistry has in fact generated the whole area of "bioinorganic chemistry", which has been almost synonymous with studies of how metal ions interact with biological molecules. Our interest has been from another perspective—how the biological systems acquire the metal ion for incorporation into macromolecules.

Kenneth N. Raymond was born in Astoria, Oregon, in 1942. He completed his B.A. degree at Reed College and his Ph.D. at Northwestern University and joined the faculty at the University of California, Berkeley, in 1968. His research interests are in coordination chemistry and include microbial iron metabolism, specific sequestering agents for toxic metal ions, and the structure and bonding of transition-metal and organometallic complexes.

Carl Carrano was born in New York, in 1950. He received his B.S. degree from the University of California, Santa Barbara, and his Ph.D. from Texas A&M University while working with Minoru Tsutsui. He is at present a postdoctoral fellow in the laboratory of Professor Raymond, and will be joining the faculty at the University of Vermont in the Fall.

Specifically, we have been concerned with the coordination chemistry of low-molecular-weight chelating agents called siderophores: molecules which are manufactured by microbes to obtain the iron they need to grow.<sup>4</sup>

Since the evolution of an oxidizing atmosphere on this planet, the availability of iron to aerobic organisms in an aqueous environment has been limited by the extreme insolubility of ferric hydroxide ( $K_{sp} \approx 10^{-38}$ ).<sup>5</sup> With the equilibrium concentration of ferric ion at pH 7 about  $10^{-18}$  M, even diffusion limited transport would

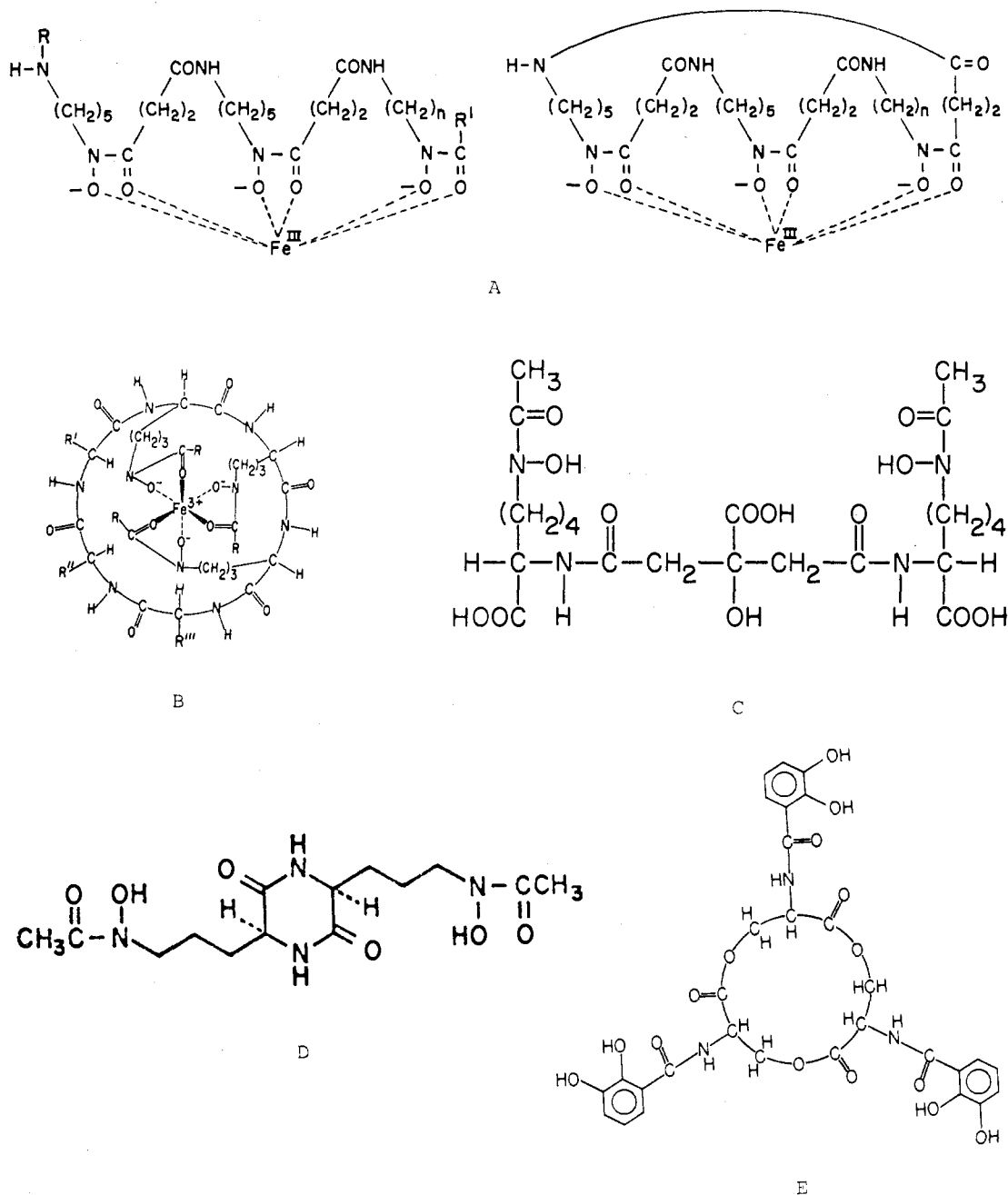
(1) J. B. Neilands in "Microbial Iron Metabolism", J. B. Neilands, Ed., Academic Press, New York, 1974.

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(5) W. M. Latimer, "Oxidation Potentials", Prentice-Hall, Englewood Cliffs, N.J., 1952.



**Figure 1.** Structural formulas of representative siderophores. (A) Linear and cyclic ferrioxamines. When linear and  $R = H$ ,  $R' = CH_3$ , the compound is ferrioxamine B; when cyclic and  $n = 5$ , the compound is ferrioxamine E. (B) The ferrichromes. For  $R' = R'' = R''' = H$  and  $R = CH_3$ , the compound is ferrichrome itself. (C) Aerobactin. (D) Rhodotorulic acid. (E) Enterobactin.

be many orders of magnitude too slow to provide sufficient amounts of iron to a microbial cell. This problem has been circumvented by microbes through the production of the siderophore (previously also called siderochrome) chelating agents which solubilize ferric iron and facilitate its transport into bacteria, molds, yeasts, and fungi. In the past 10 to 15 years a number of such compounds have been found—first isolated as microbial growth factors.<sup>6</sup>

In addition to the interesting questions of how these organisms acquire iron via a siderophore-mediated system there are several areas of practical clinical importance in which siderophores play a part. The importance of iron in the pathogenicity of certain bacterial infections has been emphasized by various

workers.<sup>7,8</sup> As little as 1 to 5 mg/kg of iron injected into mice challenged with strains of *P. aeruginosa*, *S. typhimurium*, or *E. coli* decreased the number of bacterial cells required to kill 50% of the test animals by 3–5 log units, and the ultimate number of cells found in these animals was increased several thousand-fold.<sup>9</sup> It has also been suggested that siderophores are an important means for pathogenic bacteria to acquire iron from their hosts.<sup>10</sup> Furthermore, iron is the sixth most common household poison in the United States, and several siderophores or their analogues are already in use as potential antidotes to acute iron toxicity.<sup>11</sup>

(7) I. Kochan, *Curr. Top. Microbiol. Immunol.*, **60**, 1 (1973).

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Most, if not all, of these siderophores use hydroxamate, thiohydroxamate, or phenolate groups as ligands (Figure 1). Every siderophore characterized to date forms extremely stable, highly specific octahedral complexes containing high-spin ferric ion. The high-spin  $d^5$  electronic configuration of ferric ion in the siderophore complexes has two important consequences for any proposed studies of their mode of action and stereochemistry: (1) The absence of any crystal-field stabilization energy makes the complexes kinetically labile with respect to isomerization and ligand exchange in aqueous solution, thus limiting the usefulness of simple labeling experiments in cell transport studies. (2) There are no spin-allowed  $d-d$  optical transitions; thus the visible-UV and circular dichroism (CD) spectra, which are dominated by ligand to metal or ligand-ligand charge-transfer bands, are much less amenable to simple theoretical analysis and comparison.

In our research we have used metal-substituted siderophore complexes to elucidate the mechanisms of iron transport into microbial cells and have studied the physicochemical properties of the iron siderophore complexes to elucidate and delimit their iron release and exchange mechanisms.<sup>4</sup> In the first case, the substitution of chromic ion for ferric ion (which has the same size and charge) avoids the problems cited above, since (1) the kinetically inert  $d^3$  chromic ion complexes preclude any isomerization or ligand exchange during transport experiments; thus chromic siderophore complexes can be used as "biological probes" in studies of cellular iron transport; (2) the major features of the vis-UV and CD spectra of the chromic complexes are due to the well-characterized  $d-d$  transitions of the metal ion, thus allowing the use of the chromic complexes as "stereochemical probes" in assigning the geometry at the metal site. In the second case, we have examined the electrochemistry and formation constants of the siderophore iron complexes to address such questions as: Is the siderophore thermodynamically able to remove iron from transferrin (the iron transport protein in humans)? Is reduction a plausible mechanism for intracellular iron release by the siderophore? The answers to some of these questions are presented in this Account. Our studies include the trihydroxamate-containing siderophores ferrichrome and ferrioxamine B, the dihydroxamates, rhodotorulic acid and aerobactin, and the catechol-containing siderophore, enterobactin.

### Kinetically Inert Siderophores—Use as Chemical Probes

All of the hydroxamate siderophores show a characteristic broad absorption band at 420–400 nm ( $\epsilon \approx 3000 \text{ L mol}^{-1} \text{ cm}^{-1}$ ). The iron in these complexes has been shown to be high-spin  $d^5$  by magnetic susceptibility, EPR, and Mössbauer spectroscopy.<sup>1,4</sup> As such there can be no spin-allowed  $d-d$  transitions, and the visible absorption band must be assigned as charge transfer in character. This precludes detailed analysis and interpretation of the spectra of the natural ferric complexes in all but a few cases.

The ferrichromes have natural optical activity associated with the ligand; accordingly there will be

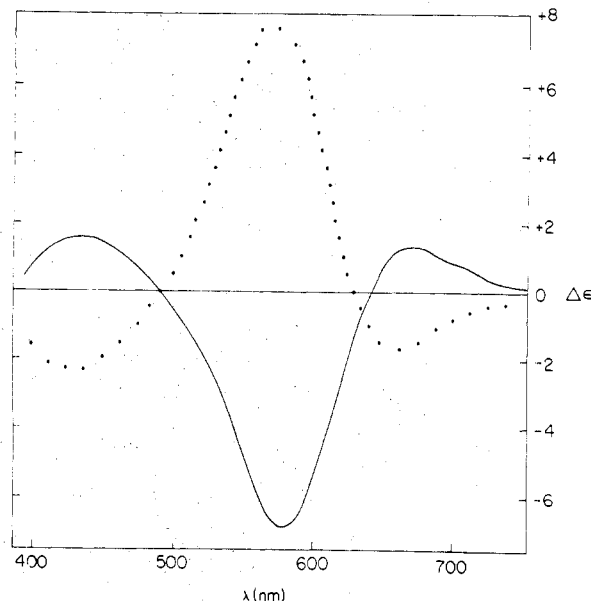


Figure 2. Circular dichroism spectra of  $\Delta$ -( $\text{NH}_4$ )<sub>3</sub>[Cr(enterobactin)] (—) and chromic desferriferrichrome (···) ( $\Delta\epsilon$  in  $\text{L mol}^{-1} \text{ cm}^{-1}$ ).

overall optical activity of the metal chelate. For an octahedral complex formed with three equivalent but optically active hydroxamate anions, there are two possible geometric isomers, cis and trans, each of which consists of  $\Delta$  and  $\Lambda$  optical isomers. These are diastereomers due to the optical activity of the ligand. Thus a total of four possible isomers are expected,  $\Delta$ -cis,  $\Delta$ -trans,  $\Lambda$ -cis, and  $\Lambda$ -trans, where the  $\Delta$  isomers have a right-handed propeller configuration looking down the  $C_3$  axis while the  $\Lambda$  isomers has the left-handed configuration. (For a more detailed explanation of the naming of these isomers, see ref 12.) In the case of the ferrichromes, examination of molecular models indicates that, while the trans isomers are improbable on steric grounds, both diastereoisomers of the cis type could form.

The molecular structures of ferrichrome A,<sup>13</sup> ferrichrysin,<sup>14</sup> ferrichrome,<sup>15</sup> and alumichrome A<sup>15</sup> have been determined by X-ray crystallographic analysis. In all cases the metal is bound by three hydroxamate groups in an octahedral cis "left-handed propeller" configuration. Although crystallization of all members of the series yields only the  $\Lambda$ -cis isomers, it is possible that both isomers of this labile ferric complex are in solution with only the less soluble  $\Lambda$ -cis isomer preferentially crystallizing out of the equilibrium mixture. However, a comparison of the circular dichroism spectrum with that of the resolved ferric tris(benzohydroxamato) complexes indicated that only the  $\Lambda$  isomer is present in solution as well.<sup>16</sup>

Further support comes from replacement of the ferric ion with the kinetically inert chromic ion, for which the large ligand-field stabilization energy of the  $d^3$  electronic configuration leads to slow ligand-substitution and

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(16) K. Abu-Dari and K. N. Raymond, *J. Am. Chem. Soc.*, **99**, 2003 (1977).

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isomerization reactions, allowing separation of the coordination isomers that might exist in an equilibrium mixture. The CD spectrum of chromic desferriferriochrome (Figure 2) is dominated by the metal-centered d-d transitions; these data can be used to identify the coordination isomers of the siderophore complexes. The chromic complexes of desferriferriochrome and desferriferriochrysin have identical CD spectra,<sup>17</sup> which are the same as that of the  $\Delta$ -cis isomer of tris(*N*-methyl-*l*-menthoxyacetylhydroxamato)chromium(III), confirming the previous assignments.<sup>18</sup>

The ferrioxamines are another series of trihydroxamates, produced by various species of the bacteria *Nocardia* and *Streptomyces*. In contrast to the ferrichromes, the linear and cyclic ferrioxamines (Figure 1) have the three hydroxamate groups as part of a polyamide chain like beads on a string, a characteristic structural feature being two repeating units of 1-amino-5-hydroxaminopentane and succinic acid, such that a stable octahedral ferric complex can be formed. One other major difference is that the ligands themselves are optically inactive; only if a substituent group has a chiral center, as in the ferrimycins, is there optical activity for the molecule. Ferrioxamine E, a cyclic ferrioxamine, crystallizes as a racemic mixture of  $\Delta$ -cis and  $\Delta$ -cis isomers as determined by X-ray diffraction methods.<sup>19</sup>

We have prepared the chromium complexes of desferriferrioxamines B and D<sub>1</sub> as possible biological and chemical probes for this series of siderophores. It was hoped to be able to resolve both optical isomers of these kinetically inert complexes; unfortunately repeated attempts to resolve these compounds have not been successful. However, the *cis* geometric isomer was separated from one or more of the four possible *trans* isomers by ion-exchange chromatography.<sup>12</sup> The isomers were assigned by comparison with the isomers of the known Cr(men)<sub>3</sub>, tris(*N*-methyl-*l*-menthoxyacetylhydroxamato)chromium(III).<sup>18</sup> The more abundant isomer (~65% of the total) has visible absorption maxima similar to those of *cis*-Cr(men)<sub>3</sub>, which has spin-allowed d-d transitions  $^4A_{2g} \rightarrow ^4T_{1g}$  and  $^4A_{2g} \rightarrow ^4T_{2g}$  at 425 (66) and 592 (70) nm ( $\epsilon$ ), respectively. The less abundant product (~35%) has visible absorption maxima very similar to those of the *trans* optical isomer of Cr(men)<sub>3</sub>, which has bands at 416 (50) and 596 (70) nm ( $\epsilon$ ). Both the *cis* and *trans* geometrical isomers of chromic desferriferrioxamine B isomerize with half-lives of several days in solution at room temperature. The observed rate of isomerization is slower than that reported for simple model chromic complexes, as expected.

The well-characterized siderophores such as the ferrichromes and ferrioxamines are trihydroxamates which coordinate 1 mol of ferric ion/mol of ligand. Low iron cultures of the yeast *Rhodotorula pilimanae* produce large amounts of rhodotorulic acid (RA), a cyclic dipeptide containing two hydroxamate groups.<sup>20</sup> The compound is produced in truly prodigious yields—up to 10 g/L of culture and a 50% yield of this one compound based on all sources of nitrogen.<sup>20</sup> Since there are only two hydroxamate groups per molecule

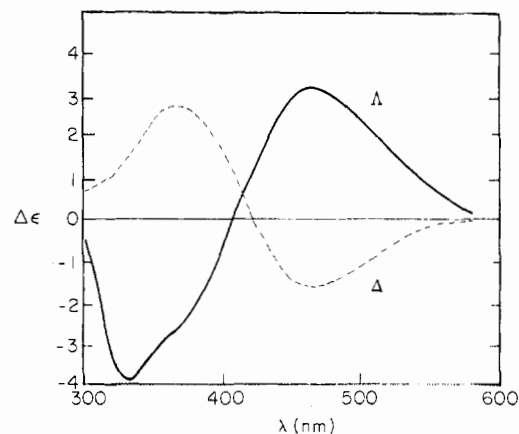


Figure 3. Circular dichroism spectra of ferrichrome A (—) and ferric rhodotorulate (---) ( $\epsilon$  in L mol<sup>-1</sup> cm<sup>-1</sup>).

of RA, a single RA molecule cannot satisfy the six-coordination requirement of ferric ion. Accordingly, we have found that the iron, aluminum, and chromium complexes of rhodotorulic acid are dimeric with the stoichiometry M<sub>2</sub>RA<sub>3</sub>,<sup>21</sup> a formulation supported by analytical results and molecular weights. At pH 7 the orange dimeric iron complex has  $\lambda_{\max}$  425 nm ( $\epsilon$  2700), and at pH 2 the complex turns red ( $\lambda_{\max}$  480 nm ( $\epsilon$  1750)). This red species has been identified as the monomeric FeRA<sup>+</sup>, with the charge on the monomer established by ion-exchange and electrophoresis techniques. Ferric rhodotorulate thus undergoes a monomer-dimer equilibrium which is unique among the natural siderophores. The stoichiometry of the dimer is perhaps not surprising in view of the fact that it is the simplest structure consistent with a tris(hydroxamate) octahedral coordination geometry about the iron, given a dihydroxamate ligand.

The CD spectrum of the dimeric ferric complex (Figure 3) and the kinetically inert Cr(III) complex indicate both exist as the  $\Delta$ -cis isomer, the opposite enantiomer found in the ferrichromes and mycobactins. Thus the  $\Delta$  coordination isomer is not the only one recognized in microbial iron transport systems. Moreover a *cis* geometric isomer could be separated from one or more isomers by silica gel chromatography.

Another class of dihydroxamate siderophores is represented by aerobactin. Aerobactin, first isolated and described by Gibson and Magrath in 1969, is a conjugate of 6-(*N*-acetyl-*N*-hydroxyamino)-2-amino-hexanoic acid and citric acid.<sup>22</sup> It is produced by *Aerobacter aerogenes* under conditions of low-iron stress. The CD spectrum of the ferric complex is similar to that of ferrichrome A, suggesting that the configuration about the metal is the same as in ferrichrome,  $\Delta$ -cis. The chromic complex has been prepared, and its CD spectrum is consistent with the  $\Delta$ -cis configuration in solution.<sup>23</sup> The close similarity of the CD spectra of the ferrichromes, the simple tris(benzohydroxamato)iron(III) complexes, and ferric aerobactin also indicates that the iron is octahedrally coordinated by six oxygen atoms. Since there are only two hydroxamate groups in aerobactin, the nature of the additional two

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oxygens needed to be identified. Examination of molecular models and equilibrium titration studies identified these as the citrate carboxyl and hydroxyl moieties.<sup>23</sup>

A second ligand functional group commonly found in the siderophores is *o*-dihydroxybenzene, or catechol, which although a very weak acid, can be deprotonated to give a dianion which acts as a powerful bidentate ligand. The isolation and characterization of the cyclic triester of 2,3-dihydroxy-*N*-benzoyl-L-serine was independently reported by O'Brien and Gibson<sup>24</sup> and Pollock and Neilands;<sup>25</sup> its synthesis has recently been reported by Corey and co-workers.<sup>26</sup> This tricatechol siderophore was isolated from low-iron cultures of *Salmonella typhimurium* and *Escherichia coli* and given the names enterobactin and enterochelin, respectively. Enterobactin (ent) can also be isolated from the enteric bacteria *Aerobacter aerogenes*, which also produces the hydroxamate chelate aerobactin. While the hydroxamate siderophores are common among various molds, fungi, and yeast, and apparently less abundant in the prokaryotes, the catechols are found only among the true bacteria.

Nuclear magnetic resonance and optical spectra have shown that enterobactin chelates metal ions through the oxygens of the catechol moieties and not through the amide oxygen.<sup>27</sup> However, examination of space-filling models suggests that the two diastereomers of metal enterobactin complexes,  $\Delta$ -cis and  $\Lambda$ -cis, are both possible. Since little was known regarding transition-metal catechol complexes and since enterobactin is relatively unstable and relatively difficult to obtain, we initially examined the chemistry of model catechol complexes.

The simple model complex, tris(catecholato)chromate(III), was prepared as a kinetically inert model complex for enterobactin. Although only partial resolution of solutions of  $[\text{Cr}(\text{cat})_3]^{3-}$  was achieved at neutral pH, complete resolution could be achieved at pH 13.<sup>28</sup> The optical spectrum of  $[\text{Cr}(\text{cat})_3]^{3-}$  is similar to that of chromium enterobactin (prepared from a chromium(II) salt followed by careful air oxidation) although the ligand-centered  $\pi \rightarrow \pi^*$  transition in enterobactin masks the  ${}^4\text{A}_{2g} \rightarrow {}^4\text{T}_{1g}$  d-d band found in the simple model complex. The CD of both  $[\text{Cr}(\text{ent})]^{3-}$  and  $\Delta$ - $[\text{Cr}(\text{cat})_3]^{3-}$  are essentially identical, however, because the interfering ligand band is not associated with the chiral center and hence does not contribute to the optical activity. The known crystal and molecular structure of the potassium salt of  $[\text{Cr}(\text{cat})_3]^{3-}$ ,<sup>29</sup> together with the established absolute configuration, allows the following assignment: the predominant isomer of the monomeric chromium enterobactin complex has a  $\Delta$ -cis absolute configuration. This assignment is further supported by a comparison of the CD spectra of chromic desferriferriochrome and chromic enterobactin;

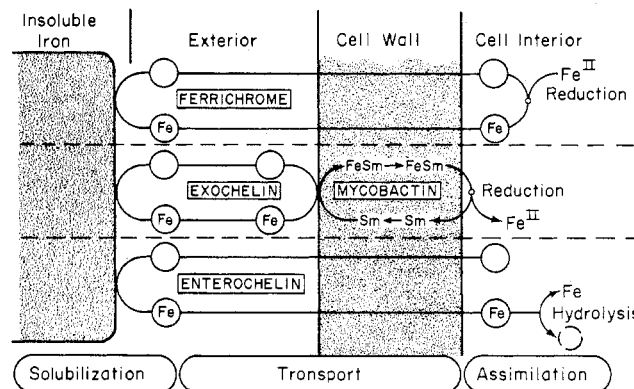


Figure 4. Three limiting mechanisms for microbial iron transport.

they are clearly enantiomorphic (Figure 2). The similarity of the chromic and ferric complexes<sup>29,30</sup> allows this assignment to be extended to the ferric complex as well. This is the opposite isomer found for the optically active hydroxamate siderophores with the exception of rhodotorulic acid.

Unfortunately the usual oxidation sensitivity of the catechol dianion is substantially increased in the chromium complexes, which precludes their use as biological probes. In an effort to circumvent this problem, we have prepared the rhodium catechol and enterobactin complexes.<sup>31</sup> The enterobactin complex, which is air stable and also a  $\Delta$ -cis isomer, has proven to be difficult to synthesize and purify; thus biological studies have not yet been attempted for this derivative.

### Kinetically Inert Siderophores—Use as Biological Probes

Several microbial iron transport systems have been investigated in recent years, and three limiting mechanisms have been recognized. These are outlined schematically in Figure 4. Mechanism 1 consists of uptake of the intact metal complex followed by internal release of the iron (presumably via reduction; vide infra) and reexcretion of the ligand for additional shuttles. Donation of iron to a cell membrane bound chelating agent without penetration of the complex or ligand is the salient feature of mechanism 2. Mechanism 3 involves uptake of the intact complex as in 1, but the internal release of iron destroys the ligand so that it is not reused. These mechanisms involve details that are often difficult to determine with the native, labile ferric complexes. The use of chromic-substituted siderophores often can allow a distinction between mechanisms as well as elucidating the detailed steps involved in each; several examples follows.

Emery first studied the uptake of ferrichrome, in the smut fungus, *Ustilago sphaerogena*.<sup>32</sup> Using both radiolabeled iron and ligand he was able to show that initially the iron and the ligand were taken up at the same rate, but eventually the ligand uptake slowed to a steady-state value considerably lower than the maximum uptake for the iron (Figure 5). This was interpreted as indicating that the complex initially is taken up intact, following which the iron is removed

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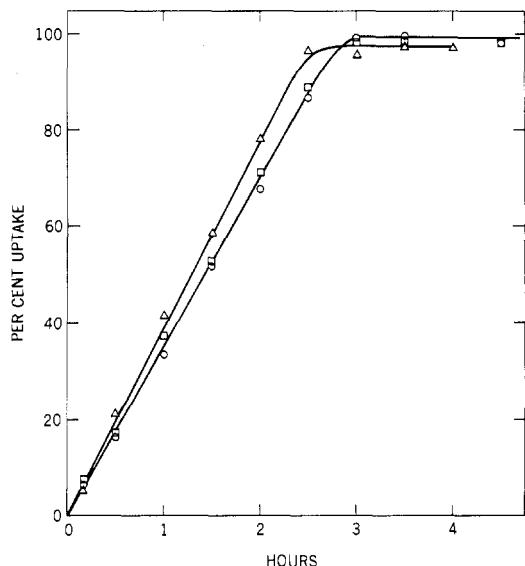
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**Figure 5.** Uptake of ferrichrome ( $^{59}\text{Fe}$ ,  $\Delta$ ) and  $\Lambda$ -cis-chromic [ $^{14}\text{C}$ ]desferriferrichrome ( $^{14}\text{C}$ ,  $\square$ ; Cr,  $\circ$ ) by *Ustilago sphaerogena*.

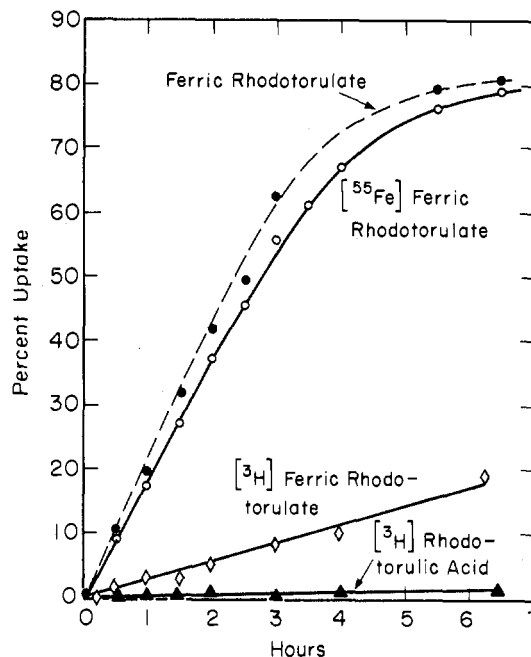
[presumably by reduction to Fe(II); vide infra] and the ligand reexcreted for additional shuttles (i.e., mechanism 1). This system is conformation and structure dependent, with other siderophores, in particular desferriferrichrome, generally being inactive. However other metal-substituted ferrichromes such as the Al(III) and Ga(III) complexes are transported, indicating that in this system the conformation of the metal complex is a primary factor in transport. By using the inert chromic complex as a biological probe we found that this complex is transported at a rate identical with that of the iron complex<sup>33</sup> (Figure 5) and that both the desferriferrichrome and the Cr(III) are transported at the same rate throughout the experiment (Figure 5). These data lead to the following conclusions: (1) Since the chromic complex of desferriferrichrome is inert and found to be transported by *Ustilago*, the  $\Lambda$ -cis optical isomer must be at least one of the biologically active forms. (2) Since the rates of uptake of the iron and chromium complexes are identical, they probably share the same transport system. (3) This specific iron transport system cannot rely on isomerization or partial dissociation of the complex during transport through the membrane. (4) Since no mechanism (such as reduction) is available to the cells to remove the chromium from transported chromic desferriferrichrome, the complex is held within the cells and the ligand is not excreted again as in ferrichrome.

Uptake of the ferric aerobactin complex has been examined in *A. aerogenes*.<sup>34</sup> The transport of the  $^{59}\text{Fe}$  label is very rapid—it is quantitative within 2 min—while the  $^3\text{H}$  label on the ligand initially is rapidly incorporated and then declines to a low steady-state level after about 8 min. Thus it resembles the transport of ferrichrome by *U. sphaerogena*. Studies of the chromium-substituted chelate have not been done.

Transport of the ferrioxamines into biological systems is less well established than it is for the ferrichromes. In fact, the transport of the natural iron or the synthetic chromium complexes has not been demonstrated in the

(33) J. Leong, J. B. Neilands, and K. N. Raymond, *Biochem. Biophys. Res. Commun.*, **60**, 1066 (1974).

(34) J. E. L. Arceneaux, W. B. Davis, D. N. Downer, A. H. Haydon, and B. R. Byers, *J. Bacteriol.*, **115**, 919 (1973).



**Figure 6.** Uptake of ferric rhodotorulate (intact chromophore,  $\bullet$ ; [ $^{55}\text{Fe}$ ] Ferric Rhodotorulate,  $\circ$ ; [ $^3\text{H}$ ] Ferric Rhodotorulate,  $\diamond$ ) and [ $^3\text{H}$ ]rhodotorulic acid by *Rhodotorula pilimanae*.

organisms which produce ferrioxamines. Such work is presently in progress. Several other organisms which do not produce ferrioxamines are nonetheless capable of using them as iron transport agents. The uptake of the iron and chromium complexes of desferriferrichrome has been studied in *Salmonella typhimurium*.<sup>35</sup> Although the ferric ion in the labeled complex was taken up efficiently, the uptake of the ligand was slower but reached a similar percentage total after a longer time. No uptake of a  $^3\text{H}$  label of either *cis*- or *trans*-chromic [ $^3\text{H}$ ]desferriferrichrome B was observed, which eliminates a mechanism of transport involving the intact complex, as occurs with ferrichrome in *Ustilago*. However, more meaningful results should be obtained from transport studies of ferrioxamine in the producing organisms.

An example of the second mechanism of transport is provided in RA-mediated iron uptake, where the transport of both the ferric and chromic rhodotorulate complexes into *R. pilimanae* has been examined.<sup>36</sup> Uptake of the  $^{55}\text{Fe}$  label of the ferric complex is rapid (complete within 4 h), while uptake of the  $^3\text{H}$  label on the ligand is extremely slow (Figure 6). The chromic complexes (*cis* or *trans*) are not transported to any appreciable extent. The system most consistent with these results is a shuttle mechanism, whereby iron is donated by the RA to the membrane but the ligand itself never enters the cell. The chromic complex is not transported, presumably because the kinetic inertness of the complex precludes such exchange. The RA molecule thus acts as a kind of "iron taxi" in shuttling iron from solution to the membrane by simply solubilizing the iron.

Uptake of iron by enterobactin occurs via the final mechanism proposed. The iron enterobactin complex apparently enters the cell intact, as shown by parallel uptake studies using  $^{55}\text{Fe}$  and [ $^{14}\text{C}$ ]enterobactin.<sup>37</sup>

(35) J. Leong and J. B. Neilands, *J. Bacteriol.*, **126**, 823 (1976).

(36) C. J. Carrano and K. N. Raymond, *J. Bacteriol.*, **136**, 69 (1978).

(37) G. Frost and H. Rosenberg, *Biochim. Biophys. Acta*, **330**, 90 (1973).

Table I  
Values of p[M] (Defined as  $-\log [\text{Fe}^{3+}]$ ) for Solutions of  
a Series of Ferric Complexes of Biological Ligands

ligand	$\log K_f$	ref	p[M] <sub>1</sub> <sup>a</sup>	p[M] <sub>2</sub> <sup>b</sup>
enterobactin	52	39	33.3	31.4
ferrioxamine E	32.5	40	29.7	27.7
ferrioxamine B	30.6	40	28.6	26.6
N-acetylferriox- amine B	30.6	40	28.5	26.4
ferrichrysin	30.0	40	27.9	25.8
ferrichrome	29.1	40	27.2	25.2
transferrin			25.6	23.6
aerobactin	22.9	23	25.4	23.3
rhodotorulic acid	62.3 (31.2) <sup>c</sup>	41	25.0	21.9

<sup>a</sup> Total metal =  $10^{-6}$  M; total ligand =  $10^{-3}$  M; pH 7.40.

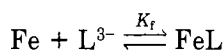
<sup>b</sup> Total metal =  $10^{-6}$  M; total ligand =  $10^{-5}$  M; pH 7.40.

<sup>c</sup> Stability constant per iron for the dimeric rhodotorulic acid complex.

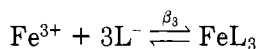
However, once in the cell the ligand is enzymatically destroyed and is not reused.<sup>38</sup> A more detailed account of iron-release mechanisms will be found later in this Account.

### Thermodynamics of Siderophore Iron Binding

The possibility of using the metal-free siderophores as therapeutic agents for the treatment of iron-overload-associated syndromes such as Cooley's anemia or acute iron toxicity from poisoning<sup>10</sup> has prompted investigations into the formation constants of the siderophore ligands with ferric ion. The reaction of these trihydroxamate ligands with iron can be represented by a formation constant  $K_f$ , where  $L^{3-}$  is a triply deprotonated siderophore.



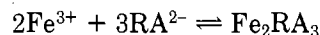
This compares with the overall formation constant for a monohydroxamate.



The overall stability constants,  $K_f$ , for several members of the trihydroxamate class are shown in Table I.<sup>39-41</sup> The values obtained are very large, making these compounds among the best iron chelators known. Interestingly very little "chelate effect" is seen, as shown by the only modest gain of  $K_f$  over the  $\beta_3$  for simple acetohydroxamic acid. Based on the excellent chelating ability of these compounds for iron, Desferal (desferrioxamine B mesylate salt, manufactured by Ciba) has seen use as a clinical agent in Europe, and more recently in the U.S., in the treatment of acute iron toxicity in man.<sup>42</sup>

Rhodotorulic acid is already undergoing limited clinical trials in the U.S. as a drug for the treatment of iron toxicity.<sup>43</sup> It has proven to be more efficacious than Desferal and can be produced at a much lower

cost. We have examined equilibrium titration data to determine the overall stability constant of the ferric RA complex. These data confirm our formulation of the ferric complex at neutral pH as the dimer,  $\text{Fe}_2\text{RA}_3$ , and the red species formed at low pH as the monomer,  $\text{FeRA}^+$ .<sup>41</sup> The overall stability constant



obtained by titration of both the ligand and the metal-ligand complex was found to be  $2 \times 10^{62}$ . Since the species in question is a dimer, the overall stability constant cannot be directly compared with that of the monomeric species ferrichrome or ferrioxamine due to the different units of  $K$ . However, values of free ferric ion (p[M]) obtained at pH 7.4,  $1 \mu\text{M}$  ferric ion, and  $10 \mu\text{M}$  ligand are compared in Table I.

The solution chemistry of aerobactin proved to be quite complex, with six species present over the pH range from 1 to 7.<sup>23</sup> However, above approximately pH 6 only one species is present, the fully formed complex. The value of the formation constant,  $\log K_f$ ,  $\text{Fe}^{3+} + L^{3-} \rightleftharpoons \text{FeL}$ , is 22.9. Aerobactin is compared with the other siderophores in Table I.

The formation constant for enterobactin recently has been determined by spectrophotometric competition with EDTA.<sup>39</sup> The value of  $K^*$  was found to be  $10^{-9.7}$ , where  $K^*$  is given by

$$K^* = \frac{[\text{Fe}(\text{ent})^{3-}][\text{H}^+]^6}{[\text{Fe}^{3+}][\text{H}_6(\text{ent})]}$$

Since the ligand protonation constants of enterobactin are unknown, the more common formulation,  $K_f$ , is not precisely known.

$$K_f = \frac{[\text{Fe}(\text{ent})^{3-}]}{[\text{Fe}^{3+}][(\text{ent})^6]}$$

Assuming typical  $\text{p}K_a$  values from the model ligand 2,3-dihydroxybenzamide of 8.4 and 12.1 for the two OH groups yields  $\log K_f = 52$ , which is the largest formation constant of any iron chelate ever determined.

### Electrochemistry and the Siderophore Iron Release Mechanism

Despite the similarity in function between the hydroxamate siderophores and enterobactin, there exists a striking difference in the mechanism of iron release. The hydroxamate siderophores such as ferrichrome are known to be reused following intracellular iron release<sup>32</sup>—the free siderophore is reexcreted by the cell for another cycle of iron transport (we have called this the "European approach" to microbial iron transport). The mechanism of iron release, thought to involve reduction of Fe(III) to the ferrous state, has been given experimental support with the determination of the redox potential of several of these complexes.<sup>44</sup> The redox potentials of all of the hydroxamates are within the range of known physiological reductants (Figure 7).

Enterobactin, however, is known not to be reused, the cyclic triester linkages of the ferric chelate being cleaved by a specific esterase.<sup>45</sup> Thus only a single ferric ion is transported by a molecule of enterobactin before it

(38) I. G. O'Brien, G. B. Cox, and F. Gibson, *Biochim. Biophys. Acta*, **237**, 537 (1971).

(39) W. R. Harris, C. J. Carrano, and K. N. Raymond, *J. Am. Chem. Soc.*, **101**, in press.

(40) A. E. Martell and R. M. Smith, "Critical Stability Constants", Vol. 3, Plenum Press, New York, 1977.

(41) C. J. Carrano, S. R. Cooper, and K. N. Raymond, *J. Am. Chem. Soc.*, **101**, 599 (1979).

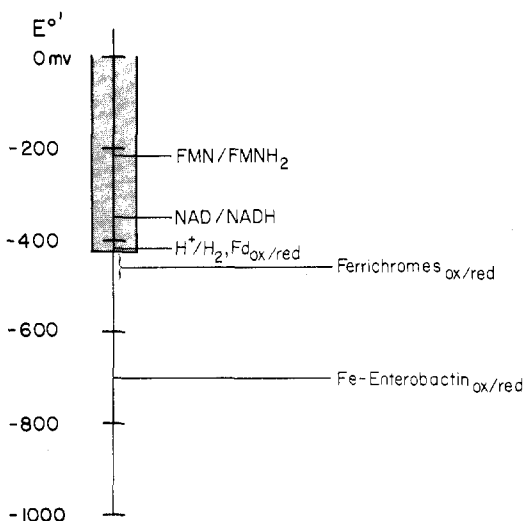
(42) See the articles by R. D. Propper, S. B. Shurin, and D. G. Nathan (p 83) and by A. W. Nienhuis and W. F. Anderson (p 115) in ref 11.

(43) R. L. Rawls, *Chem. Eng. News*, **55**, 24 (1977).

(44) S. R. Cooper, J. V. McArdle, and K. N. Raymond, *Proc. Natl. Acad. Sci. U.S.A.*, **75**, 3551 (1978).

(45) K. T. Greenwood and R. K. J. Luke, *Biochim. Biophys. Acta*, **525**, 209 (1978).





**Figure 7.** A schematic comparison of the reduction potential of the hydroxamate and catecholate siderophores (ferrichrome and enterobactin, respectively). The shaded area represents the potential range of physiological reductants.

is destroyed—it is used once and thrown away (we have called this the “American approach” to microbial iron transport). The waste of metabolic energy entailed in such a scheme suggests that this destruction must be an essential step in the iron-transport mechanism. It has been proposed that this destruction may be due to the inordinately low redox potential for the ferric complex of enterobactin. We have measured the redox potential of ferric enterobactin using cyclic voltammetry and found it to be  $-986$  mV vs. NHE at pH 10.<sup>44</sup> Below pH 10 a proton transfer is involved with the reduction process, leading to irreversible waves in the cyclic voltammograms, preventing direct determination of the pH 7 potential. However, based on the observed dependence of the half-wave potential with pH it is possible to estimate the pH 7 value as  $-750$  mV vs. NHE. This redox potential is well out of the range of any physiological reductants (Figure 7). The tris(dihydroxybenzoylserine)iron complex (the product of enzymatic cleavage) has an irreversible reduction wave at  $-350$  mV vs. NHE. Therefore reduction is possible only *after* cleavage of the internal ester linkages.

Despite the considerable expenditure of metabolic energy necessary for such a “once-through” mechanism of iron uptake, a distinct evolutionary advantage is achieved via enterobactin-mediated iron transport due to the enormous stability constant for the ferric enterobactin complex.

### Concluding Remarks

In summary, the siderophore coordination chemistry that has been reviewed in this Account has established the following:

(1) The chromic-substituted siderophore complexes can be prepared and are kinetically inert to isomerization or ligand substitution.

(2) The structure and bonding of the chromic and ferric complexes (as determined by the spectroscopic properties and single-crystal structure analyses of simple model compounds) are sufficiently alike to regard them as identical for biological systems.

(3) The chromic siderophore complexes can be used as chemical probes to assign the geometry of the metal coordination site by comparison of the IR, vis-UV, and CD spectra with model compounds. The biologically relevant isomers of enterobactin, ferrichrome, rhodotorulic acid, and aerobactin have been so assigned.

(4) The chromic-substituted siderophores can be used as biological probes in studies of microbial iron transport. These studies, which rely on the kinetic inertness of the chromic complex, have established two limiting roles for the siderophores. In some cases (such as ferrichrome in *Ustilago sphaerogena*) the siderophore acts as a true cell permease in transporting iron into the cell via an intact, immutable complex. In other cases (such as rhodotorulic acid in *Rhodotorula pilimanae*) the siderophore simply acts as an iron “taxi” by solubilizing the metal ion and facilitating its diffusion to the cell surface where it is presumably released to a cell-bound iron chelating agent.

(5) The formation constants and redox potentials of the ferric siderophore complexes establish thermodynamic limits to their iron transport and release mechanisms. For those siderophores which actually enter the cell as an intact iron complex, the redox potential determines the feasibility of an iron release mechanism based on reduction of  $\text{Fe}^{3+}$  to  $\text{Fe}^{2+}$ . The trihydroxamate siderophores such as ferrichrome and ferrioxamine B have redox potentials near the lower limit of the redox potential range of physiological reducing agents, which explains the recycling in which a siderophore ligand can be used several times to transport iron into the cell. In contrast, the formation constant of enterobactin is so large that its redox potential precludes physiological reduction and requires hydrolytic destruction of the siderophore ligand as a precursor to iron release.

While much progress has been made in our understanding of the coordination chemistry of microbial iron transport, many interesting and important questions remain. Ironically, one of these is what originally prompted our interest in this field—that is, in cases where the siderophore is specifically recognized at the cell surface and transported against a large concentration gradient into the cell, is the recognition so specific that only one isomer is accepted ( $\Delta$ -cis or  $\Lambda$ -cis, for example)? We look forward to the future resolution of this and many other questions about the coordination chemistry of the siderophores.

*We wish to acknowledge the contributions by our co-workers, whose names appear in our referenced papers. We thank Professor J. B. Neilands, whose seminal research began this field of biochemistry, for his continuing interest and collaboration. Our research is supported by the National Institutes of Health.*