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 dicarbonate, specific binding of $\text{Fe}^{3+}$ to transferrin may occur. Nitritotriacetate, ethylenediaminetetraacetate, and oxalate were found capable of replacing dicarbonate 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-nitritotriacetate 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 dicarbonate to form ternary complexes with transferrin and iron. Azide and thiocyanate were found ineffective, whereas a 100-fold excess of cyanide did not perturb the spectrophotometric properties of iron-transferrin-bicarbonate. These findings suggest that dicarbonate 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.

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

Ternary complex formation among $\text{Fe}^{3+}$, 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 dicarbonate 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 dicarbonate 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$^1$ 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 carbon dioxide 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).

$^1$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, \( E_{15}^{280} \) as 11.4 for apotransferrin, and \( F_{15}^{280} \) 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% NH\(_4\)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 A 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**

**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 Fe\(^{3+}\) by Transferrin in Absence of Bicarbonate or Complex Forming Agents**—Fe\(^{3+}\) (as FeC\(_3\)) was anaerobically 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 Fe\(^{3+}\) complexes studied, was numerically integrated, and the relative 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 pH of a 4 \times 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 Fe\(^{3+}\) (as FeC\(_3\)) at a concentration equal to that of the specifically bound metal. As the 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 equimolar amount of FeC\(_3\) was added to a 7 \times 10^{-4} M solution of human serum albumin at pH 4.9. After the pH was 81,000 as the molecular weight of the protein, \( E_{15}^{280} \) as 11.4 for apotransferrin, and \( F_{15}^{280} \) 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.

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A. Leibman and P. Aisen, unpublished observations. Sedimentation equilibrium measurements in the laboratory of Professor Charles Tanford have also yielded values for molecular weight near 80,000.
Fig. 3. Optical absorption spectrum of the anaerobic iron-transferrin complex. The spectrum was taken at an Fe\(^{3+}\) concentration of 6.2 \times 10^{-4}\ M and normalized to 5 \times 10^{-4}\ M; the pH was 9.1; the reference cell contained apotransferrin at the same concentration as the sample cell.

Fig. 5. Optical absorption spectra of -- , iron-transferrin-bicarbonate (pH 9); ---- , 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 \times 10^{-4}\ M, and normalized to a concentration of 1.2 \times 10^{-4}\ M, at which the other spectra were obtained. The reference cells contained apotransferrin in the same concentrations as the sample cells.

Binding of Iron Chelates—The addition of ferric ion to apo-transferrin 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 mu, 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 9.0 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.

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color. 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 at 240 mp, where Fe³⁺-transferrin-EDTA read against an apo-transferrin blank has little absorption. We have calculated the apparent gram atomic extinction coefficient at 520 mp. The apparent gram atomic extinction coefficient at 240 mp, referred to the concentration of Fe³⁺, was 120 gauss.

When the 2:1 EDTA complex of Fe²⁺ 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 wavelength, referred to the concentration of Fe²⁺, 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 Fe²⁺-EDTA to apotransferrin must be almost complete, since the simple chelate has an absorption maximum at 240 mp, where Fe²⁺-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 nitritotriacetate 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-nitritotriacetate 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).

In one experiment, transferrin was saturated with iron oxalate equivalent to that specifically added to the contents of each tube (approximately 0.5 ml). The EPR spectrum gradually reverted to that of native transferrin in all experiments. Less than 5 min was required for the spectra of the iron-transferrin and iron-transferrin-nitritotriacetate complexes to be replaced by the iron-transferrin-bicarbonate EPR signal; at 60 hours the spectrum of the oxalate 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.

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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 nitritotriacetate, 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 Fe²⁺ 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 X 10⁻⁴ M in Fe²⁺) 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.

![EPR spectra obtained at 77°K of A, Fe3+-nitrilotriacetate, pH 9.0; and B, Fe3+-EDTA, pH 9.5; each in 2 M NaClO4. Fe3+ concentration in both samples was about 1.5 × 10−3 M. The modulation amplitude was 5 gauss, the microwave frequency was 9153 MHz, and the microwave power was 20 milliwatts.](http://www.jbc.org)

**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
equilibrium concentration of bicarbonate is less than 1.5 compared to 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 complex. 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 structure. Until spectra are obtained at a different frequency we have foregone attempts to fit spin Hamiltonian operators to $g' = 4.2$, 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 Fe$^{3+}$ solely from analysis of EPR spectra. Although very recently an attempt has been made to explain the Fe$^{3+}$, $g' = 4.2$, EPR signals by assuming strong ligand fields of tetragonal symmetry we still believe that assumptions made earlier about rhombic fields are essentially correct (2). A suggestion made by Blumberg, that Fe$^{3+}$ coordinations producing either completely axial or completely rhombic ligand fields have the lowest energies, seems to be very attractive, especially since a number of Fe$^{3+}$ 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 Fe$^{3+}$ ions, and (b) changes in the ligand field. Most of the EPR spectra recorded during this work give no evidence of any Fe$^{3+}$-Fe$^{3+}$ coupling, corroborating earlier work on iron transferrin-bicarbonate (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. This 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 $g' = 4.2$ components. 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) arise 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 Fe$^{3+}$ 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 Fe$^{3+}$ 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-transferrin-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 nitrilotriacetate and EDTA closely resemble those of the corresponding simple chelates, while the optical spectra of these ternary complexes 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 Fe$^{3+}$ ions. The homogeneous appearance of the Cu$^{2+}$-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 coordination sites of the iron are satisfied by the chelating agent

*G. Harris, personal communication.

4 W. E. Blumberg, personal communication.
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 charge-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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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 DCC in hydrogen transfer has also been established in the methylmalonyl-CoA isomerase (25, 26) and glutamate isomerase4 reactions.

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4 H. A. Barker, personal communication.

CORRECTION

In the paper by Philip Aisen, Roland Aasa, Bo G. Malmström, and Tore Vännård (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.
Bicarbonate and the Binding of Iron to Transferrin
Philip Aisen, Roland Aasa, Bo G. Malmström and Tore Vänngård


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