Peisach, J., Stern, J. O., & Blumberg, W. E. (1973) *Drug Metab. Dispos. 1*, 45–57.
Peterson, J. A. (1971) *Arch. Biochem. Biophys. 144*, 678–693.
Philson, S. B., Debrunner, P. G., Schmidt, P., & Gunsalus, I. C. (1979) *J. Biol. Chem. 254*, 10173–10179.
Sharrock, M. (1978) 23rd Annual Meeting of the Biophysical Society, February 25–28, 1978, Atlanta, GA.
Sharrock, M., Münck, E., Debrunner, P., Lipscomb, J. D., Marshall, V. P., & Gunsalus, I. C. (1973) *Biochemistry 12*, 258–265.
Sharrock, M., Debrunner, P. G., Schultz, C., Lipscomb, J. D., Marshall, V., & Gunsalus, I. C. (1976) *Biochim. Biophys. Acta 420*, 8–26.
Sligar, S. G. (1976) *Biochemistry 15*, 5399–5406.
Sligar, S. G., Debrunner, P. G., Lipscomb, J. D., & Gunsalus, I. C. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 5399–5406.
Smith, D. W., & Williams, R. J. P. (1970) *Struct. Bonding (Berlin) 7*, 1–45.
Tang, S. C., Koch, S., Papaefthymiou, G. C., Foner, S., Frankel, R. B., Ibers, J. A., & Holm, R. H. (1976) *J. Am. Chem. Soc. 98*, 2414–2434.
Tsai, R. L., Yu, C.-A., Gunsalus, I. C., Peisach, J., Blumberg, W. E., Orme-Johnson, W. H., & Beinert, H. (1970) *Proc. Natl. Acad. Sci. U.S.A. 66*, 1157–1163.
Tyson, C. A., Lipscomb, J. D., & Gunsalus, I. C. (1972) *J. Biol. Chem. 247*, 5777–5781.
Ullrich, V., Ruf, H. H., & Wende, P. (1977) *Croat. Chem. Acta 49*, 213–222.
Wickman, H. H., McCann, S. W., Sorrell, T. N., & Collman, J. P. (1977) *Bull. Am. Phys. Soc. 22*, 337.
Williams-Smith, D. L., Bray, R. C., Barber, M. J., Tsopanakis, A. D., & Vincent, S. P. (1977) *Biochem. J. 167*, 593.
Yu, C.-A., & Gunsalus, I. C. (1974) *J. Biol. Chem. 249*, 102–106.
Yu, C.-A., Katagiri, M., & Gunsalus, I. C. (1974) *J. Biol. Chem. 249*, 94–101.

Binding of Iron from Nitrilotriacetate Analogues by Human Transferrin†

Edward J. Zapolski and Joseph V. Princiotto*

ABSTRACT: At neutral pH, human transferrin firmly binds iron at two specific iron-binding sites which lose affinity for metal under moderately acidic or alkaline conditions. Ferric nitrilotriacetate (FeNTA), at neutral pH, will efficiently provide iron to transferrin but in a sequential, rather than random, fashion. It initially directs the metal to the transferrin iron-binding site located closest to the protein's C terminus, while other commonly employed iron reagents, for reasons which are not yet clear, provide more iron to the alternate site. We studied the transferrin iron-donating characteristics of two analogues of FeNTA, where one and two of the chelator's acetic acid ligand groups were hydroxyethyl ligands. Although there is little difference in the iron-binding strengths of these chelators, there were marked differences in the rate of iron exchange to transferrin as well as differences in apportionment of metal between sites. Apportionment was studied by measuring the acidic dissociation (pH 5.8) of fractionally ^{59}Fe-labeled diferric transferrin prepared by sequential binding of chelated ^{59}Fe and ^{56}Fe isotopes and by 6 M urea–polyacrylamide gel electrophoresis of partially iron-saturated transferrin solutions that were prepared by using these chelates. Replacement of one NTA ligand had a small effect upon site allocation of iron but enhanced the exchange rate. Substitution of two alcoholic ligands resulted in an iron chelate which provided iron nearly randomly to both sites, but it was sluggishly reactive. Between pH 9 and 10, iron dissociates from transferrin but, in contrast to behavior under acidic conditions where the site closer to the N terminus loses iron affinity at a more neutral pH than the C-terminal site, iron was randomly released from both sites.

Ferric nitrilotriacetate (FeNTA) will furnish its iron completely and efficiently to transferrin's specific iron-binding sites. The chelate does not hydrolyze or form a ternary complex with the protein when bicarbonate is present and circumvents problems encountered when iron salts are used to adjust iron saturation levels or provide a radioiron label to transferrin (Bates & Schlabach, 1973; Workman et al., 1975).

As a reagent, however, FeNTA is not entirely faultless. When less than saturating quantities of FeNTA are added to human apotransferrin, the iron is not equally apportioned to each site but instead is predominately bound to one transferrin iron-binding site (Zapolski & Princiotto, 1977a; Harris, 1977a; Aisen et al., 1978; Van Eijk et al., 1978). The site is located closest to the protein's C terminus (Evans & Williams, 1978) and, among several iron compounds that have been studied in this respect, transferrin binding of FeNTA iron was atypical; all the other compounds provided more iron to the site closest to the N terminus.

The present study was undertaken to determine what effect a slight modification of NTA's iron ligand structure would have upon the characteristics of iron donation to transferrin. Two readily available NTA homologues which form stable and well characterized ferric chelates are Bicine [*N*,*N*-bis(2-hydroxyethyl)glycine] and *N*-(2-hydroxyethyl)iminodiacetic acid (Toren & Koltoff, 1955; Anderegg & Schwarzenbach, 1955; Chaberek & Martell, 1959). We compared the iron-donating characteristics of these chelates to the well-known iron reagents FeNTA and iron citrate. Transferrin loses affinity for iron at high pH as well as low pH (Laurell, 1952). We studied transferrin dissociation with respect to the behavior

† From the Department of Physiology and Biophysics, Schools of Medicine and Dentistry, Georgetown University, Washington, DC 20007. *Received September 17, 1979.* This work was supported in part by National Institutes of Health Research Grant 5R01AM19030 and the American Heart Association (Nation's Capital Affiliate).

0006-2960/80/0419-3599$01.00/0

of each site under alkaline conditions.

Materials and Methods

All glassware used in this study was rendered iron free by soaking it overnight in 6 N HCl.

Human transferrin, purchased from Behringwerke, was further purified by chromatography and dialysis prior to use (Zapolski & Princiotto, 1976) and was finally dialyzed against buffered saline–bicarbonate (0.15 M NaCl, 0.001 M $NaHCO_3$, and 0.001 M Hepes, pH 7.4). The apotransferrin content was determined by titration with ^{59}FeNTA as previously described (Princiotto & Zapolski, 1975). The chelating agents nitrilotriacetic acid (NTA), *N*-(2-hydroxyethyl)iminodiacetic acid (HEIDA), *N,N*-bis(2-hydroxyethyl)glycine (DHEG), and citric acid were used without further purification (Sigma Chemical Co., St. Louis, MO). Stock solutions (1.0×10^{-2} M) were prepared by dissolving free acid in 0.1 N NaOH, and excess base was then neutralized with 0.1 N HCl before final adjustment to volume with water. A solution of ferric chloride was prepared from electrolytic iron (Zapolski et al., 1974) and diluted with 0.1 N HCl to provide a 1.00×10^{-3} M Fe(III) solution.

Iron–chelate solutions of desired ligand/iron ratios were prepared by mixing appropriate volumes of these solutions each time they were used. Chelator solution was added to stirred ferric chloride (with or without $^{59}FeCl_3$ tracer) and carefully adjusted to pH 5 by dropwise addition of 0.1 M $NaHCO_3$ before dilution with water to yield 1.0×10^{-3} or 1.0×10^{-4} M ferric chelate solutions. This procedure provided pale green solutions (colorless at 10^{-4} M). Rapid neutralization or neutralization with a strong base such as NaOH invariably yielded yellow-amber-colored solutions which usually precipitated iron upon standing. FeNTA or FeHEIDA chelate solutions prepared in the described manner appeared to be stable for weeks, but FeDHEG or iron citrate solutions were not. They slowly turned yellow upon standing for 1–2 days.

Transferrin Iron-Binding Studies. To 5 mL of 1.0×10^{-5} M human apotransferrin in buffered saline–bicarbonate, we added 1.10 mL of 1.0×10^{-4} M ^{59}Fe-labeled ferric chelate solution. At appropriate time intervals, 0.2-mL aliquots were removed and placed upon small columns (0.6 × 2.5 cm bed volume) of AG 1 × 4 resin previously equilibrated to pH 7.4 with buffered saline–bicarbonate solution. (The samples entered the resin with 5 s.) This was followed immediately by 0.5 mL of saline wash, and the columns were then washed 3 times with the buffered saline–bicarbonate to yield a final eluate of 3 mL before the columns were placed into clean tubes. Non-transferrin-bound iron which was retained by the resin was then eluted with 1 N HCl. Each eluate was counted in a well-type γ scintillation detector, and the amount of iron bound to transferrin was calculated.

Preparation of Fractionally ^{59}Fe-Labeled Diferric Transferrin. Fractionally ^{59}Fe-labeled diferric transferrin solutions were prepared from iron chelate and apotransferrin solutions. Initially, 0.5, 1.0, or 1.5 equiv of ^{59}Fe or ^{56}Fe ferric chelate (1.00×10^{-3} M) was mixed with 1 mL of 1.0×10^{-4} M apotransferrin in buffered saline–bicarbonate and allowed to stand for at least 1 h (in some cases overnight) to assure complete binding before being passed through columns of AG 1 × 4 resin. The eluates (3 mL) were diluted with 3 mL of buffered saline–bicarbonate before a second increment of iron chelate was added to provide a total of 2.2 equiv of Fe per mol of transferrin. After being allowed to stand another hour, the solutions were again passed through resin columns and finally diluted to 10 mL. Ferric NTA or HEIDA was employed for the second iron addition. Fractionally ^{59}Fe-labeled diferric transferrins were thus obtained by altering the sequence of radioisotope addition, and four samples were prepared for each iron chelate: 0.5 ^{59}Fe + 1.5 Fe, 1.5 + 0.5 ^{59}Fe, 1.0 ^{59}Fe + 1.0 Fe, and 1.0 Fe + 1.0 ^{59}Fe (moles of Fe per mole of transferrin). A control, uniformly ^{59}Fe-labeled differic transferrin, was also prepared from each ferric chelate in a similar manner.

Transferrin Iron Dissociation Studies. Dissociation of human ^{59}Fe-labeled diferric transferrin was studied in the alkaline pH range 7.4–10.0, in the same manner described previously, by utilizing a series of 0.1 M Tris buffers (Princiotto & Zapolski, 1975). Dissociation of fractionally ^{59}Fe-labeled transferrin solutions was studied at pH 9.6 (0.1 M Tris buffer) and at pH 5.8 (0.1 M Tris–maleate buffer). Iron released from transferrin was removed by passing a mixture of diferric transferrin and buffer through a small column of anion-exchange resin as described previously or by membrane ultrafiltration. The mixture was added to a centriflow CF25 filter cone (Amicon, Lexington, MA) and centrifuged. Radioactivity in the resin eluate or ultrafiltrate was measured to determine the percent radioiron still bound by protein.

Polyacrylamide Gel Electrophoresis. Upon isoelectric focusing (IEF) in polyacrylamide gel (pH 5.5–8.5), diferric transferrin prepared from purified Behringwerke apotransferrin separated into 3 to 4 multiple red-brown bands. We therefore isolated human transferrin from 200 mL of human plasma (obtained from a unit of whole blood) by a combination of ammonium sulfate precipitation, column chromatography, and dialysis (Regoeczi et al., 1974). The final product yielded a single red-brown band of transferrin after gel IEF but still contained a trace amount of protein which did not bind iron.

Partially iron-saturated human transferrin preparations were studied by electrophoresis (Makey & Seal, 1976) to determine the distribution of metal between sites. Polyacrylamide gel electrophoresis in 6 M urea was performed by following the procedure described by Aisen et al. (1978). For this purpose, samples were prepared by mixing apotransferrin (1×10^{-4} M) with ferric chelate (1×10^{-3} M) and dialyzing the sample overnight against 6 M urea. Samples (0.010 mL) were applied, and electrophoresis was conducted for 4 h at 300 V. The gel was stained with Coomassie Blue R-250 (Bio-Rad, Richmond, CA) in ethanol–acetic acid–water (5:1:5).

Results

Transferrin iron binding from the chelates employed in this study is illustrated in Figure 1. The time required to completely saturate both transferrin iron-binding sites varied with each ferric chelate, being fastest for FeHEIDA and slowest for iron citrate. The exchange of iron from ferric citrate or ferric DHEG to transferrin was influenced by the ligand/iron ratio. It was accelerated at high ratios, but increasing the ratio had no effect upon iron exchanged from FeNTA and FeHEIDA chelates.

N,N'-Ethylenebis[2-(*o*-hydroxyphenyl)]glycine (EHPG) forms an intensely red-colored, very stabile ferric chelate (Frost et al., 1958). When we mixed FeHEIDA into EHPG, the red FeEHPG chelate would form immediately. With FeNTA, there was a noticeable transient shift from an initial violet color to the red FeEHPG color within 1 s of mixing. Color formation from FeDHEG or iron citrate was much slower and required nearly 30 min before maximum red color intensity developed. Aged solutions of FeDHEG or iron citrate reacted even more slowly, but the reactivity of 1–2-day-old FeHEIDA or FeNTA solutions was unchanged. The same relative order of reactivity that we observed for the exchange of iron to apotransferrin was also seen during the exchange of ferric ion

Table I: Percent of ^{59}Fe Recovered in Resin Eluates after Partial Dissociation at pH 5.8 of Fractionally ^{59}Fe-Labeled Human Diferric Transferrin[a]

	% ^{59}Fe: no. of equiv and addition sequence				
transferrin:	1.0 ^{59}Fe	0.5 ^{59}Fe	1.5 Fe	1.0 ^{59}Fe	1.0 Fe
iron donor:	1.0 ^{59}Fe	1.5 Fe	0.5 ^{59}Fe	1.0 Fe	1.0 ^{59}Fe
NTA	50.0	84.5	22.0	77.1	27.2
HEIDA	48.4	74.0	25.6	71.1	32.3
DHEG	49.9	55.0	38.1	54.9	44.2
citrate	48.5	45.9	54.3	44.1	51.7

[a] Results are expressed as mean values ($N = 7$). In all experiments the standard deviation was less than 4%.

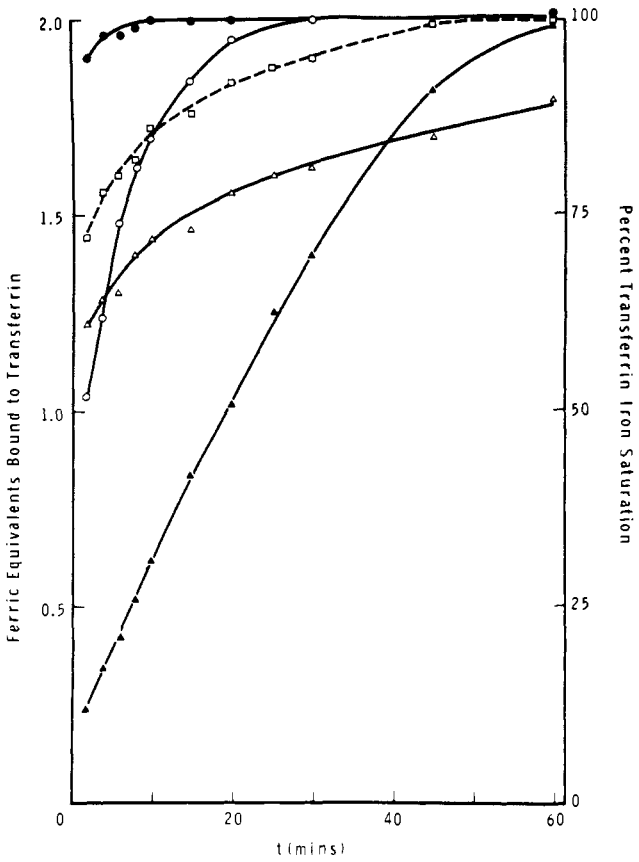

FIGURE 1: Rate of formation of diferric transferrin from apotransferrin and ferric chelates. Ligand/iron 2:1: (○) FeNTA, (●) FeHEIDA, (△) FeDHEG, (▲) iron citrate. Ligand/iron 20:1: (□) FeDHEG and iron citrate.

to this chelator (FeHEIDA > FeNTA > FeDHEG > iron citrate).

Data obtained from dissociation studies of the fractionally ^{59}Fe-labeled diferric transferrin solutions are presented in Table I where the percentage of radioiron recovered in the eluate, still bound to transferrin, is listed. Values obtained by measuring the radioactivity recovered in ultrafiltrates were identical with those obtained from resin eluates (in controls, 50.3 ± 0.6% by resin and 51.8 ± 1.2% by ultrafiltrates). This indicates that the loss of iron was not due to competitive binding by the resin. For each of the four iron complexes, five preparations were tested. Uniformly labeled diferric transferrin preparations lost half of their radioisotope (second column) as iron was released from the N-terminal site under mildly acidic conditions. The last two columns are data collected from preparations initially half iron saturated with ^{59}Fe (column 5) or unlabeled iron (column 6) before being completely iron saturated by the addition of an alternate isotope. Similarly, in columns 3 and 4, each preparation was one-quarter ^{59}Fe saturated either initially (column 3) or subsequent to the binding of unlabeled iron (column 4).

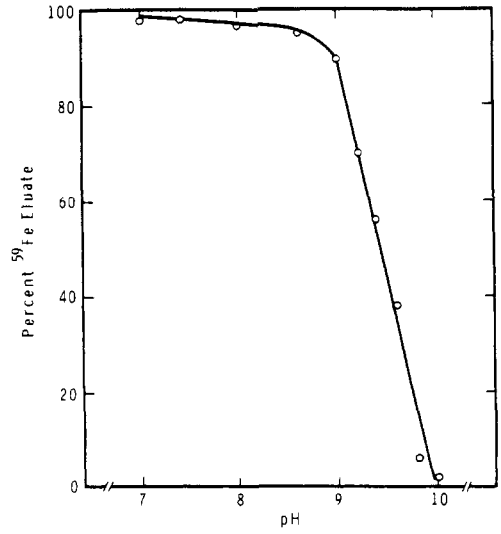

FIGURE 2: Dissociation of human diferric transferrin at alkaline pH (0.05 M Tris-HCl buffer and 5×10^{-6} M transferrin).

When ^{59}Fe from FeNTA was initially bound to transferrin (either 25 or 50% iron saturated), little radioiron dissociated from the protein upon pH reduction (84 and 77% remained bound). However, only 22 or 27% remained undissociated when ^{59}Fe occupied sites left vacant after transferrin first bound unlabeled FeNTA iron. Comparing data from FeHEIDA and FeDHEG to those from FeNTA, we note that if ^{59}Fe was initially added to protein, progressively less radioiron remained bound to transferrin (columns 3 and 5). The opposite effect was seen when radioisotope was added secondly (columns 4 and 6). Comparison of paired values for each ^{59}Fe fractional saturation level (columns 3 and 4 or columns 5 and 6) indicates that for these three iron vehicles, ^{59}Fe was more resistant to dissociation at acid pH when it was the first isotope that was bound to transferrin. In agreement with previous reports (Harris, 1977a; Zapolski & Princiotto, 1977b), more radioiron dissociated from transferrin when iron-59 citrate iron was bound initially (columns 3 and 5).

The pH-dependent, iron-dissociating characteristics of diferric transferrin iron in the alkaline range are shown in Figure 2. Above pH 9, affinity for metal decreased sharply in a monophasic linear fashion.

The same fractionally labeled diferric transferrin compounds used to provide data in Table I were also studied under conditions of alkaline dissociation in Tris buffer at pH 9.6. In marked contrast to the results obtained after dissociation at acid pH, very nearly half of the ^{59}Fe was released from each of the diferric transferrin preparations (47.2 ± 1.51%). This suggests that iron was randomly lost from each site under alkaline conditions.

Polyacrylamide gel electrophoresis in 6 M urea separates partially iron-saturated human transferrin into four compo-

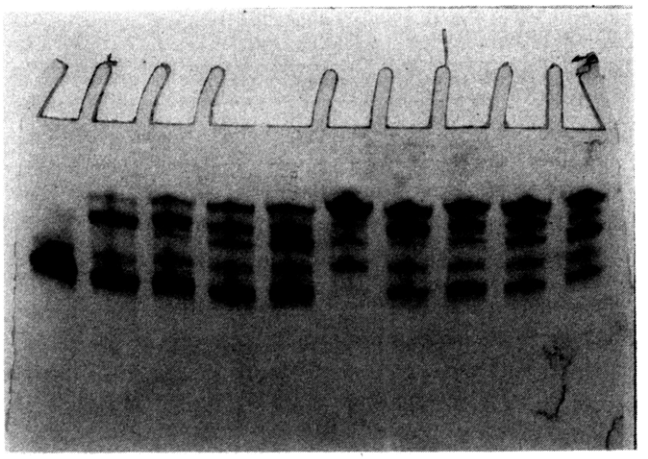

FIGURE 3: Polyacrylamide–6 M urea slab gel electrophoresis of partially iron-saturated human transferrin preparations. Left to right: (1) 100% iron saturated diferric transferrin; (2–5) 60% iron saturated with (2) FeNTA, (3) FeHEIDA, (4) FeDHEG, and (5) iron citrate; (6) apotransferrin; (7–10) 30% iron saturated with (7) FeNTA, (8) FeHEIDA, (9) FeDHEG, and (10) iron citrate.

nents (Makey & Seal, 1976). The results we obtained with this technique are shown in Figure 3. Apotransferrin migrates the slowest (top band); diferric transferrin is the fastest. Monoferric transferrin bearing iron at the C-terminal site is less mobile than the other form of monoferric transferrin and is closer to apotransferrin (second from the upper band); this is the monoferric transferrin produced when transferrin is partially iron saturated with FeNTA iron (Evans & Williams, 1978). There are no differences among the electrophoretic migrations of monoferric or diferric transferrins prepared from each ferric chelate. If the ligand occupied carbonate anion binding sites, the charge and migration of the protein might be altered. At the left of Figure 3 (position 1), diferric transferrin was applied, followed by 60% iron-saturated transferrins prepared with FeNTA (2), FeHEIDA (3), FeDHEG (4), and iron citrate (5). Apotransferrin was applied in the next sample slot (6). It has apparently acquired traces of metal from the 6 M urea dialysis, the electrophoresis buffer, or the gel. Present in all samples (including the next four which are 30% iron saturated with FeNTA, FeHEIDA, FeDHEG, and iron citrate) is a small amount of contaminating protein which migrates slightly slower than diferric transferrin.

Clearly, the relative proportions of C- and N-terminal monoferric transferrin produced by the reaction of apotransferrin with different iron chelates vary. FeNTA iron yields very little N-terminal monoferric transferrin, while for FeDHEG nearly equivalent amounts of N- and C-terminal monoferric transferrins are formed. With iron citrate, the C-terminal species predominates. The polyacrylamide gel electrophoresis results are in excellent agreement with the dissociation data of Table I.

Discussion

When human apotransferrin is made partially iron saturated by the addition of FeNTA at neutral pH, ferric ion is completely exchanged to the protein and almost exclusively bound at one iron-binding site which has been identified by virtue of its unique chemical and biological properties. The site retains affinity for iron at lower pH than its counterpart (Princiotto & Zapolski, 1975; Lestas, 1976), does not surrender its iron to rabbit reticulocytes when both sites bear iron (Princiotto & Zapolski, 1976), and is located closer to the C-terminal portion of the molecule (Evans & Williams, 1978). The monoferric form of transferrin bearing iron at this site has a slower electrophoretic mobility in 6 M urea polyacrylamide gels than diferric transferrin or the species of monoferric transferrin bearing iron bound to the alternate site (Aisen et al., 1978; Evans & Williams, 1978; Makey & Seal, 1976). Van Eijk et al. (1978) reported that in phosphate-buffered saline, FeNTA does not exchange iron human apotransferrin at pH 6.7–7.0 and phosphate also interferes with the exchange of FeNTA iron to ovotransferrin (Rogers et al., 1977). These properties, peculiar to FeNTA, impose serious limitations if it is used to provide a radioiron label to transferrin solutions.

In the present study, we examined the effects produced by small modifications of the NTA ligand upon exchange of iron to transferrin, and we compared iron binding from ferric HEIDA (one $-CH_2COOH$ ligand of NTA replaced by $-CH_2CH_2OH$) and ferric DHEG (two acetate ligands changed to alcohols) to that from ferric NTA and ferric citrate whose properties in this respect have been well characterized. (Trihydroxyethyl-substituted NTA, *triethanolamine*, precipitated iron even at acid pH and could not serve as a transferrin iron donor.)

The NTA-type compounds form 1:1 ferric chelates which are stable at acid pH but differ with respect to alkaline stability. All release three protons when binding ferric ion. Ferric DHEG, once known as Fe(III) specific, has the highest formation constant of the series, log $K = 30.1$ (for FeNTA log $K = 24.3$ and for FeHEIDA log $K = 14.1$), but it disproportionates above pH 7 to form a binuclear complex and precipitate iron (Chaberek & Martell, 1959). At near neutral pH, Warner & Weber (1953) estimate that the formation constant for ferric citrate approximates that of FeNTA (10^{25}).

The same order of reactivity which we observed for transferrin binding of chelated iron (Figure 1) was also seen for the exchange of ferric ion to another chelator, EHPG. Ferric EHPG shows Raman resonance spectral characteristics that are strikingly similar to human transferrin spectra and has been employed as a model compound for transferrin iron binding (Gaber et al., 1974) and to investigate human iron metabolism in vivo (Korman, 1960). Even at a high ligand/iron ratio, which stabilizes ferric citrate from dimerization or polymerization (Bates et al., 1967), FeDHEG and iron citrate were slower in exchanging their iron to EHPG or transferrin than were FeNTA or FeHEIDA. Their sluggish reactivity may be related to this tendency to polymerize or form binucleate complexes. Iron exchange was unrelated to the strength of the chelates. Hegenauer et al. (1979) suggest that strengths of metal–chelate complexes are best compared by looking at the concentration of free (uncomplexed) metal ion in equilibrium with an excess of chelator. At pH 7 the pFe values for FeNTA and iron citrate are identical (16.8) (Hegenauer et al., 1979) and are very nearly the same for FeDHEG (16.2) and FeHEIDA (17.3) (Chaberek & Martell, 1959). Since all the ferric chelates are equivalent in this respect, the differences we observed for the exchange reactions, both with EHPG and with apotransferrin, must be due to the characteristics of the chelate ligands.

Random binding dictates that each transferrin iron-binding site binds half the radioiron added during preparation of the fractionally ^{59}Fe-labeled diferric transferrin solutions. The order of isotope addition and iron saturation level will not alter the distribution but would affect radioisotope distribution if binding was nonrandom (Zapolski et al., 1974). Modification of the NTA ligand structure markedly influenced apportionment of iron between the two transferrin iron-binding sites (Table I and Figure 3). Changing acetic acid liganding groups

to hydroxyethyl ligands shifted the C-terminal site binding pattern observed for NTA iron to an almost random distribution. The change in distribution was most evident for the bis alcoholic compound, but even modification of a single carboxyl group altered the metal distribution between sites.

In concurrence with earlier reports (Zapolski & Princiotto, 1977a; Aisen et al., 1978), we observed that more citrated iron was bound to N-terminal than to C-terminal binding sites. It is interesting that Harris (1977b) noted that chromium(III) citrate complexes also furnished metal selectively to this site. Obviously, whether iron was predominately bound to one site was not determined by the relative iron-binding strengths of the iron chelates since pFe values are similar for all the compounds.

The dissociation of iron from transferrin at alkaline pH (Figure 2) does not reflect any of the characteristics associated with acid dissociation. Iron binding is reported to be irreversibly destroyed above pH 10 (Laurell, 1952). We observed that dissociation occurred between pH 9 and 10, and diferric transferrin lost half its iron near pH 9.6. Unlike dissociation at acid pH, under alkaline conditions metal randomly dissociated from each binding site.

Chasteen et al. (1977) reported that divanadyltransferrin EPR spectral differences varied with pH and suggested that the spectra arise from conformational states of the metal-binding sites where the geometrical arrangement and/or identity of one or more ligands might differ. A similar EPR observation has been reported for transferrin copper binding (Zweier, 1978). At neutral pH, the sites are in A and B conformations but at pH 9, both sites exist in the B conformation. Chasteen et al. (1977) suggested that the loss of iron binding at alkaline pH is associated with the loss of B-conformation signals. Our observation that both sites relinquish iron simultaneously at pH 9.6 may reflect that both sites are in the same conformational state at alkaline pH but are each in different states at neutral pH.

References

Aisen, P., Leibman, A., & Zweier, J. (1978) *J. Biol. Chem. 253*, 1930.

Anderegg, G., & Schwarzenbach, G. (1955) *Helv. Chim. Acta 38*, 1904.

Bates, G. W., & Schlabach, M. R. (1973) *J. Biol. Chem. 248*, 3228.

Bates, G. W., Billups, C., & Saltman, P. (1967) *J. Biol. Chem. 242*, 2810.

Chaberek, S., & Martell, A. E. (1959) *Organic Sequestering Agents*, p 586, Wiley, New York.

Chasteen, N. D., White, L. K., & Campbell, R. F. (1977) *Biochemistry 16*, 363.

Evans, R. W., & Williams, J. (1978) *Biochem. J. 173*, 543.

Frost, A. E., Freedman, H. H., Westerback, S. J., & Martell, A. E. (1958) *J. Am. Chem. Soc. 80*, 530.

Gaber, B. P., Miskowski, V., & Spiro, T. G. (1974) *J. Am. Chem. Soc. 96*, 6868.

Harris, D. C. (1977a) *Biochemistry 16*, 560.

Harris, D. C. (1977b) in *Proteins of Iron Metabolism* (Brown, E. B., Aisen, P., Fielding, J., & Crichton, R., Eds.) p 197, Grune & Stratton, New York.

Hegenauer, J., Saltman, P., & Nace, G. (1979) *Biochemistry 18*, 3685.

Korman, S. (1960) *Ann. N.Y. Acad. Sci. 88*, 460.

Laurell, C. B. (1952) *Pharmacol. Rev. 4*, 371.

Lestas, A. N. (1976) *Br. J. Haematol. 32*, 341.

Makey, D. G., & Seal, U. S. (1976) *Biochim. Biophys. Acta 453*, 250.

Princiotto, J. V., & Zapolski, E. J. (1975) *Nature (London) 255*, 87.

Princiotto, J. V., & Zapolski, E. J. (1976) *Biochim. Biophys. Acta 428*, 766.

Regoeczi, E., Hatton, M. W. C., & Wong, K. L. (1974) *Can. J. Biochem. 52*, 155.

Rogers, T. B., Feeney, R. S., & Meares, C. F. (1977) *J. Biol. Chem. 252*, 8108.

Toren, P. E., & Koltoff, I. M. (1955) *J. Am. Chem. Soc. 77*, 2061.

Van Eijk, H. G., Van Noort, W. L., Kroos, M. J., & Van der Heul, C. (1978) *J. Clin. Chem. Clin. Biochem. 16*, 557.

Warner, R. C., & Weber, I. (1953) *J. Am. Chem. Soc. 75*, 5086.

Workman, E. F., Jr., Graham, G., & Bates, G. W. (1975) *Biochim. Biophys. Acta 399*, 254.

Zapolski, E. J., & Princiotto, J. V. (1976) *Biochim. Biophys. Acta 421*, 80.

Zapolski, E. J., & Princiotto, J. V. (1977a) *Biochem. J. 166*, 175.

Zapolski, E. J., & Princiotto, J. V. (1977b) in *Proteins of Iron Metabolism* (Brown, E. B., Aisen, P., Fielding, J., & Crichton, R., Eds.) p 205, Grune & Stratton, New York.

Zapolski, E. J., Gantz, R., & Princiotto, J. V. (1974) *Am. J. Physiol. 226*, 334.

Zweier, J. L. (1978) *J. Biol. Chem. 253*, 7616.