Bicarbonate and the Binding of Iron to Transferrin*

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SUMMARY

The binding of iron to transferrin has been studied by electron paramagnetic resonance and optical absorption spectroscopy and by measurements of proton magnetic relaxation rates under conditions in which carbon dioxide (and therefore bicarbonate) is excluded. Even in the absence of bicarbonate, specific binding of Fe^{3+} to transferrin may occur. Nitritotriacetate, ethylenediaminetetraacetate, and oxalate were found capable of replacing bicarbonate to form colored complexes with iron and transferrin. Since the nitritotriacetate and oxalate complexes, in particular, have optical absorption spectra similar to those of the usual iron-transferrin-bicarbonate complex, their recognition depends on correlating electron paramagnetic resonance and optical studies. The electron paramagnetic resonance spectrum of the iron-transferrin-nitrilotriacetate complex consists of a line, 30 gauss in width, centered near the apparent g value, 4.25, whereas the oxalate complex shows a broad absorption, from 700 to 1400 gauss. In contrast, the bicarbonate complex has a spectrum centered near the apparent g value, 4.22, with a total width of 120 gauss. This spectrum is not influenced by changes in pH in the range, 6.1 to 10.5.

Of a number of metal-complexing agents tested, only those with 2 or more carboxyl groups were found capable of replacing bicarbonate to form ternary complexes with transferrin and iron. Azide and thiocyanate were found ineffective, while a 100-fold excess of cyanide did not perturb the spectroscopic properties of iron-transferrin-bicarbonate. These findings suggest that bicarbonate is not simply coordinated to iron in iron-transferrin-bicarbonate. This view is corroborated by proton relaxation rate measurements, which show little change between iron-transferrin and iron-transferrin-bicarbonate.

Cu^{2+} was also found to bind specifically to apotransferrin in the absence of bicarbonate, as evidenced by the electron paramagnetic resonance spectrum of the Cu^{2+}-transferrin complex, which is quite different from the spectrum of the Cu^{2+}-transferrin-bicarbonate complex.

Ternary complex formation among Fe^{3+}, transferrin, and bicarbonate was observed almost immediately after addition of the ferric chelates to apotransferrin in air. On standing in the presence of an excess of bicarbonate these complexes were gradually replaced, the time depending on the chelate, by the bicarbonate complex. It appears likely, then, that the initial and rapid step in the formation of iron-transferrin-bicarbonate is the formation of the ternary complex, iron-transferrin-chelate, while the slow reaction entails displacement of the chelate by bicarbonate.

A remarkable property of the metal-combining proteins, transferrin and conalbumin, is the apparent dependence of metal complex formation on the binding of bicarbonate ion. Thus, the characteristic colors of the iron-conalbumin and copper-conalbumin complexes were not observed by Warner and Weber in the absence of bicarbonate although oxalate seemed able to replace bicarbonate to some extent in the formation of a colored complex (1). Furthermore, the inclusion of bicarbonate concentration in the equilibrium equations for metal binding led to consistent results at various bicarbonate concentrations for both conalbumin (1) and transferrin (2). Although the participation of carbon dioxide as the bidentate carbonate ion in inorganic complex formation of transition metal ions has long been recognized (3), the involvement of carbon dioxide in the binding of metal by proteins seems to be unique in transferrin and conalbumin.

The sensitivity of electron paramagnetic resonance spectroscopy to changes in the ligand fields of transition metal ions offers a powerful method for the study of transition metal-protein complexes (4). In the experiments reported in this paper we have correlated EPR studies with spectrophotometric and proton magnetic relaxation measurements of a number of transferrin complexes. By so doing we have been able to establish the existence of a variety of complexes of transferrin with ferric and cupric ions. These offer some insight into the role of the carbonate ion in the specific binding of metals by this protein.

EXPERIMENTAL PROCEDURE

Materials—Transferrin and apotransferrin were prepared from Cohn Fraction IV-7 by methods previously described (5).

† The abbreviations used are: EPR, electron paramagnetic resonance; g', apparent g value.
Recent estimations of the molecular weight and extinction coefficients of transferrin have yielded values slightly different from those utilized earlier. In the experiments now reported, we have taken 81,000 as the molecular weight of the protein, \( E_{15\text{cm}}^{1\text{%}} \) at 280 \( \mu \text{m} \) as 11.4 for apotransferrin, and \( E_{15\text{cm}}^{1\text{%}} \) at 280 \( \mu \text{m} \) as 14.1 for iron-saturated transferrin.  

To avoid problems with hydrolysis, all iron complexes were freshly prepared by adding the complexing agent to a solution of ferric chloride, then adjusting the pH of the preparation to 4.5 to 5.0 with sodium hydroxide. Except when otherwise specified, the molar ratio of complexing agent to iron was 2:1 or 3:1. Distilled, deionized water and reagent grade chemicals were used in all experiments.

**Methods**—Absorption spectra were measured in a Cary model 15 spectrophotometer with the use of cuvettes with a 1-cm light path. EPR spectra were obtained at 77° K and 90° K with a Varian model E-3 spectrometer. Measurements of proton relaxation rates were made by the method of adiabatic fast passage in a Varian model DP60 nuclear magnetic resonance spectrometer, operating near 60 MHz (some experimental details are given in Reference 6).

An adaptation of the method of Fraenkel-Conrat (7) was used to obtain bicarbonate-free complexes for spectroscopic studies. Two Thunberg tubes, \( A \) and \( B \), were connected as shown in Fig. 1, and a calibrated quartz EPR sample tube (Varian), \( C \), was joined to one of them with a graded seal. To tube \( A \) were added 1 ml each of 10% NaOH and 25% \( \text{NH}_4\text{OH} \); it was then flushed with hydroxide-washed nitrogen through tube \( B \), evacuated with an oil pump, and sealed. Tube \( B \) was used to carry out the reaction to be studied. A 4% solution of apotransferrin in water, adjusted to pH 4.9 to 5.0, was placed in the lower portion of tube \( B \) and, to ensure complete binding, slightly less than a stoichiometric amount of the appropriate iron complex was placed in the bulb. After the second tube had been flushed and evacuated, the contents of base and bulb of tube \( B \) were mixed. At this point, ammonia was allowed to diffuse from tube \( A \) to tube \( B \) for about 1 min, a time determined by trial and error to be sufficient to raise the pH of the iron-protein solution to 8.5 to 9.5 as measured at the end of the experiment. The preparation was then ready for examination in the EPR spectrometer. A similar apparatus was utilized for spectrophotometric measurements, with a Thunberg type spectrophotometer cell substituted for the EPR-Thunberg tube.

**RESULTS**

*\( \text{pH Dependence of EPR Spectrum of Native Transferrin} \)*—The EPR spectrum of the iron-transferrin-bicarbonate complex ("native" transferrin) did not vary between pH 6.1 and 10.5, and was identical with one obtained from freshly prepared human blood serum (Fig. 2). Thus, variations in the EPR spectra to be presented subsequently cannot be attributed to changes in pH.

*Binding of \( \text{Fe}^{3+} \) by Transferrin in Absence of Bicarbonate or Complex forming Agents—\( \text{Fe}^{3+} \) (as \( \text{FeCl}_3 \)) was anachronically added to apotransferrin at pH 4.9, with the use of the double Thunberg tube described above. The resulting complex was pale yellow, and did not change color appreciably on the diffusion of ammonia into the tube. The optical spectrum is shown in Fig. 3. The EPR spectrum, which is shown in Fig. 4C, is centered at \( g' = 4.30 \), with a width of 50 gauss. This EPR spectrum, as well as the EPR spectra of other \( \text{Fe}^{3+} \) complexes studied, was numerically integrated, and the relative \( \text{Fe}^{3+} \) concentrations were found to be those expected within a factor of 2.*

In order to determine whether this spectrum was due to nonspecific binding of iron by protein, the \( \text{pH} \) of a 4 x 10⁻⁴ M solution of transferrin in which the two specific metal-binding sites were saturated with iron was lowered to 4.9 by the addition of HCl, with disappearance of most of the red color. This solution was then treated with \( \text{Fe}^{3+} \) (as \( \text{FeCl}_3 \)) at a concentration equal to that of the specifically bound metal. As the \( \text{pH} \) was then restored to 9.0 by addition of NaOH, the characteristic color reappeared and the EPR spectrum showed only the signal of the iron-transferrin-bicarbonate complex, with about the same intensity as that of the original preparation. In another experiment, an equimolecular amount of \( \text{FeCl}_3 \) was added to a 7 x 10⁻⁴ M solution of human serum albumin at pH 4.9. After the pH was...
Binding of Iron Chelates—The addition of ferric ion to aptransferrin as the 1:3 ferric-nitrilotriacetate complex or the 1:3 ferric-oxalate complex in the double Thunberg tube resulted in the immediate formation of a red complex. The color did not appreciably change as ammonia was allowed to distill over into the tube containing the protein preparation. The optical absorption spectra of these complexes (Fig. 5) each showed a peak near 470 μm, and were very similar to that of native transferrin in shape and intensity, particularly in the visible region. When frozen for the EPR studies, there was no apparent change in color in these complexes or in the other complexes studied.

The EPR spectrum of the nitrilotriacetate complex (Fig. 4A) has $g' = 4.25$ and a line width of about 30 gauss. The oxalate complex gives a spectrum (Fig. 6) with broad absorptions ranging from 700 to 1400 gauss; the narrow absorption centered near 1550 gauss is due to a small amount of the bicarbonate complex (corresponding to less than 2% of the iron present). These two spectra differ from the spectrum of the bicarbonate complex raised to 0.6 the EPR spectrum of this preparation showed a narrow line (width, about 40 gauss) at $g' = 4.27$, but with an intensity corresponding to less than 0.5% of that of the corresponding line from an equal concentration of iron in native transferrin.

Fig. 3. Optical absorption spectrum of the anaerobic iron-transferrin complex. The spectrum was taken at an Fe$^{3+}$ concentration of 6.2 × $10^{-4}$ M and normalized to 5 × $10^{-4}$ M; the pH was 9.1; the reference cell contained apotransferrin at the same concentration as the sample cell.

Fig. 4. EPR spectra obtained at 77° K of iron-transferrin-nitrilotriacetate (Curve A), iron-transferrin-EDTA (B), and iron-transferrin (C), all being about 1.5 × $10^{-4}$ M in Fe$^{3+}$. The relative amplifier gain factors were 1.5, 5, and 4 for Curves A, B, and C, respectively. The modulation amplitude was 5 gauss, the microwave frequency was 9140 MHz, and the microwave power was 20 milliwatts.

Fig. 5. Optical absorption spectra of ——, iron-transferrin-bicarbonate (pH 9.0); ——, iron-transferrin-nitrilotriacetate (pH 9.5); and ——, iron-transferrin-oxalate (pH 9.4). The spectrum of the oxalate complex was taken at an Fe$^{3+}$ concentration of 8 × $10^{-4}$ M, and normalized to a concentration of 1.2 × $10^{-4}$ M, at which the other spectra were obtained. The reference cells contained spectrally pure NaCl in the same concentrations as the sample cells.
At first, the EPR spectrum showed the iron-transferrin-transferrin at pH 6.1 (in air), there was a rapid development of that not over 15% of the ferric-EDTA chelate is unbound in our experiments. We have calculated complete, since the simple chelate has an absorption maximum at 240 mp, where Fe³⁺-transferrin-EDTA read against an apo-transferrin blank has little absorption. We have calculated the simple iron-transferrin-EDTA complex to be replaced by the iron-transferrin-bicarbonate complex still showed both forms present. The same changes were observed, but much more slowly, when the tubes were simply opened and allowed to stand in air.

In one experiment, transferrin was saturated with iron oxalate in the Thunberg apparatus. After the apparatus was opened to air, an amount of iron oxalate equivalent to that specifically bound was added to the preparation. No changes in optical EPR spectra were produced by this addition.

When the 2:1 EDTA complex of Fe³⁺ was added to apotransferrin in the double Thunberg apparatus, a violet color developed promptly as ammonia was allowed to diffuse into the protein-containing tube. The visible spectrum of the complex is given in Fig. 7; there is a broad absorption with a maximum at 520 mp. The apparent gram atomic extinction coefficient at this wave length, referred to the concentration of Fe³⁺, was estimated as 400, compared to 2500 at 470 mp for iron-transferrin-bicarbonate (2). The EPR spectrum of the iron-transferrin-EDTA complex is shown in Fig. 4B. About 24 hours were required after the addition of bicarbonate for the EPR signal to be replaced by that of the iron-transferrin-bicarbonate complex.

The binding of Fe³⁺-EDTA to apotransferrin must be almost complete, since the simple chelate has an absorption maximum at 240 mp, where Fe³⁺-transferrin-EDTA read against an apotransferrin blank has little absorption. We have calculated that not over 15% of the ferric-EDTA chelate is unbound in our experiments.

When the iron nitroltriecarate chelate was added to apotransferrin at pH 6.1 (in air), there was a rapid development of color. At first, the EPR spectrum showed the iron-transferrin-

- nitroltriecarate complex as the principal component. On standing, this gradually converted to the bicarbonate form, until after several days the EPR spectrum was identical with the spectrum of iron-transferrin-bicarbonate.

A series of similar experiments was attempted with ferric citrate complexes, but the EPR spectra obtained were variable and not readily reproducible. Undoubtedly, this is due to the complex solution chemistry of ferric citrate with the formation of polynuclear iron complexes, as described by Spiro et al. (8).

Formation of Iron-Transferrin-Chelate Complexes at High pH without Exclusion of Carbon Dioxide—In order to determine whether the binding of the iron complexes to transferrin would also be observed if direct additions to apotransferrin at high pH were made, a series of experiments was undertaken in which the nitroltriecarate, EDTA, and oxalate complexes of iron were added in air to apotransferrin at pH 9. The resulting preparations were frozen in liquid nitrogen as rapidly as possible for study in the EPR apparatus; no more than 1 or 2 min elapsed between the addition of the iron complex to the protein and the freezing. In each case, the EPR spectrum showed predominance of the bicarbonate-free complex, with somewhat variable intensity of the bicarbonate signal in different experiments.

Effect of Nitrate, Acetate, Azide, Thiocyanate, and Cyanide—When Fe³⁺ was anaerobically added to apotransferrin in the presence of a large excess of nitrate (20:1), acetate (20:1), azide (10:1), or thiocyanate (15:1) in the double Thunberg apparatus, as described under “Methods,” the complexes formed in each case showed the pale yellow color and typical EPR spectrum of the simple iron-transferrin complex. A similar experiment could not be done with cyanide, because of the volatility of hydrogen cyanide at low pH. However, the addition of a 100-fold excess of cyanide over iron to iron-transferrin-bicarbonate in air did not affect the EPR spectrum.

Proton Relaxation Rate Studies—The longitudinal relaxation rate (T₁⁻¹) of water protons in the iron-transferrin complex (7.9 × 10⁻³ M in Fe³⁺) was 1.15 sec⁻¹. After conversion to the iron-transferrin-bicarbonate complex the T₁⁻¹ was 0.96 sec⁻¹.
The addition of a large excess of cyanide over iron to a transferrin solution gave no change at all in the relaxation rate.

**EPR Spectra of Ferric Chelates**—The nitrilotriacetate and EDTA chelates of Fe³⁺ at the pH of the preceding experiments were also studied in the EPR spectrometer. These solutions were made 2 M in sodium perchlorate in order to improve resolution (cf. Reference 6). Attempts to prepare the ferric oxalate complex under these conditions were unsuccessful, since precipitation, presumably due to hydrolysis, occurred.

The EPR spectra of Fe³⁺-nitrilotriacetate and Fe³⁺-EDTA are shown in Fig. 8. Besides the line at \( g' = 4.2 \) in this figure, there is also a line at about \( g' = 9.3 \) in both spectra, as well as in the other iron EPR spectra. The intensity and shape of this low field line differ very substantially for different complexes (cf. Reference 2). The iron-nitrilotriacetate complex also shows weak lines similar to the lines between 700 and 1400 gauss in the iron-transferrin-oxalate complex shown in Fig. 6. The same lines can also be observed in the iron-transferrin spectrum.

**Copper Complex of Transferrin**—With the aid of the double Thunberg tube, an experiment was performed to determine whether Cu²⁺ (as CuSO₄) could also specifically bind to apotransferrin in the absence of bicarbonate. The EPR spectrum (Fig. 9A) of the anaerobic preparation at pH 9.2, which was colorless, was distinctly different from that of nonspecific protein-bound copper, as well as from that of the Cu²⁺-ammonia complex (Fig. 9C) and from that of the usual copper-transferrin-bicarbonate complex (2). Within 30 min of opening the tube to air the characteristic yellow color appeared and the EPR spectrum (Fig. 9B) became indistinguishable from that of copper-transferrin-bicarbonate.

**DISCUSSION**

Bicarbonate has generally been considered essential for the specific binding of iron to transferrin (2) and conalbumin (1), although some discussion about this point has arisen (1). In the study reported now, with Fe³⁺ (as FeCl₃) added to apotransferrin under conditions in which bicarbonate is excluded, specific binding of iron still occurs. This is shown most clearly by the EPR spectrum of the resulting metal-protein complex at pH 9.2 (Fig. 4C). Unbound iron at this pH is essentially completely hydrolyzed and produces too broad an EPR absorption to be seen. Furthermore, nonspecifically bound iron, such as iron bound to serum albumin, produces a distinctly different EPR absorption line of much smaller intensity, as does iron bound to denatured transferrin (2). Finally, when the specific sites of transferrin are saturated by Fe³⁺ and bicarbonate, no further increment or change in EPR signal is produced by the addition of more iron.

The simple iron-transferrin complex, however, lacks the visible absorption band so characteristic of the usual iron-transferrin-bicarbonate complex. That bicarbonate is not essential for the development of this color, however, is shown by the experiments in which the ferric chelates of oxalate and nitrilotriacetate are complexed with transferrin. The optical
absorption spectra of these complexes (Fig. 5) are very similar to
the spectrum of iron-transferrin-bicarbonate, the most apparent
difference being the enhanced absorption by the oxalate complex
in the ultraviolet region. At these concentrations, the simple
eritic chelates themselves have almost no absorption in the
visible region. We depend now on EPR spectroscopy to show
that the ternary complexes of oxalate or nitrilotriacetate with Fe³⁺
and transferrin (Figs. 6 and 4A) are distinctly different from the
one with bicarbonate (Fig. 2). It is clear, then, that oxalate
and nitrilotriacetate can replace bicarbonate in the formation of
a colored complex. Bicarbonate must be much more strongly
bound than nitrilotriacetate, however, since the latter form is
gradually replaced by the bicarbonate form at pH 6.1, where the
equilibrium concentration of bicarbonate is less than 10⁻⁴ M
(9), and the nitrilotriacetate concentration is close to 10⁻⁴ M.
The iron-transferrin-EDTA complex is of some interest since it
has a distinctive violet color resulting from a shift of the visible
absorption band to longer wave lengths. The apparent extinction
coefficients of both the visible and ultraviolet bands are
substantially less than in the bicarbonate complex.
The EPR spectra obtained from direct addition of the ferric
chelates to the apoprotein at high pH are composed principally
of the iron-transferrin-chelate spectra identified in the Thunberg
tube experiments. This shows, then, that the ammonia in the
Thunberg experiments functions simply as a base to raise pH,
and does not participate directly in the binding of iron to the
protein. Furthermore, the formation of the iron-transferrin-
chelate complex appears to be the initial and rapid step in the
ultimate development of the iron-transferrin-bicarbonate com-
plex. The slower reaction, the rate of which varies depending
upon the chelate, is the replacement of the chelate by bicarbonate.
It is noteworthy that this conclusion follows only from the
correlation of EPR and optical spectra of the ternary complexes.
Since there is no simple and consistent relationship between the
development of color and the specific binding of iron in the
systems studied, recognition of the different complexes requires
both optical absorption and EPR spectroscopy.

All iron EPR spectra recorded in this study show a line of
g' = 4.2 with either no structure or very poorly resolved struc-
ture. Until spectra are obtained at a different frequency we
have foregone attempts to fit spin Hamiltonian operators to
them. Despite this lack of detailed description the more gen-
eral question concerning the origin of the Fe²⁺, g' = 4.2, EPR
spectra in terms of ligand configuration is still of great interest.
Unfortunately, we are still far from able to draw any conclusions
about the coordination of Fe²⁺ solely from analysis of EPR
spectra. Although very recently an attempt has been made to
explain the Fe²⁺, g' = 4.2, EPR signals by assuming strong
ligand fields of tetragonal symmetry we still believe that assump-
tions made earlier about rhombic fields are essentially correct
(2). A suggestion made by Blumberg,⁴ that Fe²⁺ coordinations
producing either completely axial or completely rhombic ligand
fields have the lowest energies, seems to be very attractive,
especially since a number of Fe²⁺ complexes show an EPR signal
with a g' value of about 4.2.

Two different mechanisms may change a g' = 4.2 line: (a)
exchange or dipole coupling between 2 or more Fe²⁺ ions, and
(b) changes in the ligand field. Most of the EPR spectra re-
corded during this work give no evidence of any Fe²⁺-Fe²⁺
coupling, corroborating earlier work on iron transferrin-bicarbon-
ate (2). The EPR spectra of native transferrin (Fig. 2), iron-
transferrin-EDTA, iron-transferrin-nitrilotriacetate (Fig. 4, B
and A), and iron-EDTA (Fig. 8B) all show typical g' = 4.2
spectra with an accompanying low field line at g' = 9.3. The
differences may be attributed to small changes in the ligand field.
These result in corresponding changes in the excited states which,
in turn, give rise to changes in the zero field splitting (the
parameters D and E in the spin Hamiltonian formulation) of the
ground state through the intermediate of spin orbit coupling.
The EPR spectrum of iron-transferrin-EDTA (Fig. 4B) is
further complicated because it consists of two g' = 4.2 com-
ponents. The narrow part in the middle is derived from a
different molecular species than the rest of the line, as evidenced
by their different relative magnitudes in separate experiments.
It does not seem unlikely that the narrow signal comes from
unbound iron-EDTA, i.e. the narrow parts in the EPR signals
of iron-transferrin-EDTA (Fig. 4B) and iron-EDTA (Fig. 8B)
are derived from similar molecular species. This further implies that
the iron-EDTA spectrum consists of at least two different spectra,
which is not too surprising as Fe³⁺ evidently can form many
strongly pH-dependent complexes with EDTA (10). The narrow
part of the iron-transferrin-EDTA signal can account only for
a very small fraction of the total iron concentration, so that
this interpretation is consistent with the optical measurements,
which indicate that no more than 15% of the total Fe³⁺ is in
the form of the simple EDTA chelate.

Some of our EPR results are not easily understood in terms of
a rhombic ligand field with zero field splitting much larger than
the Zeeman term. This is particularly true with iron-trans-
ferrin-oxalate (Fig. 6). Very similar broad lines (but much
weaker and not shown in Figs. 4C and 8A) are also observed in
the region between 700 and 1400 gauss in the EPR spectra of the
chemically quite different systems, iron-transferrin and iron
nitrilotriacetate. As iron nitrilotriacetate is known to form
dimers (10), the possibility that these lines can be caused by
iron-iron interaction arises. This possibility seems unlikely,
however, since if significant dimer formation occurred a number
of binding sites should be available for further binding of iron
oxalate when equivalent concentrations of binding sites and
iron oxalate are present. The addition of more iron oxalate
would then be expected to increase the intensity of the EPR and
optical spectra, but this does not occur.

An interesting feature of our study is the apparently poor
correlation between EPR and optical spectra for some of the
complexes formed. There is a paradoxical situation in that the
EPR spectra of the iron-transferrin complexes with nitrilotriacet-
ate and EDTA closely resemble those of the corresponding
simple chelates, while the optical spectra of these ternary com-
plexes are much more similar to the spectrum of iron-transferrin-
bicarbonate than to the spectra of the iron chelates. A rather
trivial explanation for these findings would be that the two iron-
binding sites in transferrin are different, one giving rise to the
color with no EPR signal, the other giving an EPR signal but no
color. This possibility has already been discussed in Reference 2
and found most improbable because of the equal binding strengths
for both Fe³⁺ ions. The homogeneous appearance of the Cu²⁺-transferrin EPR spectrum further substantiates the
identity of the two binding sites. With identical sites, the EPR
findings might be explained by saying that most of the coordina-
tion sites of the iron are satisfied by the chelating agent.
in the ternary complex. On the other hand, the optical findings suggest the reverse, namely, that the protein supplies most of the ligands of iron. Possibly the actual situation lies somewhere between these extremes.

There may be several reasons for the finding that the correlation between optical and EPR spectra is absent or only very weakly displayed. The excited levels which are mixed into the ground state to produce the zero-field splitting probably have lower energies than the levels involved in the observed optical transitions (11). Also, these transitions are so intense that they may well be transfer transitions. In fact, even for the case of Cu²⁺, which is much simpler than Fe³⁺, similar effects have been observed (12), namely, changes in optical properties without any change in EPR behavior.

The nature of the bicarbonate-binding site remains to be considered. Transferrin is remarkable for both the strength and the specificity of bicarbonate binding. Of all anions tested, only those with the structurally similar carboxyl groups were capable of substituting for bicarbonate. Even the isoelectronic nitrate ion showed no evidence of binding to the iron-transferrin complex, while in carbonic anhydrase, which also binds bicarbonate, nitrate competes strongly with bicarbonate for the anion-binding site (13).

Does bicarbonate enter into complex formation by binding directly to the metal, or only to nearby groups on the protein? We are unable to answer this question with certainty, but for a number of reasons we favor the latter view. If bicarbonate were bound directly to the metal, water might be expected to replace it in the iron-transferrin complex; the proton relaxation rate studies do not support the hypothesis that an additional water molecule is bound to Fe³⁺ when no anion is bound, since there is little difference between iron-transferrin and iron-transferrin bicarbonate. Both the copper and iron complexes bind bicarbonate, and it is difficult to see how both metals, with their different coordination properties, could specifically bind bicarbonate in the same way at the same binding sites. Finally, the findings that such metal poisons and metalloenzyme inhibitors as azide and thiocyanate do not affect the EPR spectrum of the bicarbonate-free iron transferrin complex, and that a great excess of cyanide does not perturb the iron-transferrin-bicarbonate spectrum, suggest that the coordination requirements of the iron molecule are satisfied in both the bicarbonate and the bicarbonate-free forms. This makes it difficult to understand how bicarbonate could be directly complexed to the metal.

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REFERENCES

tions are known in which partial reactions do not occur unless all reaction components are present, including those which do not directly participate in the partial reaction. The most recent example is provided by the convincing work of Eggerer, who has shown that the condensing enzyme catalyzes tritium exchange between solvent and acetyl-CoA only in the presence of (S)-malate (24).

Participation of the carbon atom 5' position of DBCC in hydrogen transfer has also been established in the methylmalonyl-CoA isomerase (25, 26) and glutamate isomerase4 reactions.

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REFERENCES

H. A. Barker, personal communication.

CORRECTION

In the paper by Philip Aisen, Roland Aasa, Bo G. Malmström, and Tore Vångård (Vol. 242, No. 10, Issue of May 25, 1967, page 2484), on page 2488, the magnetic field scale given on the abscissa of Fig. 9 as "2700, 2800, 2900, 3000, 3100, 3200" should be "2500, 2700, 2900, 3100, 3300, 3500," respectively.
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