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Role of Tyrosyl Groups in Metal Binding Properties of Transferrins*

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ABSTRACT: Chicken ovotransferrin and human serum transferrin, and their iron complexes, were modified chemically with reagents which reacted with the tyrosine moieties of the protein. When the metal-free proteins and the iron complexes were treated with triiodide ion, the metal-free proteins lost their chromogenic capacities with added metal ion, but the iron complexes did not. Also the extent of iodination was greatest with the metal-free proteins. The metal binding sites were also protected in the copper complexes. Acetylation of the hydroxyl groups of both transferrins was done with *N*-acetylimidazole; both metal-free transferrins lost their chromogenic capacity, while the iron complexes retained this capacity. Approximately six more *O*-acyl groups were formed on modification of the metal-free transferrins than on modification of the iron complexes. When the acetylated transferrins were *O*-deacylated with hydroxylamine, metal binding capacity was restored. Thus, chemical modifications of transferrins gave strong support for an essential role of tyrosyl groups in the metal binding property of the transferrins.

The transferrins[1] are naturally occurring chelating agents which form highly associated colored complexes with metal ions (Feeney and Komatsu, 1966). Ovotransferrins[2] are present in avian egg whites. Lactotransferrins are present in the milk of mammals. Serum transferrins are present in the bloods of vertebrates.

Because the metal-free transferrins are unstable under the usual conditions used for chemical modification, definitive modification of the metal-free transferrins without loss of their metal binding properties apparently was not accomplished in earlier studies (Fraenkel-Conrat and Feeney, 1950; Azari and Feeney, 1958, 1961). Several chemical modifications, including iodination, destroyed the chromogenic capacities of the metal-free proteins but not those of the iron complexes (Azari and Feeney, 1961; Jones and Perkins, 1965). Buttkus *et al.* (1965) have recently modified chicken ovotransferrin, human serum transferrin, and human lactotransferrin by treatment with acetic anhydride, succinic anhydride, and KCNO without loss of chromogenic capacity with either copper or iron. They considered this evidence that the more readily available amino groups are not essential for binding. A shift in the absorption maximum of the iron complex to shorter wavelength occurred, however, indicating at least an indirect effect of the modifications. Buttkus *et al.* (1965) concluded that the groups most probably involved in the chelation are the nitrogens of the histidyl residues and the hydroxyls of the tyrosyl residues.

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[1] Human serum transferrin is sometimes named siderophilin or β_1-metal binding globulin.

[2] Ovotransferrin is also named conalbumin.

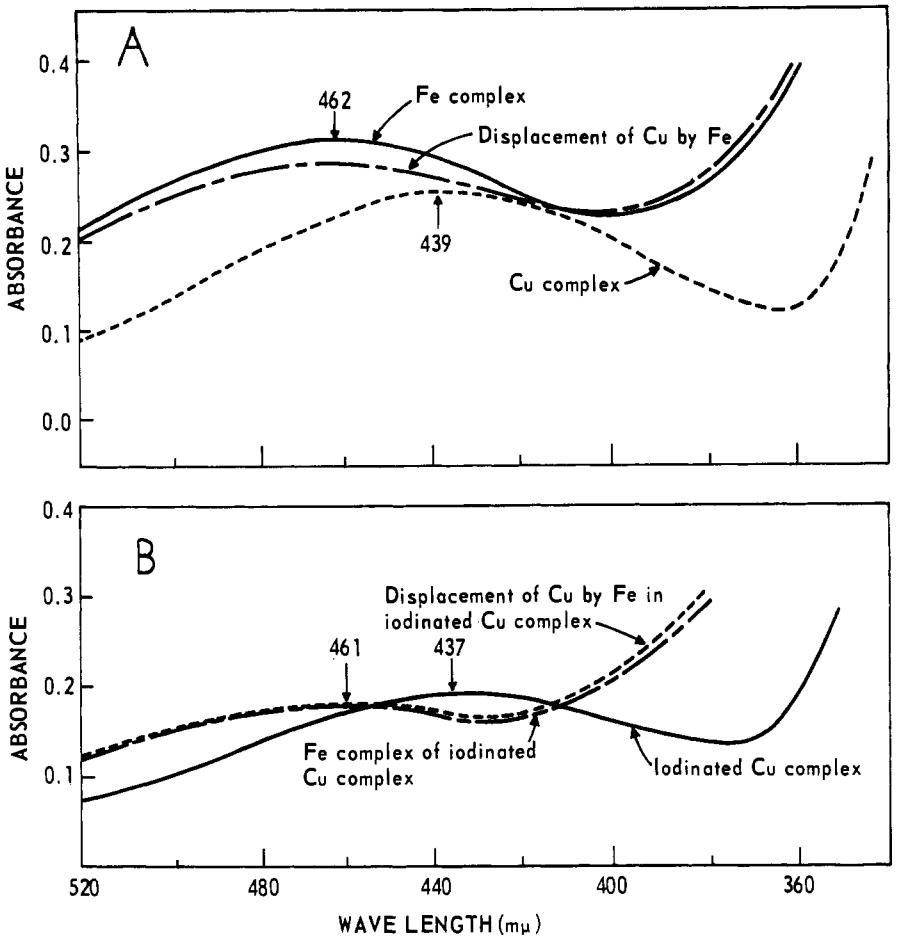

FIGURE 1: Visible spectra of copper and iron complexes of ovotransferrin and iodinated copper complex of ovotransferrin. Iodination was done with excess iodine as described in text. Samples were 5 mg/ml in a 0.02 M citrate–0.1 M HCO_3^- buffer (pH 8.1). Displacement of copper was done by making the solution 0.135 mM in $FeCl_3$. (A) Unmodified ovotransferrins. (B) Iodinated copper ovotransferrins.

We are presently reporting studies on chemical modifications directed at the tyrosines in chicken ovotransferrin and human serum (HS)[3] transferrin. The first apparently successful inactivation and reactivation of the chromogenic capacity is described.

Materials and Methods

Materials. Crystalline chicken ovotransferrin prepared according to Warner (1954) was purified further by DEAE-cellulose column chromatography (Clark *et al.*, 1963). The principal preparations of human serum transferrin were isolated from Cohn fraction IV-7, obtained from Cutter Laboratories, Berkeley, Calif. The transferrin was separated by sequential chromatography on DEAE-cellulose and CM-cellulose and by gel filtration on Sephadex G-200 columns by an adaptation of the method described for the purification of transferrin from rat serum (Gordon and Louis; 1963; Charlwood, 1963). Because such preparations were heterogeneous when examined by electrophoresis in starch gel, small amounts of more homogeneous human serum transferrin were prepared for reference standards and for checking initial modifications. These were prepared by identical procedures, but the starting material was fresh human serum from a single donor.

Radioactive iodine, as $Na^{131}I$ solution (1 mc/ml), was obtained from Volk Radiochemical Co. *N*-Acetylimidazole was obtained from K & K Laboratories. The cellulose exchangers were obtained from the Whatman Co., and the Sephadex G-200 was obtained from Pharmacia Fine Chemicals, Inc.

Chemical Modifications. Conditions for iodination were similar to those described by Azari and Feeney (1961). The transferrins were dissolved in 0.1 M carbonate buffer (pH 9.5). The samples were chilled to 1–2° on an ice bath before the reaction was started. The reagent used for iodination was 0.053 M I_2 solution,

[3] Abbreviation used: HS, human serum.

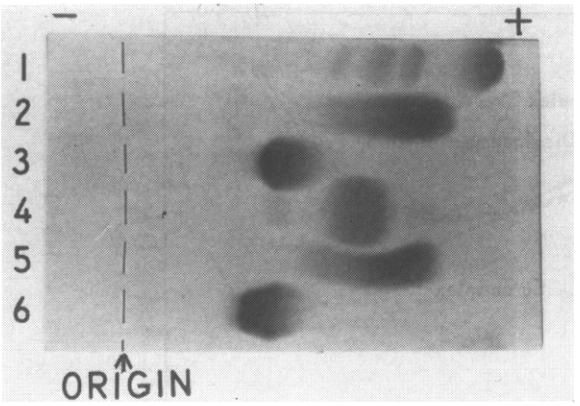

FIGURE 2: Starch gel electrophoretic patterns of ovotransferrins acetylated with *N*-acetylimidazole. Treatment with *N*-acetylimidazole described in text. Electrophoresis was done at room temperature in 16 hr at 8 v/cm and 25 ma, with the discontinuous buffer system: 0.076 M Tris, 0.005 M citric acid, and 2 M urea, at pH 8.6, in the gel buffer; 0.3 M boric acid and 0.06 M NaOH in the bridge buffer. The gel was stained for 2 min with a 0.5% solution of aniline blue-black. The acetylated samples appear in the data of Table I. (1) Iron complex of ovotransferrin acetylated with 100-fold excess reagent. (2) Iron complex of ovotransferrin acetylated with tenfold excess reagent. (3) Iron complex of ovotransferrin. (4) Ovotransferrin acetylated with 100-fold excess reagent. (5) Ovotransferrin acetylated with tenfold excess reagent. (6) Ovotransferrin.

which was also 0.24 M in KI. ^{131}I was used in the radioactive tracer experiments. The final concentration of protein was 10%. The method of preparation of the ^{131}I solution was that of Gruen *et al.* (1959) and Azari and Feeney (1961).

Acetylation with *N*-acetylimidazole was done in 0.02 M sodium veronal–2.0 M NaCl buffer (pH 7.5) (Simpson *et al.*, 1963). The concentration of transferrin was 0.4%. In the reactions, 10-, 50-, or 100-fold excess solid *N*-acetylimidazole, calculated on the basis of the number of residues of tyrosine per molecule of each transferrin, was used. For this purpose, a molecule of ovotransferrin was considered to have 20 tyrosines and a molecule of human serum transferrin to have 25 tyrosines. The reaction, which took place at room temperature (20–23°) over a period of 2 hr, was stopped by acidification with acetic acid to pH 4.5. The samples were dialyzed against water to remove excess reagent and salts before being lyophilized.

Free amino groups were determined routinely by the ninhydrin method (Fraenkel-Conrat, 1957), standardized against values obtained by the Van Slyke gasometric method (Stevens and Feeney, 1963). Values of 76,000 and 90,000 were used for the molecular weights of ovotransferrin and serum transferrin, respectively.

The number of *O*-acyl groups introduced into the protein by acetylation was determined by the hydroxylamine method of Lipmann and Tuttle (1945), as modified by Uraki *et al.* (1957). To 1 ml of a 2.5% solution of acetylated transferrin, 1 ml of 2.0 M hydroxylamine hydrochloride and 1 ml of 3.5 M NaOH were added. After the mixture had been incubated at 25° for 2 min, 1 ml of a 2:1 dilution of concentrated HCl and 1 ml of the ferric chloride reagent (0.37 M ferric chloride in 0.1 M HCl) were added, and the absorbance was read at 540 mμ. Ethyl acetate was used as a standard.

The *O*-acyl groups were removed with hydroxylamine as described by Grossberg and Pressman (1963). A 2% solution of acetylated transferrin was adjusted to pH 9.5 with 1 M NaOH. To this solution an equal volume of 2 M hydroxylamine hydrochloride, previously neutralized, was added. The pH of the mixture was readjusted to 9.5 and the reaction mixture kept

TABLE I: Acetylation of Tyrosine and Amino Residues of Ovotransferrin and Serum Transferrin by *N*-Acetylimidazole.[a]

Sample	*O*-Acetyl Groups Modified[b]		ε-Amino Groups Modified	
	No.[c]	%	No.[c]	%
Experiment A[d]				
Ovotransferrin	<0.10	—	—	—
Ovotransferrin (tenfold excess reagent)	3.25	16.7	12	18
Ovotransferrin (50-fold excess reagent)	9.7	48.5	19	29
Ovotransferrin (100-fold excess reagent)	10.6	53	34	52
Iron ovotransferrin (tenfold excess reagent)	2.14	10.7	4	6
Iron ovotransferrin (50-fold excess reagent)	4.45	22.2	14	21
Iron ovotransferrin (100-fold excess reagent)	4.57	23	29	43
Experiment B[d]				
HS transferrin	<0.10	—	—	—
HS transferrin (tenfold excess reagent)	13.3	59	23	38
HS transferrin (100-fold excess reagent)	18.2	70	39	65
Iron HS transferrin (tenfold excess reagent)	7.7	29.5	11	18
Iron HS transferrin (100-fold excess reagent)	13.5	52	37	62

[a] Conditions described in text. [b] This figure is for total hydroxyl groups acetylated and would include serines and threonines if they were also acetylated. [c] Number per mole. [d] Starch gel patterns of samples of expt A and B are shown in Figures 2 and 3, respectively.

TABLE II: Reactivation of *N*-Acetylimidazole-Acetylated Transferrins upon Deacylation with Hydroxylamine.

Sample	OD (465 mμ)[a] before Fe^{3+}	OD (465 mμ)[a] after Fe^{3+}	Act. (%)
Experiment A			
Ovotransferrin	0.007	0.242	100
Ovotransferrin (50-fold excess reagent)	0.027	0.067	20
Ovotransferrin (50-fold excess reagent + hydroxylamine treatment)[b]	0.038	0.211	70
Ovotransferrin (100-fold excess reagent)	0.024	0.030	3.4
Ovotransferrin (100-fold excess reagent + hydroxylamine treatment)[b]	0.035	0.185	66
Ovotransferrin (100-fold excess reagent + imidazole added)[c]	0.058	0.066	<5
Ovotransferrin (100-fold excess reagent + phenol added)[c]	0.059	0.066	<5
Experiment B			
HS transferrin	0.0123	0.261	100
HS transferrin (100-fold excess reagent)	0.068	0.068	0
HS transferrin (100-fold excess reagent + hydroxylamine treatment)	0.057	0.192	60

[a] Reading of optical densities corrected to a sample concentration of 4.5 mg/ml. Readings made before and after additions of Fe^{3+} as indicated. [b] Treatment with hydroxylamine is described in text. Deacylated samples were found to contain no detectable *O*-acetyl groups. [c] Treatment of the acetylated transferrin with 100-fold excess imidazole or phenol, as compared to the number of tyrosine residues, under the same conditions used for the original acetylation with *N*-acetylimidazole.

at 5° for about 18 hr. The product then was dialyzed and lyophilized.

Removal of Iron and Copper from the Metal Transferrin Complexes. The metal complexes were dissolved and titrated to pH 4.7 with 1 M citric acid, and then made 0.01 M in citrate with 0.1 M citrate buffer (pH 4.7). Dowex 1 (Cl) was added to the above solution and the pH was readjusted to 4.7. The solution was stirred and then filtered.

Physical Measurements. ^{131}I was counted on a Baird-Atomic Model 440 ratemeter. Optical density at 465 and 280 mμ and ultraviolet spectra were measured with a Beckman Model DB spectrophotometer.

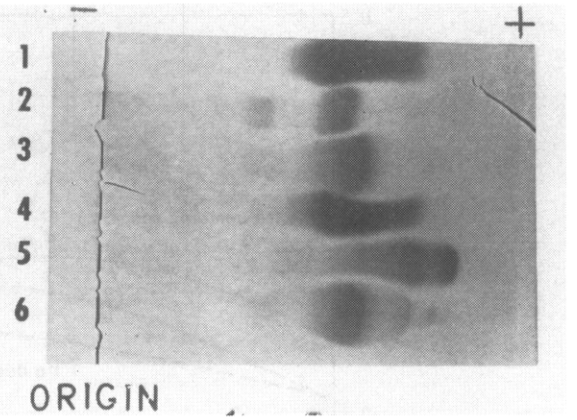

FIGURE 3: Starch gel electrophoretic patterns of human serum transferrin acetylated with *N*-acetylimidazole. Treatment with *N*-acetylimidazole described in text. Electrophoretic conditions described in Figure 2. The acetylated samples appear in the data of Table I. (1) HS transferrin. (2) HS transferrin acetylated with tenfold excess reagent. (3) HS transferrin acetylated with 100-fold excess reagent. (4) Iron complex of HS transferrin. (5) Iron complex of HS transferrin acetylated with tenfold excess reagent. (6) Iron complex of HS transferrin acetylated with 100-fold excess reagent.

Spectra in the visible range were obtained on a Cary Model 14 recording spectrophotometer.

Chromogenic capacities were done spectrophotometrically as previously described (Buttkus *et al.*, 1965). Binding capacities were expressed as percentages of the colors produced with unmodified transferrins and excess metal ions.

Starch gel electrophoresis was used to evaluate the purity of proteins during the isolation of transferrins and to follow the degree of chemical modification of the proteins. The starch gels were prepared according to Clark *et al.* (1963).

Results

Iodination. Modification of metal-free ovotransferrin or metal-free HS transferrin with excess I_3^- led to almost complete (>90%) loss of chromogenic capacity. In contrast, modification of either iron or copper transferrins occurred with almost complete retention of chromogenic capacity. The following numbers of atoms were introduced into ovotransferrin in several experiments (figures in parentheses are averages): ovotransferrin, 27–31 (29); copper ovotransferrin, 24–29 (26); and iron ovotransferrin, 19–27 (23). On reiodination, however, more iodine was introduced. For example, in one experiment the number of atoms of iodine in ovotransferrin increased from 31 to 37 and in copper ovotransferrin from 29 to 33 on reiodination.

Over 80% of the iron and copper binding capacities were retained after iodination of copper ovotransferrin

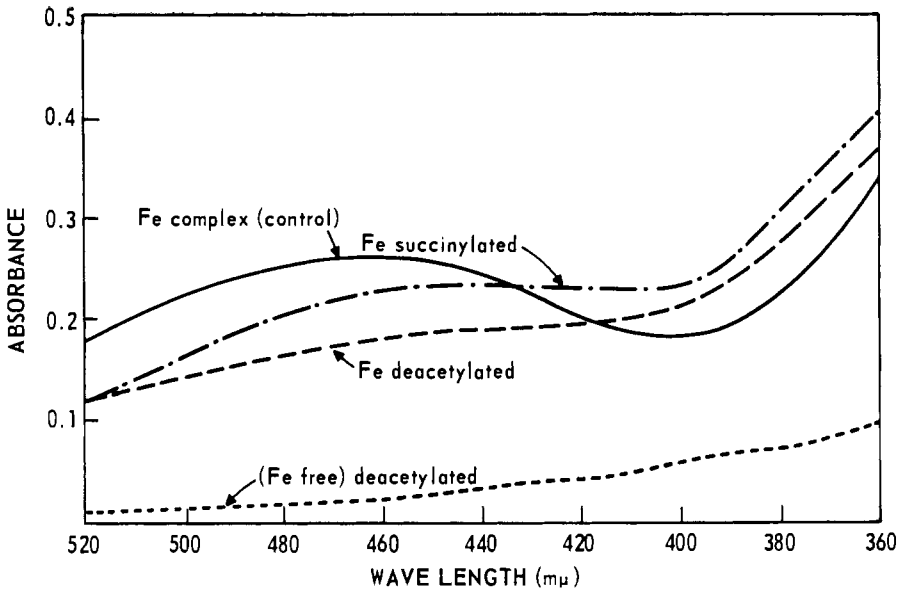

FIGURE 4: Visible spectra of iron complexes of succinylated and *O*-deacetylated ovotransferrins. Chemical modifications are given in the text. Spectra scanned at a concentration of 5 mg/ml in a 0.02 M citrate–0.1 M HCO_3^- buffer (pH 8.1).

or copper HS transferrin (Figure 1). This retention was proven by removing the copper and then adding copper or iron to the colorless or near colorless solutions or by directly displacing the copper by adding iron to the iodinated copper complex.

N-Acetylimidazole. Both hydroxyl and amino groups were approximately equally modified when ovotransferrin, HS transferrin, or their iron complexes were treated with *N*-acetylimidazole (Table I). Starch gel electrophoresis of the samples listed in Table II indicated that all treated samples had increased electrophoretic mobilities. Representative patterns are given in Figures 2 and 3. The mobility toward the anode was increased to a greater extent at low levels of modification. Also, the extent of modification was greater with the iron-free transferrins. Similar experiments with the copper complexes were unsuccessful because the copper complexes apparently were highly dissociated under the conditions of the reaction. Attempts to do the reaction at pH 9 to 10 also were unsuccessful.

Extensive inactivation of the chromogenic capacities for copper or iron was obtained with the metal-free transferrins at the two higher levels of *N*-acetylimidazole studies (Table II). At the highest level (100-fold excess based on tyrosine content) nearly complete inactivation occurred. Only small degrees of inactivation were found at the lower level (tenfold excess) of reagent. No detectable losses in chromogenic capacities were observed on treatment of the iron complexes with any of the three levels of reagent.

Table II also contains representative data on the chromogenic capacities for iron of *O*-deacetylated transferrins. In several experiments the reactivation was 60–75%. The chromogenic capacities for copper were similarly reactivated. Reactivation, however, was not obtained by treatment of the acetylated ovotransferrin with a relatively large level of imidazole or phenol under the same conditions as used for the original acetylation with *N*-acetylimidazole.

Comparisons of the properties of unmodified and modified transferrins revealed that they had several properties not shared in common. The differences in chromogenic capacities and starch gel electrophoretic patterns were discussed above. The iron complexes of the *O*-deacetylated transferrins had the spectral changes and yellow color previously reported for succinylated transferrins and to a lesser extent for acetylated (with acetic anhydride) transferrins (Buttkus *et al.*, 1965). The spectra of the *O*-deacetylated appeared to differ even more extensively from the unmodified than did the succinylated (Figure 4). The starch-gel electrophoretic patterns of the *O*-deacetylated were not significantly different from those of the acetylated.[4]

Discussion

The results of this study support a direct role for tyrosyl residues in the binding site of the transferrins. The specificity of chemical reagents for individual groups in proteins must, of course, always be questioned. In the present study tyrosyls would appear the ones most probably affected by both reagents employed. The inactivation of the metal-free transferrin by iodination confirms previous studies (Azari and Feeney, 1961; Jones and Perkins, 1965). The binding

[4] A study is underway on the effects of various chemical modifications on the physical properties of ovotransferrins of different species.

of iron by the iodinated copper transferrin indicates that the binding of copper protects the entire binding site. The loss of metal binding activity following acetylation with *N*-acetylimidazole, and recovery of the activity after removal of the *O*-acyl groups with hydroxylamine, indicate strongly that several tyrosines are involved per atom of iron bound to the transferrin. The estimate of 60–80% recovery of the initial chromogenic capacity is low. A more realistic estimate probably is that 80–100% of the chromogenic capacities was recovered, since the calculation is based on the extinction at 465 mμ used for unmodified transferrin. The value most likely is low because the absorption maximum of the modified transferrin is at 420–440 mμ rather than at 465 mμ. Measurements were made at 465 mμ because no primary standard was available for the lower wavelength. The quantitative data on numbers of *O*-acyl groups introduced indicated that three hydroxyl groups were involved in the chelation of each atom of iron. The data with iodination indicated that, while the metal complexes were less reactive to iodine, the differential reactivity was a relative one and that no more than three tyrosines are involved in the chelation of one atom of iron.[5]

The binding of iron by iodinated copper complexes might also be explained on the basis of a conformational factor as well as on the protection of an additional ligand (third tyrosine) necessary for the iron complex. The conformational factor could be an indirect masking in the copper complex of the hypothetical additional ligand necessary for iron binding. Still other interpretations, however, might be that iodination of a third essential tyrosine does not affect significantly its chromogenic capacity for iron, or that a third group, believed to be necessary for the binding of iron, might not be a tyrosine.

Another important aspect possibly related to conformational factors concerns the spectra of the *O*-deacetylated transferrins (acetylated by *N*-acetylimidazole) and the succinylated transferrins. Buttkus *et al.* (1965) suggested that the shift in the absorption spectra of the iron complexes of the succinylated derivative to shorter wavelengths might be due to decreased charge transfer in the complex. The present study confirms the spectral shift on acylation but does not supply further insight into the cause of the shift.[4]

The present results do not allow the presentation of formulas for the copper and iron complexes significantly different from those previously presented by Windle *et al.* (1963). These formulas contained two nitrogens (histidines) and two hydroxyls (tyrosines) for the copper complex, and these same four ligands plus a third tyrosine and an extraneous bicarbonate for the iron complex (Aasa *et al.*, 1963; Warner and Weber, 1953; Windle *et al.*, 1963; Wishnia *et al.*, 1961). Feeney and Komatsu (1966) have recently outlined the experimental bases for these formulas and have discussed certain inconsistencies.

[5] After this work was completed, an abstract appeared (Michaud and Woodworth, 1966), in which it was reported that iodination yielded only three more diiodotyrosines per molecule of the iron-free transferrins than per molecule of the iron-saturated transferrins. A value of only four tyrosyls (2/atom of iron) can also be calculated from the data of Azari and Feeney (1961).

Acknowledgment

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