The Reduction and Release of Iron from Fe$^{3+}$·Transferrin·$\text{CO}_3^{2-}$

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This paper examines the reaction in which the iron of Fe$^{3+}$·transferrin·$\text{CO}_3^{2-}$ is reduced to the Fe$^{2+}$ state by a reducing agent, released from the protein, and bound by a chromogenic ferrous ion acceptor. When sodium dithionite, Na$_2$S$_2$O$_4$, is used as the reducing agent and bathophenanthroline sulfonate (BPS) is used as the Fe$^{2+}$ acceptor, the reaction has a first order dependence on Fe$^{3+}$·transferrin·$\text{CO}_3^{2-}$ and dithionite, and is zero order with regard to BPS. These kinetic data are consistent with a reaction sequence in which the iron of Fe$^{3+}$·transferrin·$\text{CO}_3^{2-}$ is reduced in the rate-limiting step following which Fe$^{3+}$ is rapidly released and sequestered by BPS. In the presence of excess dithionite (pseudo-first order conditions) a curvilinear first order plot is obtained suggesting the two transferrin sites have somewhat different reactivities. A plot of reaction velocity versus pH exhibits a slope of −2 in the range from pH 6.7 to 4.3, consistent with the protonation of two groups during the iron release reaction. Several chelating agents were found to enhance the rate of the reaction. Their effect is greater than would be anticipated from their chelating effectiveness alone. Factors other than the oxidation-reduction potential influence the rate at which various reducing agents act in this reaction. Thioglycolic acid is unusually effective. Kinetic studies suggest the formation of a complex of Fe$^{3+}$·transferrin·$\text{CO}_3^{2-}$ and thioglycolic acid. Thioglycolate can act as a carbonate substitute at the anion binding site of transferrin (8). As our laboratories, however, show that under the conditions employed the reductive release of iron from Fe$^{3+}$·transferrin·$\text{CO}_3^{2-}$ is of interest in the design and development of chelation therapy aimed at the treatment of iron overload diseases.

There are several chemical factors that may be involved in promoting iron release from Fe$^{3+}$·transferrin·$\text{CO}_3^{2-}$. These include: chelation, reduction, protonation, disruption of the carbonate-iron stabilization interactions, and possible conformational factors. The reduction of the iron from Fe$^{3+}$ to Fe$^{2+}$ offers a potential pathway of iron release. Studies have shown that the binding of Fe$^{2+}$ to apotransferrin is weak and in rapid equilibrium (2, 4). Thus, the reduction process would lead to rapid release of iron from transferrin. Reports from other laboratories, however, show that under the conditions employed the reductive release of iron from Fe$^{3+}$·transferrin·$\text{CO}_3^{2-}$ may be slow and incomplete (5–7). In an initial publication we presented evidence that reductive iron release can occur at reasonable rates, especially at acidic pH and in conjunction with substitutions at the carbonate binding site of transferrin (8).

The experiments reported here are designed to test the susceptibility of Fe$^{3+}$·transferrin·$\text{CO}_3^{2-}$ toward reduction with special emphasis on a search for those conditions which enhance the rate and extent of the reductive iron release reaction. In this regard, the effects of the reducing agent, chelating agent, ferrous chrogenes, pH, and other factors have been investigated. The results suggest that the reduction of Fe$^{3+}$·transferrin·$\text{CO}_3^{2-}$ could be an important pathway in the biological iron exchange reaction and could provide new approaches to chelation therapy.

MATERIALS AND METHODS

General—Methods, materials, and instrumentation utilized are described in related papers from our laboratory (3, 8, 9). Reagents, including iron, chelating agents, and reducing agents were of the highest purity commercially available and were not further purified. Human apotransferrin was obtained from Behring-Diagnostics. The

The chemistry of transferrin, the serum iron transport protein, has been reviewed recently by Chasten (1). As our understanding of the protein grows so does our appreciation of its complexity. We are beginning to see new relationships between the physiological requirements of iron transport and the chemical properties of transferrin. Apotransferrin exhibits a high reactivity toward labile Fe$^{2+}$ (2) and Fe$^{3+}$ complexes (3), and thus protects the organism from the toxic effects of this element. Fe$^{3+}$·transferrin·$\text{CO}_3^{2-}$ is inert toward the myriad of nonspecific complexing agents of serum; yet, readily recognizes membrane-bound receptors and quickly relinquishes its iron to these in a series of concerted interactions.

A most challenging problem in transferrin chemistry is the description of the mechanisms by which Fe$^{3+}$·transferrin·$\text{CO}_3^{2-}$ is induced to release its iron. This multifaceted subject is relevant to our understanding of the chemistry of metal-binding proteins and the mechanisms by which metal ions are exchanged in biological systems. Further, iron release from Fe$^{3+}$·transferrin·$\text{CO}_3^{2-}$ is of interest in the design and development of chelation therapy aimed at the treatment of iron overload diseases.

The experiments reported here are designed to test the susceptibility of Fe$^{3+}$·transferrin·$\text{CO}_3^{2-}$ toward reduction with special emphasis on a search for those conditions which enhance the rate and extent of the reductive iron release reaction. In this regard, the effects of the reducing agent, chelating agent, ferrous chrogenes, pH, and other factors have been investigated. The results suggest that the reduction of Fe$^{3+}$·transferrin·$\text{CO}_3^{2-}$ could be an important pathway in the biological iron exchange reaction and could provide new approaches to chelation therapy.
buffers and bathophenanthroline sulfonate (BPS) were also obtained from Sigma Chemical Co.

Preparation of Fe\textsuperscript{3+}.transferrin-CO\textsubscript{3}\textsuperscript{2-} and Fe\textsuperscript{2+}.transferrin-Thioglycolate—Fe\textsuperscript{3+}.transferrin-CO\textsubscript{3}\textsuperscript{2-} was prepared for the experiments as follows. Apotransferrin was solubilized in a buffer containing 10 mM Hepes, 0.15 M NaCl, and 10 mM NaHCO\textsubscript{3}, pH 7.4. An aliquot was analyzed for unsaturated iron-binding capacity by spectrophotometric titration with Fe\textsuperscript{2+}.nitrilotriacetate as previously described (10). To the batch of apotransferrin was then added an equivalent amount of Fe\textsuperscript{2+}.nitrilotriacetate (10). The resulting Fe\textsuperscript{2+}.transferrin-CO\textsubscript{3}\textsuperscript{2-} plus nitrilotriacetate mixture was then applied to a Sephadex G-25 column equilibrated and eluted with 10 mM Hepes, 0.15 M NaCl, and 10 mM NaHCO\textsubscript{3}. The purpose of the gel filtration is to remove the nitrilotriacetate which may cling tenaciously to the Fe\textsuperscript{3+}.transferrin-CO\textsubscript{3}\textsuperscript{2-} complex. The final buffer concentrations are described under "Results" and in the legends to the figures.

Fe\textsuperscript{2+}.transferrin-thioglycolate was prepared by a slight modification of the "low pH purge" method described earlier (9, 10). Fe\textsuperscript{3+}.transferrin-CO\textsubscript{3}\textsuperscript{2-} was taken to pH 3.2 with a stream of nitrogen containing volatilized HCl. The mixture was then stirred and the surface of the solution swept for 45 min with a stream of CO\textsubscript{2}-free air which had been passed through three Ascarite columns for CO\textsubscript{2} removal and finally scrubbed with a concentrated KOH solution. Air, rather than nitrogen, was employed since we did not wish to alter the oxygen concentration of our reaction solution. A 4-fold excess of thioglycolate was added before acidification. The pH was restored to 7.4 with an NH\textsubscript{3} and nitrogen gas mixture.

Monitoring the Reaction—Slow reactions were monitored with a Cary 118C spectrophotometer. Initial rates were determined using an absorbance scale and chart speed selected to optimize slope determinations. A coefficient of variation in initial rate determination of approximately 4% was determined in successive trials of identical reactions. Rapid reactions were examined using a Durrum model 110 stopped flow spectrophotometer (3).

In the experiment exhibited in Fig. 4, reaction rates at pH values below 6.5 were examined by stopped flow spectrophotometry. Fe\textsuperscript{2+}.transferrin-CO\textsubscript{3}\textsuperscript{2-} was placed in one syringe. This solution was at pH 7.4 weakly buffered with 10 mM Hepes. Dithionite and BPS were contained in the other stopped flow syringe. These reagents were in a much more concentrated buffer solution adjusted to a point near the desired pH value. The pH of the reaction mixture was determined by emptying the contents of the stop syringe into a small plastic vial and measuring with a pH electrode. In this fashion the Fe\textsuperscript{3+}.transferrin-CO\textsubscript{3}\textsuperscript{2-} was maintained at physiological pH until the exact start of the reaction.

RESULTS

The Reductive Release Reaction—The reaction in which the ion of Fe\textsuperscript{3+}.transferrin-CO\textsubscript{3}\textsuperscript{2-} is reduced to Fe\textsuperscript{2+}, and released from the protein, and bound by a ferrous iron chromogen is depicted by the chemical equation:

$$\text{Fe}^{3+}.\text{TRF-CO}_3^{2-} + \text{Rd} + \text{Chrm} \rightleftharpoons \text{Fe}^{2+}.\text{Chrm} + \text{TRF} + \text{Ox} + \text{CO}_3^{2-}$$

where Rd is the reducing agent and Ox, its oxidized product; TRF is transferrin and Chrm is a Fe\textsuperscript{2+} chromogen. The reaction can be reversible, that is the binding of ferrous iron ion by apotransferrin and its subsequent oxidation to the trivalent form to yield Fe\textsuperscript{3+}.transferrin-CO\textsubscript{3}\textsuperscript{2-} is known to occur in aerobic solutions (2). In order to obviate complexities resulting from the reverse reaction and to provide a sensitive spectrophotometric probe, we employed BPS as a chromogenic ferrous ion acceptor. Fe\textsuperscript{2+}.BPS has a molar absorptivity of 22,140 liters mol\textsuperscript{-1} cm\textsuperscript{-1} (12) and it is completely inert toward apotransferrin. BPS is known to form a 3:1 BPS-iron complex.

Accordingly, the BPS concentration in our reaction solutions always exceeded the iron by a factor of 3 or greater.

Sodium dithionite (Na\textsubscript{2}S\textsubscript{2}O\textsubscript{4}) was chosen as the reducing agent for much of our work since it is not expected to substitute at the carbonate binding site of transferrin (9), it is a strong reducing agent, and it is relatively free from interfering side reactions.

A problem encountered with the use of dithionite is the acidity of its oxidation products. These are generated initially in the reaction with molecular oxygen dissolved in the reaction solution and during the reductive iron release reaction. In order to maintain a constant pH, a high buffer capacity is required. We used 0.5 M Hepes at pH 7.4 except where noted. This buffer stabilized the pH to within 0.1 to 0.2 pH unit over a 3-day period. Control experiments showed that less than 2% of the iron of Fe\textsuperscript{3+}.transferrin-CO\textsubscript{3}\textsuperscript{2-} was reduced under the solution conditions employed in the absence of reducing agents.

Several control experiments were carried out to assure the validity of monitoring the reaction using the 538 nm peak of Fe\textsuperscript{3+}.BPS. The ferrous chromogen complex is stable in the presence of dithionite for a period of at least 3 days in the pH range studied. BPS by itself appears to be acted upon slowly by dithionite; however, this reaction does not interfere with the reductive release reaction or the 538 nm peak. In long term reactions, requiring 3 to 4 days, precipitation of protein is noted under certain conditions as the reaction nears completion. This turbidity leads to an increase in absorbance at 538 nm. Thus, we were only able to accurately monitor the first 75% to 80% of the reaction. This turbidity did not interfere with those studies in which we examined initial reaction rates. BPS is unable to remove iron from Fe\textsuperscript{3+}.transferrin-CO\textsubscript{3}\textsuperscript{2-} in the absence of reducing agents. The addition of Fe\textsuperscript{3+} to BPS results in no absorbance increase at the 538 nm. Other workers have used 535 nm as the absorbance maximum of Fe\textsuperscript{2+}.BPS (13). The exact maximum appears to be somewhat dependent on BPS concentration and we chose to use 538 nm as the monitoring wavelength under our conditions.

Reduction Rate as a Function of Reactant Concentrations—The rate of the reaction in which the iron of Fe\textsuperscript{3+}.transferrin-CO\textsubscript{3}\textsuperscript{2-} is reduced by dithionite and complexed by BPS was observed as a function of reactant concentrations. In the experiment depicted in Fig. 1 the Fe\textsuperscript{3+}.transferrin-CO\textsubscript{3}\textsuperscript{2-} concentration was varied over a 30-fold range while the concentrations of dithionite and BPS were maintained constant. A linear plot is observed indicating that the reductive release reaction is first order with regard to the concentration of Fe\textsuperscript{3+}.
The exact cause of this spectral change is not understood. The slope of this plot and the dithionite concentration yields an apparent second order rate constant of $1.3 \times 10^{-3}$ liter eq$^{-1}$ s$^{-1}$.

The initial velocity of the reductive release reaction over a 1000-fold range of dithionite concentration is shown in Fig. 2. The plot is linear, however, it has a non-zero intercept. The experiment was repeated several times with the same result. The reason for the non-zero intercept is ascribed to a change in the absorbance of the reaction mixture upon addition of dithionite. The exact cause of this spectral change is not understood. The slope of this plot together with the concentration of Fe$^{3+}$-transferrin-CO$_3^{2-}$ yields a calculated second order rate constant of $1.3 \times 10^{-3}$ liter eq$^{-1}$ s$^{-1}$.

A 40-fold range of BPS concentration resulted in less than a 5% variation in the initial velocity of the reductive iron release reaction. The reaction rate is thus zero order with respect to BPS. An apparent second order rate constant of $1.3 \times 10^{-3}$ liter eq$^{-1}$ s$^{-1}$ was observed in the experiment in excellent agreement with the values reported above.

The results described above can be interpreted in terms of the reaction sequence shown here:

**Reaction Sequence I**

where Rd is dithionite and Ox, its oxidized products. Other abbreviations are stipulated above. The first step is assumed to be rate-limiting. It is a bimolecular interaction between dithionite and the Fe$^{3+}$-transferrin-CO$_3^{2-}$, resulting in the formation of Fe$^{2+}$-transferrin-CO$_3^{2-}$ and inorganic oxidation products. Since the binding of Fe$^{3+}$ to transferrin is weak and in rapid equilibrium (2, 4) it is anticipated that a rapid release of the Fe$^{3+}$ from the protein would occur. The third and final step is the complexing of Fe$^{2+}$ by BPS, a reaction which is extremely rapid. Since BPS is not involved in the rate-limiting step of the reaction sequence its concentration does not affect the reaction velocity.

These results are important since they indicate that the observed kinetics are a manifestation of Step 1 and not subsequent iron release or binding to the chromogen. We also examined the reductive release reaction using 2,2'-bipyridine as the ferrous chromogen. As observed with BPS, the reaction rate shows only slight variation with the concentration. The average velocity value together with the concentrations of Fe$^{3+}$-transferrin-CO$_3^{2-}$ and dithionite yielded an observed second order rate constant of $1.3 \times 10^{-3}$ liter eq$^{-1}$ s$^{-1}$ in agreement with those reported above.

**Reaction Time Course under Pseudo-first Order Conditions**

![Graph](http://www.jbc.org/)

**Fig. 2.** The effect of dithionite concentration on the initial velocity of the reaction in which the iron of Fe$^{3+}$-transferrin-CO$_3^{2-}$ is reduced by dithionite and sequestered by BPS. The dithionite concentration was varied over a 1000-fold range while the other reactants and solution components were maintained constant at the concentrations indicated in the legend to Fig. 1. Fe$^{3+}$-transferrin-CO$_3^{2-}$ concentration was 73 μeq liter$^{-1}$ (in iron).

![Graph](http://www.jbc.org/)

**Fig. 3.** A first order kinetic plot of the time course of the reaction in which the iron of Fe$^{3+}$-transferrin-CO$_3^{2-}$ is reduced by dithionite and sequestered by BPS. Initial concentrations of reactants were Fe$^{3+}$-transferrin-CO$_3^{2-}$, 73 μeq liter$^{-1}$ (in iron); BPS, 0.25 mM; and dithionite, 30 mM. The buffer was 0.5 M Hepes, 0.15 M NaCl, and 10 mM NaHCO$_3$, pH 7.4. The solid line represents the curve anticipated for two independent simultaneous first order reactions taking place with rate constants of $3.3 \times 10^{-3}$ s$^{-1}$ and $5.3 \times 10^{-3}$ s$^{-1}$. The deviation of the data from the curve at times greater than 35 h may be due to incipient precipitation. The data is interpreted in the text in terms of the possibility of a differential reactivity of the two sites of Fe$^{3+}$-transferrin-CO$_3^{2-}$.

![Graph](http://www.jbc.org/)

**Fig. 4.** The logarithm of the initial velocity (in eq 1$^{-1}$ s$^{-1}$) of the reaction in which the iron of Fe$^{3+}$-transferrin-CO$_3^{2-}$ is reduced by dithionite and sequestered by BPS as a function of pH. The concentration of the solution components were as indicated in Fig. 3 with the exception that the dithionite concentration was 26 mM and the buffers were altered according to the pH. For determinations between pH 7.9 and 6.9, the buffer was 0.5 M Hepes. For determinations between pH 7.3 and 6.7, the buffer was 0.5 M Heps plus 0.1 M sodium acetate. At pH values greater than 6.5, reaction rates were determined using the Cary 118C. The more rapid reactions below pH 7.3 were monitored using a Durrum model 110 stopped flow spectrophotometer as described above under "Experimental Procedures." At pH values below 4.2 precipitation was observed and that rates could not be determined.
The reductive iron release reaction was examined at a dithionite concentration 411 times that of Fe²⁺-transferrin-CO₃⁻. Dithionite concentration thus becomes a constant factor in the rate equation, and the time course of the reduction can be plotted according to first order kinetics. This plot is shown in Fig. 3. The solid line is calculated for two independent simultaneous first order reactions with rate constants of 0.12 h⁻¹ (3.3 × 10⁻⁵ s⁻¹) and 0.019 h⁻¹ (6.3 × 10⁻⁶ s⁻¹). The close fit of the data to this line suggests that the two binding sites of transferrin may differ by a factor of approximately 6 in their susceptibility to reduction by dithionite. Deviation from the curve after about 35 h is attributed to incipient precipitation of the protein.

In a communication, Harris et al. (6) described the reductive release of iron from Fe³⁺-transferrin-CO₃⁻ labeled with ⁵⁹Fe and ⁵⁸Fe. They reported no difference in the rate of iron release from the two transferrin binding sites. Their methodology and conditions were quite different from ours (e.g. no Fe²⁺ acceptor was present, the pH varied during the experiment) and it is not possible to explain the discrepancy in our results.

The Effect of pH on the Reductive Release Reaction—The affinity of transferrin for iron decreases at acidic pH (7, 14–16). A series of reactions was carried out to examine the effect of pH on the susceptibility of Fe²⁺-transferrin-CO₃⁻ to reduction by dithionite. In Fig. 4 the results are plotted as the log of the velocity of the reductive iron release reaction as a function of pH. The slope of the line between pH 6.7 and 4.3 has a value of -2.0. Within this range the rate equation is second order with regard to hydrogen ion concentration. This is consistent with, but not limited to a system in which the protonation of two groups increases the susceptibility of the iron to reduction.

The Effect of NaHCO₃ and Ca²⁺—We reported that Ca²⁺ and NaHCO₃ decrease the rate and extent of iron removal from Fe³⁺-transferrin-CO₃⁻ by nitritolactate (17). The effect of Ca²⁺ and NaHCO₃ on the reductive iron release reaction was studied. In contrast to the decreased rate observed in the chelate-mediated iron removal reaction, a 30% increase in the rate of reductive iron removal is observed on increasing added NaHCO₃ from 0 to 50 mM.

Before the effects of Ca²⁺ on the reaction can be studied it is necessary to remove any contaminating Ca²⁺ from Fe³⁺-transferrin-CO₃⁻. This was done by incubation of the iron protein with 0.5 mM EDTA and subsequent chromatography of the mixture on a Sephadex G-25 column equilibrated and eluted with the standard buffer. The addition of Ca²⁺ led to a slight increase in the rate of the reaction. This effect appeared to plateau at approximately 10 mM Ca²⁺ and resulted in an increase of only 13% in the rate of the reaction. Thus, both NaHCO₃ and Ca²⁺ tend to slightly enhance the reductive iron release reaction.

The Effect of Guanidine Hydrochloride—It is possible that conformational changes associated with iron binding by transferrin (18) may be utilized in enhancing the rate of release of iron to cellular transferrin receptor sites. Kennah (19) has provided evidence that the denaturation of Fe²⁺-transferrin-CO₃⁻ by guanidine hydrochloride does not fit a simple two step mechanism. Intermediate conformational states may be present. We investigated the possibility that the presence of a denaturing agent would labilize the iron of Fe²⁺-transferrin-CO₃⁻ toward reducing agents.

In Fig. 5 is shown the rate of the reductive iron release reaction as a function of guanidine hydrochloride concentration. In the presence of guanidine hydrochloride, it was necessary to use ascorbate as the reducing agent and 2,2'-bipyridine as the ferrous ion acceptor in order to avoid precipitation in the reaction mixture. It was found that the reductive iron release reaction is first order in ascorbate and zero order with regard to 2,2'-bipyridine. It is clear that the denaturing agent, guanidine hydrochloride, enhances the rate of the reductive iron release reaction. The rate in the presence of 2.25 mM guanidine hydrochloride is some 60 times greater than in the absence of this denaturant.

The Effect of Chelating Agents—The possibility that some chelating agents and reducing agents might work in concert is examined in this section. In the absence of chelating agents the kinetics of the reductive iron removal reaction are in agreement with Reaction Sequence I postulated above. When a chelating agent is added an additional reaction pathway for iron is possible:

\[
\text{Step 1 } \text{Fe}^{3+} \cdot \text{TRF} \cdot \text{CO}_3^- + \text{Chel} \rightarrow [\text{Chel})\text{Fe}^{3+} \cdot \text{TRF} \cdot \text{CO}_3^-] + \text{Ox}
\]

\[
\text{Step 2 } [\text{Chel})\text{Fe}^{3+} \cdot \text{TRF} \cdot \text{CO}_3^-] + \text{Rd} \rightarrow [\text{Chel})\text{Fe}^{2+} \cdot \text{TRF} \cdot \text{CO}_3^- + \text{Ox}
\]

where Chel indicates the chelating agent. If Steps 2 and 3 are much faster than Step 1, the overall rate of Reaction Sequence II will be the same as the rate of iron removal from Fe²⁺-transferrin-CO₃⁻ by the chelating agent. For all chelating agents tested, with the exception of EDTA and high concentrations of thioglycolate, this was found to be true. The rate of reduction of the iron of Fe²⁺ chelate and transfer to BPS was much faster than the rate at which the chelate removed iron from Fe²⁺-transferrin-CO₃⁻.

If one assumes that Reaction Sequences I and II are the only pathways of iron removal under these conditions, then the rate of Fe²⁺-BPS formation, when the chelate is added to the reductive iron release reaction mixture, should be the combined rates of reductive iron removal and chelation iron removal. One may postulate other pathways for the removal of iron from Fe³⁺-transferrin-CO₃⁻ in the reductive iron removal reaction mixture containing added chelating agent. Another possibility is given here:

\[
\text{Step 1 } \text{Fe}^{3+} \cdot \text{TRF} \cdot \text{CO}_3^- + \text{Chel} \rightarrow ([\text{Chel})\text{Fe}^{3+} \cdot \text{TRF} \cdot \text{CO}_3^-]
\]

\[
\text{Step 2 } [([\text{Chel})\text{Fe}^{3+} \cdot \text{TRF} \cdot \text{CO}_3^-] + \text{Rd} \rightarrow ([\text{Chel})\text{Fe}^{2+} \cdot \text{TRF} \cdot \text{CO}_3^-] + \text{Ox}
\]
Reductive Release of Iron from Transferrin

where \([\text{ChelFe}^{2+} \cdot \text{TRF-CO}_3^{2-}]\) is a complex of an unspecified geometry in which the chelating agent is bound to \(\text{Fe}^{3+}\). transferrin-CO\(_3^{2-}\) to form a quaternary complex.

If the quaternary complex is more susceptible to reduction than is \(\text{Fe}^{2+}\). transferrin-CO\(_3^{2-}\), a reaction rate greater than that expected for the sum of Reaction Sequences I and II would be observed. Conversely, if the quaternary complex of chelating agent and \(\text{Fe}^{3+}\). transferrin-CO\(_3^{2-}\) represents a stable, more inert complex, then the rate of reaction would be less than expected for the combination of the chelation and the reduction pathways.

In Table I is shown the effect of chelating agents on the reductive iron removal reaction. In Column I is the rate of iron removal from \(\text{Fe}^{3+}\). transferrin-CO\(_3^{2-}\) by the chelating agent alone. This rate was determined by decrease in absorbance at 465 nm.

Column II adds the rate of the chelation reaction (Column I) to the rate of the reductive iron release reaction under these conditions \((3.0 \times 10^{-9} \text{ eq liter}^{-1} \text{ s}^{-1})\), and gives the anticipated rate of reaction if no third synergistic pathway is involved.

The observed rate of iron removal from \(\text{Fe}^{3+}\). transferrin-CO\(_3^{2-}\) in the presence of the chelating agent, dithionite, and BPS is given in Column III. In most cases this value exceeds the anticipated value given in Column II.

Column IV shows the difference between the observed reaction rate and that anticipated for Reaction Sequences I and II combined. With the exception of ATP and perhaps phosphate, there is kinetic evidence for a synergistic interaction of chelating agent and reducing agent. While Table I does not establish that Reaction Sequence III above is the actual reaction route, it is clear that cooperative interactions between chelating agents and dithionite not only occur, but may predominate in leading to the release of iron from transferrin. It must be noted that cysteine and thioglycolate can act as reducing agents as well as chelating agents as discussed below.

The Effect of Pyrophosphate—Pollack et al. (20) and others have demonstrated the unusually high efficiency of pyrophosphate in removing iron from \(\text{Fe}^{3+}\). transferrin-CO\(_3^{2-}\). Our results shown in Table I are in concert with this observation. The effect of pyrophosphate concentration on the rate of iron removal by chelation alone, and by chelation and reduction acting together is shown in Fig. 6. The curve labeled “Reduction Reaction” gives the rate of appearance of \(\text{Fe}^{2+}\). BPS. The curve labeled “Chelation Reaction” exhibits the effect of pyrophosphate concentration on the reaction in which iron is removed from \(\text{Fe}^{3+}\). transferrin-CO\(_3^{2-}\) by a competitive chelation by pyrophosphate. This reaction was monitored by the decrease in absorbance at 465 nm. Concentrations of reactants and buffer were as indicated in the legend to Fig. 3.

Reductive Release of Iron from Transferrin

\[
\text{Chelation agent} \quad \text{Rate of iron removal by chelating agent} \quad \text{Anticipated rate of iron removal by chelating agent and dithionite} \quad \text{Observed rate of iron removal by chelating agent and dithionite} \quad \text{Apparent rate of synergistic pathway}
\]

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* Column I. This rate was determined by observing the decrease in absorbance at 465 nm in a reaction mixture containing \(\text{Fe}^{3+}\). transferrin-CO\(_3^{2-}\) and the 10 mm chelating agent.

** Column II. This is the sum of the iron removal rate by chelation (Column I) and by reduction \((3 \times 10^{-9} \text{ eq liter}^{-1} \text{ s}^{-1})\).

*** Column III. This is the observed rate of appearance of \(\text{Fe}^{2+}\). BPS in the reaction mixture containing \(\text{Fe}^{3+}\). transferrin-CO\(_3^{2-}\), chelating agent, dithionite, and BPS.

**** Column IV. This is the increment in the observed rate over that anticipated. It is the rate attributable to third (synergistic) reaction pathway.

Determined by increase in absorbance at 535 nm maximum of \(\text{Fe}^{2+}\). thioglycolate.

![Fig. 6. A comparison of the dependence of two reactions on pyrophosphate concentration.](http://www.jbc.org/)

The chelation reaction follows a roughly hyperbolic dependence of rate on pyrophosphate concentration. We searched for spectrophotometric evidence of a complex of pyrophosphate and \(\text{Fe}^{3+}\). transferrin-CO\(_3^{2-}\) but found none. When one subtracts the bottom curve from the top curve of Fig. 6, the plot of the cooperative effect is obtained. This was found to be roughly hyperbolic. The results showing the synergistic action of pyrophosphate and dithionite are consistent with the formation of an intermediate involving pyrophosphate and \(\text{Fe}^{3+}\). transferrin-CO\(_3^{2-}\). The solubility of pyrophosphate limits the concentration range that can be examined. Control experiments showed that \(\text{Fe}^{3+}\). pyrophosphate is rapidly reduced by dithionite and the iron bound by BPS.

The Effect of Reducing Agents—In contributions from other laboratories, several reducing reagents were found to be ineffective in the reductive removal or iron from \(\text{Fe}^{3+}\). transferrin-CO\(_3^{2-}\). These reducing agents included ascorbate, NADIH, and thioglycolate \(5-7\). In some of these experiments a ferrous ion acceptor, such as BPS, was not provided, and the reverse reaction \((2)\) may have been as rapid as the forward reaction. We examined several reducing agents to test their effectiveness in a reaction mixture containing \(\text{Fe}^{3+}\). transfer-
The reductive iron release reaction was carried out with 15 mM reducing agent. Fe<sup>3+</sup>-transferrin·CO<sub>3</sub><sup>2-</sup>, BPS, and the reducing agent. The reaction was examined at pH 7.4 and pH 6.1 since other authors have used slightly acidic conditions in their studies (7). The results which are shown in Table II indicate there is no relationship between the rate of reductive iron removal and the standard oxidation-reduction potential of the reducing agent.

An interesting observation is that NADPH reduces the iron of Fe<sup>3+</sup>-transferrin·CO<sub>3</sub><sup>2-</sup> 3 times faster than does NADH. The standard oxidation-reduction potentials are identical. It may be that specific binding of NADPH to Fe<sup>3+</sup>-transferrin·CO<sub>3</sub><sup>2-</sup> is involved. Dithionite, which is the strongest of the reducing agents tested, is in the middle range of the rates; and thioglycolate, which is in the midrange of the standard oxidation-reduction potentials, gives an extraordinarily high rate of reaction. Several factors may be involved in the action of the reducing agents listed in Table II. Some are able to act as chelating agents and perhaps also to labilize the iron of Fe<sup>3+</sup>-transferrin·CO<sub>3</sub><sup>2-</sup> by formation of quaternary complexes, as discussed below for the case of thioglycolic acid.

Observations on the Reaction with Thioglycolate—The data of Tables I and II suggest an unusually high reactivity of thioglycolate in the removal of iron from Fe<sup>3+</sup>-transferrin·CO<sub>3</sub><sup>2-</sup> whether acting alone (Table I, Column I), with BPS (Table II), or with dithionite and BPS (Table I, Column III). A high degree of cooperativity between thioglycolate and dithionite, in the reductive iron release reaction, is suggested by Column IV of Table I.

The action of thioglycolate was studied by carrying out the reductive iron release reaction over a range of thioglycolate concentration. In Fig. 7 is shown the dependence of the rate of reaction (measured by Fe<sup>2+</sup>-BPS formation) as a function of thioglycolate concentration in the presence and absence of dithionite. Both curves clearly exhibit a saturation of the effect of thioglycolate on the reaction velocity.

This observation is consistent with the formation of a complex of thioglycolate and Fe<sup>3+</sup>-transferrin·CO<sub>3</sub><sup>2-</sup>. Since the thioglycolate can act both as a labilizing chelate and as a reducing agent, it is possible that 2 thioglycolate molecules become bound to each Fe<sup>3+</sup>-transferrin·CO<sub>3</sub><sup>2-</sup>. Replotting velocity divided by thioglycolate concentration yields a bi-phasic plot. We were not able to obtain spectrophotometric evidence for the binding of thioglycolate to Fe<sup>3+</sup>-transferrin·CO<sub>3</sub><sup>2-</sup>.

In the absence of dithionite, dissolved oxygen oxidizes some Fe<sup>2+</sup>-thioglycolate complex, thus competing with Fe<sup>3+</sup>-BPS formation. This affects the rate somewhat at thioglycolate concentrations above 20 mM, hence the line is dashed above this value on the lower curve. The enhancement of the rate in the presence of dithionite is probably related to its action in reducing the iron of Fe<sup>3+</sup>-transferrin·CO<sub>3</sub><sup>2-</sup> and in eliminating oxygen from the solution.

Studies with Fe<sup>3+</sup>-Transferrin-Thioglycolate—In previous studies of anion exchange (21), and iron removal reactions (7, 22) other authors have pointed out that substitution of "labilizing anions," at the carbonate binding site of transferrin, need be considered. Since thioglycolate can act as a carbonate substitute this could account for its labilizing activity. To test the anion substitution hypothesis with thioglycolate, we prepared Fe<sup>3+</sup>-transferrin-thioglycolate (with a 4-fold excess of thioglycolate) by methods described earlier (9, 11). The product was tested spectrophotometrically and found to have the anticipated absorbance maximum, molar absorptivity, and reactivity with NaHCO<sub>3</sub>.

The reaction of Fe<sup>3+</sup>-transferrin-thioglycolate with 26 mM dithionite in the presence of 0.25 mM BPS was observed with stopped flow spectrophotometry. The velocity of the reaction was found to be 4.2 x 10<sup>-5</sup> eq liter<sup>-1</sup> s<sup>-1</sup>. This value is 2000 times greater than would be anticipated for the reduction of Fe<sup>3+</sup>-transferrin·CO<sub>3</sub><sup>2-</sup> by identical concentrations of dithionite and thioglycolate. It thus appears that substitution of thioglycolate at the carbonate binding site of Fe<sup>3+</sup>-transferrin·CO<sub>3</sub><sup>2-</sup> yields a much more reactive complex suggesting that this is not the pathway by which thioglycolate labilizes Fe<sup>3+</sup>-transferrin·CO<sub>3</sub><sup>2-</sup> toward reduction.

### Table II

<table>
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<th>Reducing agent</th>
<th>pH 7.4</th>
<th>pH 6.1</th>
<th>pH 7.4</th>
<th>pH 6.1</th>
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<td>10&lt;sup&gt;-&lt;/sup&gt; s&lt;sup&gt;-1&lt;/sup&gt;</td>
<td>10&lt;sup&gt;-&lt;/sup&gt; s&lt;sup&gt;-1&lt;/sup&gt;</td>
<td>10&lt;sup&gt;-&lt;/sup&gt; s&lt;sup&gt;-1&lt;/sup&gt;</td>
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<td>13</td>
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<td>-0.320</td>
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</table>

*These were reported as absorbance at 455 nm in the absence of reducing agent (see also Ref. 14).

## Discussion

The reductive release of iron from Fe<sup>3+</sup>-transferrin·CO<sub>3</sub><sup>2-</sup> is clearly sensitive to conditions such as pH, the nature of the reducing agent, the presence of chelating agents, substitution at the anion binding site, and so on. The time required for the
Reductive iron release reaction ranges from over 1 year (5) to under 1 s (8) depending on the chemical factors employed. Clearly, through the use of concerted attack mechanisms cellular iron receptor sites could quickly remove iron from Fe^{3+} \cdot \text{transferrin} \cdot \text{CO}_3^{2-} via iron reduction. This discussion will consider the coupling of iron reduction with other chemical factors.

In discussing the effect of chelating agents on the reductive iron release reaction we must be careful to distinguish between the two types of chelating agent used in this study. The first type is the chromogenic ferrous ion acceptor such as BPS and 2,2'-bipyridine. These are not involved in the rate-limiting step of the reaction. Their purpose is to bind the ferrous ion that is released from transferrin, to prevent the reverse reaction, and to provide a sensitive spectrophotometric probe.

The second type of chelating agent used is that group shown in Table I. These are able to remove ferric ion from Fe^{3+} \cdot \text{transferrin} \cdot \text{CO}_3^{2-} and have been shown by Pollack et al. (20) to be active in accelerating the exchange of iron between Fe^{3+} \cdot \text{transferrin} \cdot \text{CO}_3^{2-} and desferrioxamine. It is this type of chelating agent that we shall discuss. The data of Table I shows that five of the seven chelating agents tested accelerated the rate of the reductive iron release reaction beyond that expected from their chelating ability alone. In other words the chelating agents and dithionite exhibited a synergistic effect in removing iron from Fe^{3+} \cdot \text{transferrin} \cdot \text{CO}_3^{2-}. The synergistic pathway (Column IV) was generally faster than the two independent removal routes (chelation and reduction) combined (Column II). There is no correlation between the synergistic pathway enhancing reduction (Column IV, Table I) and the enhancement of ferric ion exchange between Fe^{3+} \cdot \text{transferrin} \cdot \text{CO}_3^{2-} and desferrioxamine (Table I, Ref. 20).

Reaction Sequence III (above) proposes that the synergistic effect may arise from the formation of a quaternary complex, \([\text{Chel} \cdot \text{Fe}^{3+} \cdot \text{transferrin} \cdot \text{CO}_3^{2-}]\), which is more susceptible to reduction than is \(\text{Fe}^{2+} \cdot \text{transferrin} \cdot \text{CO}_3^{2-}\). While the kinetics cannot rule out all other possible models, this appears to be the simplest explanation that is consistent with the data and other reports in the literature.

In forming a quaternary complex the chelating agent could bind to: 1) the ferric ion; 2) an allosteric site; or 3) the anion binding site. The latter has been suggested by Aisen for anion exchange reactions (21) and by Pollack et al. (22) and Carver and Frieden (7) for iron release reactions. We suggest that binding to the ferric ion also needs to be considered.

Aisen has shown that \(\text{Fe}^{2+} \cdot \text{transferrin} \cdot \text{CO}_3^{2-}\) does not release ferric iron in the absence of chelating agents (23). Consistent with this is the fact that the rate of ferric ion removal is dependent on the concentration of the chelating agent. This dependence is linear for EDTA (24), nitrilotriacetate,\(^3\) diethyleneetriaminepentaacetic acid,\(^\text{c}\) citrate,\(^\text{c}\) and approximately hyperbolic for desferrioxamine,\(^\text{c}\) tripolyphosphate,\(^\text{c}\) pyrophosphate, and thioglycolate (see above) and ATP at pH 6.1 (7). The chemical feature that these agents have in common is an ability to complex iron. It seems a safe assumption that bond formation between the chelating agent and the iron of Fe^{3+} \cdot \text{transferrin} \cdot \text{CO}_3^{2-}\) is an important rate-limiting step in these iron removal reactions.

Pollack et al. (20) found that the ability of an anion to accelerate iron exchange between Fe^{3+} \cdot \text{transferrin} \cdot \text{CO}_3^{2-} and desferrioxamine had no relationship to the ability of the anion to form a stable \(\text{Fe}^{2+} \cdot \text{transferrin} \cdot \text{anion}\) complex. While they found no correlation between strength of iron binding and exchange enhancement, all of the effective anions are effective iron-complexing agents. The data can be interpreted in terms of theility to pH in the neutrality region does not eliminate protonation as an important aspect of biological iron exchange reactions, where specific groups rather than the solvent would require a few days, however, the reaction lifetime can be considerably shortened by lowering the pH, addition of chelating agents or denaturing agents, or substitution for carbonate at the anion binding. For example, the reduction of Fe^{3+} \cdot \text{transferrin} \cdot \text{thioglycolate} by 26 mM dithionite has a half-life of 0.2 s. It is apparent that reductive iron release involving a concerted attack on Fe^{2+} \cdot \text{transferrin} \cdot \text{CO}_3^{2-}\) offers a potential pathway for the biological iron exchange reaction.

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Reductive Release of Iron from Transferrin

The reduction and release of iron from Fe3+ .transferrin.CO3(2-).
N Kojima and G W Bates


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