The Effect of Chemical Modification on the Iron Binding Properties of Human Transferrin*

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ABSTRACT: Commercial transferrin was subjected to coupling with 5-diazonium-1H-tetrazole, nitration with tetranitromethane, and alkylation with bromoacetate.

The coupling reaction was not specific for histidine. However, alkylation was quite specific for histidyl side chains. Some loss of iron binding activity occurred when 10 residues had been alkylated and was complete upon the alkylation of 14 residues. This indicated the possible participation of two histidyl side chains in the binding of each iron atom. Nitration of iron-free transferrin produced loss of iron binding activity that was linear with the degree of the phenolic group modification. The "active site" tyrosine residues (three per one iron atom) were unequal with respect to their reactivity to tetranitromethane, and were protected against nitration by iron. Nitrated iron-saturated transferrin retained its iron binding capacity upon the removal and reconstitution of the nitrated material with iron.

Transferrin, the iron-carrying protein of human serum, is said to bind 2 moles of ferric iron/mole of protein (Koechlin, 1952). The two iron binding sites have been found to be independent and equivalent with respect to the mode of iron binding (Aasa *et al.*, 1963; Aisen *et al.*, 1966). Komatsu and Feeney (1967) have recently applied the method of chemical modification in their study of the chemical nature of the iron binding site of transferrin. These workers used *N*-acetylimidazole to acetylate the tyrosyl side chains of transferrin which resulted in the loss of iron binding activity. The metal-complexing properties of transferrin were then restored by deacetylation with hydroxylamine. Three tyrosyl groups were implicated in the binding of each mole of iron by these workers. Earlier physical studies (Aasa *et al.*, 1963; Windle *et al.*, 1963), have also implicated two nitrogen-containing residues in the iron binding properties of transferrin. Since the work of Buttkus *et al.* (1965) has shown that free amino groups were not involved, histidyl side chains have been presumed to take part in the iron binding activity of transferrin in addition to the tyrosyl residues, although the guanido groups have not been excluded.

The work reported in the present communication was designed to test the hypothesis that both histidine and tyrosine residues of transferrin are involved in the binding of iron. Tetranitromethane has been shown to be a specific reagent for the modification of tyrosine residues of proteins in the absence of free sulfhydril groups (Sokolovsky *et al.*, 1966). The nitro groups introduced into the three position of the tyrosine residues of transferrin would be expected to render the electrons of the phenolic oxygen unavailable for coordination of iron and thus inhibit the iron binding activity of transferrin. Bromoacetic acid has been shown to be specific for the modification of imidazole groups at neutrality (Banaszak *et al.*, 1963), and diazonium-1H-tetrazole has been employed as a probe into the reactivity state of histidine residues of proteins (Horinshi *et al.*, 1964, 1965). A molecular weight of 88,000 was assumed for transferrin in all the calculations employed in this paper.

Materials and Methods

Transferrin and Other Reagents. Transferrin was either prepared from pooled human serum by the method of Bezkorovainy *et al.* (1963) followed by gel filtration on Sephadex G-200 (Bezkorovainy, 1966), or was purchased from Hoechst Laboratories, Cincinnati, Ohio. The commercial preparation contained trace amounts of a rapidly sedimenting component (ultracentrifuge); however, in all other respects (molecular weight of 90,000, $E^{1\%}_{.65}$ of 0.6 of the iron-saturated moiety, and single band with β-globulin mobility by zone electrophoresis), this preparation was similar to that prepared in our laboratory (Bezkorovainy, 1966). Glycyl-L-tyrosine was purchased from Nutritional Biochemicals Corp., and TNM[1] was the product

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[1] Abbreviations used: TNM, tetranitromethane; DHT, diazotized 5-amino-1H-tetrazole.

of Aldrich Laboratories. Bromoacetic acid and 3-nitro-L-tyrosine were obtained from K & K Laboratories. All other reagents came from Fisher Laboratories and were of reagent grade quality. Ion-exchange resins and gel filtration media were obtained from Bio-Rad Laboratories.

Analytical Methods. The concentration of unmodified iron-free transferrin was frequently determined from its absorption at 278 mμ ($E^{1\%}_{278}$ 11.7; Bezkorovainy *et al.*, 1963). The concentration of unmodified iron-saturated transferrin was determined by its absorption at 465 mμ ($E^{1\%}_{465}$ 0.6; Inman *et al.*, 1961). Protein concentrations associated with modified iron-free transferrin preparations were determined by the microbiuret method of Itzhaki and Gill (1964), using the native iron-free transferrin as a standard. Identical concentrations of iron-saturated transferrin, iron-free transferrin, and nitrated iron-free transferrin gave identical absorbance values at 300 mμ by this method after the appropriate blank corrections.

Iron was determined by the nitroso-R salt (1-nitroso-2-naphthol-3,6-disulfonic acid disodium salt) method of Ness and Dickerson (1965) (E_{max} 720 mμ). The conventional 2,2′-dipyridyl method of Ramsay (1958) was less advantageous because of the required deproteinization and the interference of nitrotyrosine chromophores of the nitrated transferrin with the spectrophotometer readings at 520 mμ.

Spectral determination of iron (absorption maximum at 465 mμ) was not used because of the interference problems in case of the nitroderivatives described above and the spectral shifts encountered with carboxymethylated iron-saturated transferrin (see below).

Iron binding activity of transferrin preparations was evaluated as follows. A transferrin preparation (10–15 mg) (0.12–0.17 μmole) in 0.1 M $NaHCO_3$ was mixed with 0.8 ml of 1×10^{-3} M iron(III)–citrate complex solution. The mixture was made up to 3 ml with 0.1 M $NaHCO_3$ and was permitted to stand overnight at room temperature. It was then passed through a 1×16 cm column of Dowex 1-X8 (200–400 mesh) in the bicarbonate form. The column was then washed with 0.1 M $NaHCO_3$ whereby the protein was eluted with the void volume (7 ml) and the excess of negatively charged iron–citrate complex was retained by the resin. The protein solution was collected in a volume of 5 ml and both protein and iron were determined in this solution by the colorimetric methods stated above. A 1-ml sample of the eluate (2–3 mg protein) was used for the iron determination. These quantities of native iron-saturated transferrin gave absorbances of 0.2–0.3.

Iron was removed from iron-saturated transferrin or modified iron-saturated transferrin, whenever necessary, by dialysis of the protein against 0.1 M acetate–0.01 M EDTA at pH 4.8 overnight.

Hydrolysis of proteins for amino acid analysis was done under nitrogen with 6 N HCl (protein concentration was 1–2 mg/ml) at 110° for 24 hr. The removal of HCl was done by repeated lyophilization. The amino acid analysis was done using the methods of Moore *et al.* (1958), using the Spinco automatic apparatus. The experimental error in these analyses was 6%. The number of amino acid residues modified was calculated in all cases from the difference between the amount of a specified amino acid in the native protein and that in the modified protein. Quantitation of 3-nitrotyrosine was not reliable because of the overlapping of the hexosamine and 3-nitrotyrosine peaks in the amino acid analysis elution diagrams, and quantitation of carboxymethylated histidines was not done because of the unavailability of the appropriate standards.

All single-wavelength spectrophotometric determinations were done in the Zeiss PMQ-II apparatus. Absorption spectra were recorded in a Beckman DK-2 spectrophotometer.

Optical rotation measurements were done at room temperature in a Perkin-Elmer Model 141 polarimeter with a digital readout. The 10-cm microcells, 1-ml capacity, were used throughout. The light source was a sodium lamp and the rotations were observed at 589 mμ. Reliability of the measurements was ±2°.

Zone electrophoresis was done in a Shandon apparatus on cellulose acetate strips 5 cm wide, using 67 v/strip for 90 min at pH 8.6 (Veronal, 0.05 M). Staining was done with Procion Blue in absolute methanol.

Chemical Modification Methods. Diazotization of 5-amino-1H-tetrazole and coupling with the protein was done, with minor modifications by the method of Horinishi *et al.* (1964, 1965). Alkylation with 2-bromoacetic acid was done by the method of Banaszak *et al.* (1963), except that the buffer used was 0.5 M rather than 1 M phosphate at pH 6.8. The procedure was to incubate 186 mg (2.1 μmoles) of iron-free transferrin with 0.2 M potassium bromoacetate in 10 ml of the buffer (55-fold excess of bromoacetate over each of the 17 histidine residues of transferrin) at room temperature. A control was set up with the same amount of protein and 0.2 M sodium acetate instead of the bromoacetate. Aliquots of 2 ml of both the control and reaction mixture were then removed at 3-day intervals for 12 days. Excess bromoacetate and buffer were removed from the aliquots by dialysis against water. The progress of the alkylation reaction was routinely followed by zone electrophoresis. Each aliquot was also tested for protein concentration and iron binding activity. Amino acid analyses were performed on the alkylated samples only.

Nitration with TNM was done essentially by the method of Sokolovsky *et al.* (1966). It was frequently necessary to employ ethanol in the reaction mixture to facilitate the solution of TNM in aqueous media. For this reason, the stability of iron-free transferrin and iron-saturated transferrin was checked in the presence of various concentrations of ethanol by optical rotation and iron binding activity measurements. Ten milligrams of iron-free transferrin and iron-saturated transferrin was allowed to stand at room temperature for 30 min in the presence of various amounts of ethanol, the total volume being made up to 2 ml with 0.1 M Tris-HCl at pH 8.0. Optical rotations were then taken at 589 mμ. In addition, three samples of iron-free

transferrin of 50 mg each were permitted to stand at room temperature in solution containing 4, 12, and 30% v/v ethanol, respectively, and made up to 10 ml with the Tris-HCl buffer. These solutions were then passed through 4 × 16 cm Bio-Gel P-4 columns equilibrated with 0.1 M Tris-HCl at pH 8.0 and the proteins were collected in volumes of 50 ml. After concentrating the material by dialysis and lyophilization, the iron binding properties of these samples were determined.

The extent of nitration was followed by amino acid analysis and by the spectrophotometric determination of 3-nitro-L-tyrosine (absorption maximum at 420 mμ with a molar extinction coefficient of 3.5×10^3, determined with glycyl-L-tyrosine). In the first series of nitration experiments, 5 mg of iron-free transferrin (0.06 μmole) was incubated with 2.5–90 μmoles of TNM in the presence of 0–30% v/v ethanol, the total volume being made up to 1 ml with 0.1 M Tris-HCl (pH 8.0). After 30 min, 0.5 ml of the sample was passed through a 1 × 16 cm Bio-Gel P-4 column equilibrated with 0.1 M Tris-HCl (pH 8.0) in order to remove ethanol, excess TNM, and nitroform. The protein emerging immediately behind the void volume (5 ml) was collected in a volume of 5 ml and the absorbance at 420 mμ was determined. It must be noted that frequently the nitration experiments had to be carried out in a biphasic system in spite of the presence of ethanol in the medium. For this reason, the abcissa in Figures 4–7 is labeled micromoles of TNM added rather than concentration of TNM.

In the second series of experiments, where the authors were interested in achieving a definite degree of nitration on relatively large quantities of iron-free transferrin, 35 mg (0.4 μmole) of iron-free transferrin and iron-saturated transferrin was nitrated in a total volume of 3 ml in the presence of 0.1 M Tris-HCl (pH 8.0). Both the TNM concentration and the reaction time were manipulated to attain the desired degree of nitration. The reaction mixtures were then passed through 2 × 16 cm Bio-Gel P-4 columns (Sokolovsky *et al.*, 1966) equilibrated with 0.1 M $NaHCO_3$. The nitrated protein emerged immediately behind the void volume (15 ml) and was collected in a volume of 5 ml. It was advantageous to exchange the Tris buffer for $NaHCO_3$ because the former intefered with the protein analysis and because the $NaHCO_3$ is required for the iron binding activity of iron-free transferrin. The $E^{1\%}_{420}$ of nitrotyrosine did not change as a result of the solvent change. The nitrated protein, thus freed of excess TNM and nitroformate, was subjected to spectrophotometric analysis, iron binding capacity determinations, protein determination, and in some cases, amino acid analysis.

The time-course study of the reaction between 30 mg (0.34 μmole) of iron-free transferrin and TNM was done at room temperature with 1×10^{-2} M TNM in the presence of 4% v/v ethanol and made up to 6 ml with 0.1 M Tris-HCl at pH 8.0. Each aliquot used for the spectrophotometric reading was freed of excess TNM, nitroformate, and ethanol by the passage through 1 × 16 cm Bio-Gel P-4 column equilibrated with 0.1 M Tris-HCl at pH 8.0 and collected in a volume of 5 ml.

Results

Coupling of Iron-Free Transferrin and Iron-Saturated Transferrin with DHT. When iron-free transferrin and iron-saturated transferrin were coupled with DHT and the reaction progress was plotted by the method of Horinishi *et al.* (1964, 1965), three plateau regions were observed for both iron-free transferrin and iron-saturated transferrin. However, iron-saturated transferrin required considerably more DHT to attain the same degree of coupling than did iron-free transferrin. The specificity of the reaction was tested by treating 35 mg of iron-free transferrin (0.4 μmole) with 4.7 mmoles of DHT in 300 ml of 0.7 M $NaHCO_3$ at pH 8.8 for 30 min and subjecting the coupled protein to amino acid analysis (Table I). Whereas the spectrophotometric analysis at 480 mμ according to Horinishi *et al.* (1965) showed that all the histidine residues of iron-free transferrin had been modified, the amino acid analysis (Table I) showed that only 10 of the 17 histidine residues were altered with extensive tyrosine and lysine residue modification. These findings are in accord with those of Sokolovsky and Vallee (1966).

Alkylation with Bromoacetate. The progress of alkylation of iron-free transferrin with bromoacetate was followed qualitatively by zone electrophoresis. A representative pattern is shown in Figure 1. Table I shows the amino acid analysis results of iron-free transferrin alkylated for 12 days. This analysis shows that histidine was apparently the only amino acid modified. No loss of lysine or methionine was observed and no evidence of homoserine, its lactone, or *S*-carboxymethylhomocysteine, the breakdown products of *S*-carboxymethylmethionine, was noted in the amino acid elution diagrams. The results are represented in Figure 2. It is evident from this figure that the rate of alkylation had slowed down considerably after 9 days, and that a quantitative modification of histidyl residues would require a formidable length of time under these conditions. For this reason, the last point in Figure 2 was obtained by a 14-day alkylation with 0.4 M bromoacetate, the control being worked up with 0.4 M sodium acetate under otherwise identical conditions. This is contrasted by the alkylation time course of myoglobin (Banaszak *et al.*, 1963) where modification of all available imidazole groups was achieved after 3 days under the above conditions. The effect of alkylation on the iron binding properties of iron-free transferrin is shown in Figure 2B. This plot is designed both to demonstrate the participation of histidine in the binding of iron by iron-free transferrin from a qualitative point of view, and to estimate the number of histidyl side chains required for the binding of each iron atom. Plots similar to that shown in Figure 2B have been used for other purposes by Aisen *et al.* (1966). Nine to ten histidyl residues were apparently not required for the binding of iron by iron-free transferrin. Further alkylation, however, was accompanied by a drastic

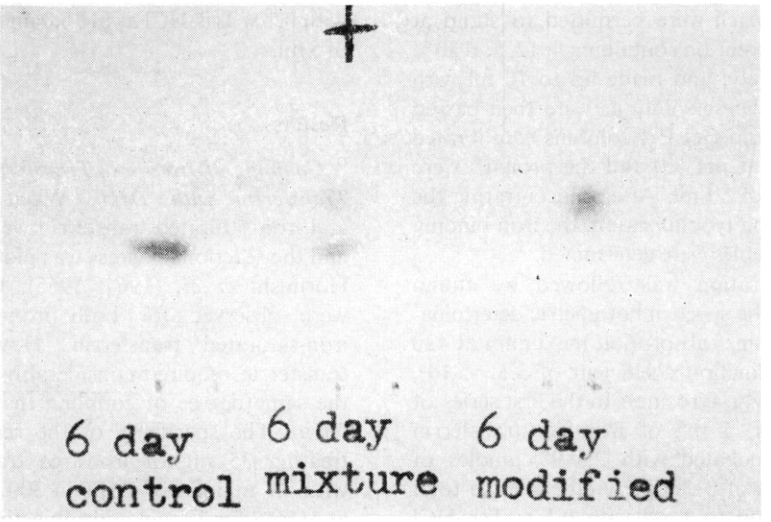

FIGURE 1: Zone electrophoresis of iron-free transferrin, modified iron-free transferrin, and a mixture of the two species. The alkylated iron-free transferrin was obtained after a 6-day reaction with 0.2 M bromoacetate.

TABLE I: Amino Acid Analysis of Native and Modified Iron-Free Transferrins (in moles of amino acyl residue per mole of protein).[a]

Amino Acid	Native	Coupled with DHT	Nitrated with TNM[b]	Carboxymethylated with Bromoacetate[c]
Lysine	53	0	55	57
Histidine	17	7	16	5.5
Ammonia	61	132	72	64
Arginine	30	29	28	28
Aspartic	79	82	76	79
Threonine	28	28	26	29
Serine	35	36	36	38
Glutamic	58	58	57	56
Proline	30	32	30	33
Glycine	47	47	46	47
Alanine	54	55	54	54
Valine	38	38	39	37
Methionine	8	5	7	8
Isoleucine	14	15	14	15
Leucine	56	56	56	56
Tyrosine	25	5	5	23
Phenylalanine	26	26	26	26

[a] To the nearest whole residue; single analyses in all cases; cysteine and tryptophan were not determined; transferrin contains no free SH groups (Bearn and Parker, 1966), hence no reaction of cysteine with TNM or bromoacetate is expected; tryptophan has been shown not to be affected by TNM (Sokolovsky *et al.*, 1966). [b] Spectrophotometric method showed 21 tyrosyl side chains nitrated. [c] Alkylation time, 12 days.

drop in the iron binding activity of iron-free transferrin. Control samples showed full iron binding activity at all time intervals tested (3–14 days).

Absorption spectra of the alkylated iron-free transferrin samples saturated with iron are shown in Figure 3. It is evident that alkylation was accompanied by a shift of the absorption maximum toward the shorter wavelength region, as was noted under different circumstances by Buttkus *et al.* (1965) and Komatsu and Feeney (1967).

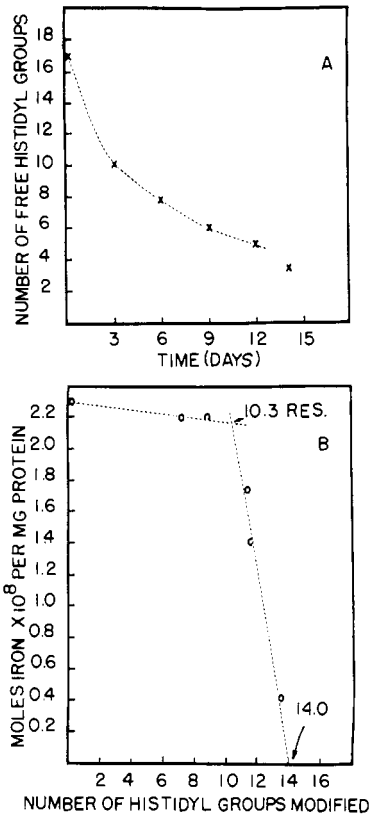

FIGURE 2: Iron-free transferrin studies. (A) Time-course study of the reaction of iron-free transferrin with bromoacetate. See text for details. (B) The relationship between the degree of alkylation and the iron binding properties of iron-free transferrin. The last point in this and Figure 3A was obtained by alkylating iron-free transferrin with 0.4 M bromoacetate for 14 days.

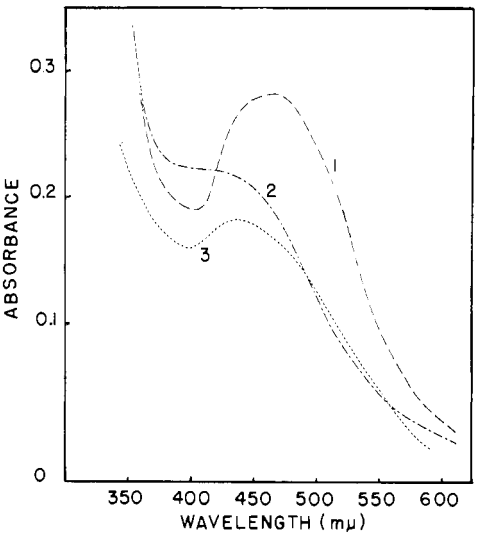

FIGURE 3: Absorption spectra of native and alkylated, iron-saturated transferrins in 0.1 M $NaHCO_3$. curve 1: Native iron-saturated transferrin, full iron binding capacity, 4.3 mg/ml; curve 2: iron-free transferrin alkylated for 12 days, 60% of the original iron binding capacity, 4.8 mg/ml; curve 3: iron-free transferrin alkylated for 6 days, 97% of the original iron binding capacity, 3.1 mg/ml.

Stability of Iron-Free Transferrin and Iron-Saturated Transferrin in Alcohol Solutions. The results of alcohol stability studies on iron-free transferrin and iron-saturated transferrin are shown in Table II. It may be seen that both iron-free transferrin and iron-saturated transferrin were apparently unaffected by alcohol concentrations of up to 20% v/v. In 30% v/v alcohol, however, significant optical rotation differences were observed, indicating alterations in the secondary or tertiary structures of these proteins. However, the iron binding capacity of these proteins, after the removal of ethanol, was apparently unaffected even by exposure to 30% v/v ethanol. When iron-free transferrin was permitted to stand at room temperature for 19 hr in the presence of 4% v/v ethanol, no apparent changes in its optical rotation or iron binding properties were observed.

Nitration of Transferrins. The titration of fixed amounts of iron-free transferrin with varying amounts of TNM and alcohol is shown in Figure 4 (curve 1). A definite plateau region may be seen in the vicinity of 12–14 tyrosine residues modified, the maximum attainable being some 24.6 nitrotyrosine residues/mole of protein. When the same reaction was run with 19% v/v ethanol at all TNM concentrations (curve 2 of Figure 4) was observed. It may be noted that a small plateau region around 12 tyrosine groups modified was present, with a maximum of 16–17 tyrosine side chains nitrated. When the experiment was done in 30% v/v ethanol at all TNM concentrations, the plateau region was abolished (Figure 4, curve 3), and the maximum number of tyrosine residues nitrated approached the value seen in curve 1.

The above experiments suggested that a certain number of tyrosine residues in iron-free transferrin are unavailable for nitration and are rendered reactive only upon the introduction of 30% v/v ethanol in the medium. The experiment represented in Figure 5 was then run to test the possibility. This time-course curve shows that some 23.6 of the 25 tyrosine side chains present in iron-free transferrin were nitrated in 19 hr under relatively mild conditions.

Figure 6 illustrates the progress of the nitration observed with iron-saturated transferrin. It may be noted that the reaction was much slower than that with iron-free transferrin, some 18 nitro residues/mole being introduced after 19 hr in the presence of 30% v/v ethanol.

In the second series of experiments, 35-mg samples of both iron-free transferrin and iron-saturated transferrin were nitrated to varying extent by manipulating TNM concentrations and reaction times. Curves seen in Figures 4–6 were used as guides in choosing the

TABLE II: Stability of Transferrin in Ethanol Solutions after Standing for 30 min at Room Temperature.

Ethanol Concn (% v/v)	Specific Rotation, $[\alpha]_D$		Fe Binding Capacity[d]	
	Fe-Free Transferrin	Fe-Satd Transferrin	Fe-Free Transferrin[a]	Fe-Satd Transferrin[b]
0	−50	−56	2.30	2.30
4[c]	−50		2.30	
11.4	−49		2.40	
19.0	−50			
30	−57	−59	2.35	2.30

[a] After ethanol removal *via* P-4 column. [b] Determined spectrally in the presence of ethanol. [c] After standing 19 hr. [d] Moles of iron $\times 10^8$/mg of protein.

TABLE III: Nitration of Iron-Free Transferrin with Tetranitromethane (TNM).

TNM Concn (M $\times 10^3$)	Reaction Time (min)	Moles of Tyrosine Residues Modified/Mole of Protein		Fe Binding Capacity		$[\alpha]_D$ (degrees)
		Spectrophotometric Anal.	Amino Acid Anal.	Moles of Fe $\times 10^8$/mg of Protein	% of Control	
Native				2.30	100	−50
1.25	30	4.8	4.8	1.85	83	−50
2.50	30	6.8	7.3	1.52	68	−48
4.15	30	8.1	7.9	1.34	58	−46
10	15	11.2	10.7	1.26	55	−54
10	30	13.9		0.97	42	−54
10	150	17.8	16.1	0.60	26	−48
10	210	20.4		0.32	14	−50
10	1140	23.6		0.12	5	−54
30[a]	30	21.0	20.0			

[a] In the presence of 28% ethanol.

appropriate set of conditions. The ethanol concentration did not exceed 4 and 20% v/v for iron-free transferrin and iron-saturated transferrin, respectively. TNM concentration did not exceed 1×10^{-2} M in either case. Table III lists the conditions used for nitration of each iron-free transferrin sample. The spectrophotometric estimate of the number of tyrosine side chains modified was in most instances checked by amino acid analysis (Table III), showing good agreement between the two methods. The nitrated protein samples were then subjected to iron binding activity determinations, the results being represented in curve 1 of Figure 7. It is evident that progressive nitration of iron-free transferrin was accompanied by corresponding loss of iron binding capacity. A plateau region was encountered between some 8 and 12 residues of tyrosine nitrated, after which the loss of iron binding capacity was again linear with the degree of modification. It may be noted that only one-half of the iron binding capacity of the native iron-free transferrin was present if 8–12 tyrosine side chains of iron-free transferrin were nitrated. Nitration of 23.6 tyrosine residues (maximum achieved) almost completely abolished the iron binding properties of iron-free transferrin.

Additional information on the number and nature of tyrosine side chains involved in the binding of iron was obtained by nitrating iron-saturated transferrin. The conditions used and results obtained are given in Table IV. The protein had apparently experienced no appreciable loss of iron, and the maximum number of tyrosine side chains thus nitrated was 17.2 (Table IV and curve 2 of Figure 7). It may also be noted that whereas the $[\alpha]_D$ of nitrated iron-free transferrin (Table III) was almost identical with that of the native

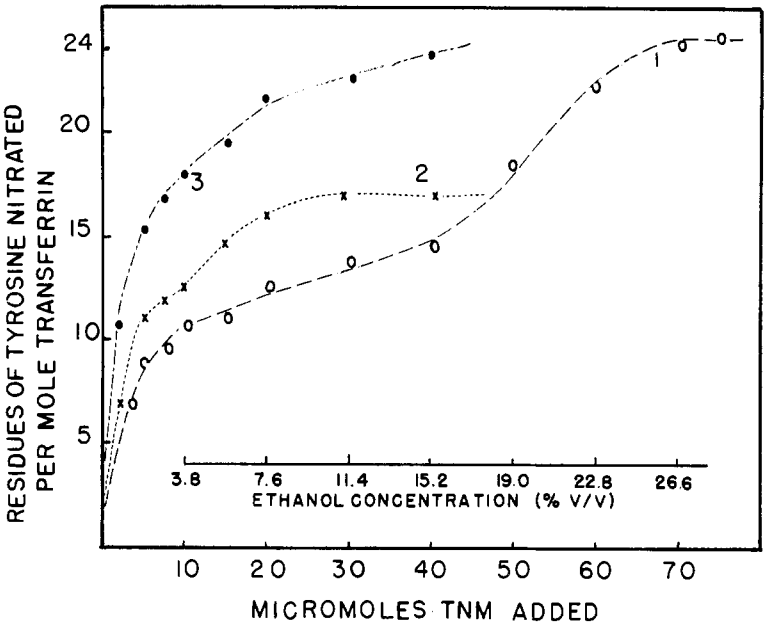

FIGURE 4: Nitration of iron-free transferrin with TNM, 30-min reaction time. curve 1: Varying ethanol and TNM concentrations; curve 2: varying TNM concentration, ethanol at 20% v/v; curve 3: varying TNM concentration, ethanol at 30% v/v.

iron-free transferrin, the $[\alpha]_D$ of nitrated iron-saturated transferrin was much lower than that of native iron-saturated transferrin. When iron was removed from the nitrated iron-saturated transferrin, the $[\alpha]_D$ returned to $-50°$. After the removal of iron from nitrated iron-saturated transferrin, an attempt was made to reconstitute the iron with the nitrated transferrin sample (17.2 tyrosine side chains modified). The results presented in Table IV and curve 2 of Figure 7 indicate that this was successfully accomplished, suggesting that the presence of iron had protected the "active" tyrosine residues against nitration. This may be contrasted with a sample of iron-free transferrin with 17.2 tyrosyl side chains modified in a random fashion and possessing only 26% of the original iron binding activity (Figure 7, curve 1). Finally, when iron was removed from the nitrated iron-saturated transferrin and the iron-free protein was subjected to further nitration, some six additional nitro groups were introduced into each protein molecule for a total of 23.6 nitrated

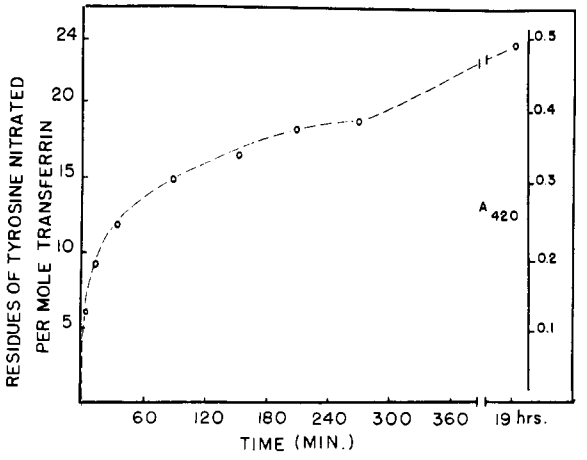

FIGURE 5: Nitration of iron-free transferrin with 1×10^{-2} M TNM at 4% v/v alcohol concentration as a function of time.

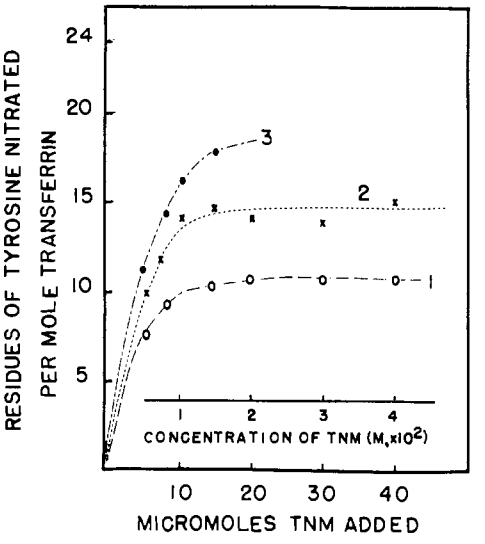

FIGURE 6: Nitration of iron-saturated transferrin in the presence of 30% v/v ethanol. curve 1: 30-min reaction time; curve 2: 90-min reaction time; curve 3: 19-hr reaction time.

TABLE IV: Nitration of Iron-Saturated Transferrin with Tetranitromethane (TNM) in 20% Ethanol.

TNM Concn ($M \times 10^2$)	Reaction Time (hr)	Moles of Tyrosine Residues Modified/Mole of Protein[a]		Fe Binding Capacity[b]		α_D (degrees)	
		Without Addnl Nitration	With Addnl Nitration (19 hr) after Fe Removal	Remaining after Nitration	Following Removal of Fe and Reconstitution	Nitrated Fe-Satd Transferrin	After Removal of Fe
Native		0	0	2.30	2.30	−56	−50
1	0.5	8.9		2.24	2.33		−46
1	2.5	12.6		2.04	2.13	−67	−51
1	19	17.2	23.6	1.78	1.82	−69	−52

[a] Spectrophotometric, following the removal of iron. [b] Moles of iron $\times 10^8$/mg of protein.

tyrosine side chains (Table IV). This figure is in good agreement with the maximum number of nitrable tyrosyl residues present in iron-free transferrin (Table III).

Discussion

Alkylation of iron-free transferrin with bromoacetate appeared to be specific for the histidine residues, without any apparent involvement of the lysine or methionine side chains. However, it is clear that the alkylation of all histidyl residues present in iron-free transferrin would require an unreasonable amount of time (see Figure 2A). No change in iron binding capacity of iron-free transferrin occurred until some nine to ten histidyl residues had been modified; however, alkylation of additional histidyl side chains was accompanied by a precipitous drop in the iron binding capacity of iron-free transferrin. A double extrapolation of the iron binding capacity *vs.* number of histidine residues modified (Figure 2B) showed that the iron binding capacity of iron-free transferrin was lost between 10 and 14 histidyl residues alkylated, suggesting the participation of two histidyl groups in the binding of each iron atom. Furthermore, it is quite apparent that these "active site" histidyl residues possess the same degree of reactivity toward bromoacetic acid, and that they are clearly differentiated from those not involved in the iron binding reaction.

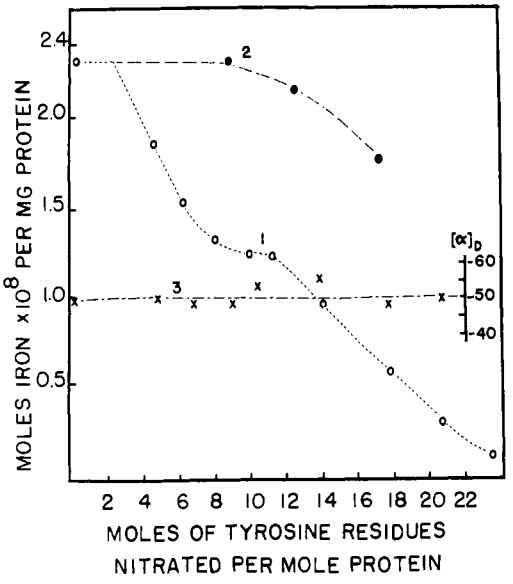

FIGURE 7: Iron binding activity and optical rotation of iron-free transferrin and iron-saturated transferrin as a function of the number of tyrosyl side chains modified (determined spectrally). curve 1: Native transferrin; curve 2: iron-saturated transferrin (20% ethanol v/v); curve 3: optical rotation at 589 mμ of native transferrin as a function of the degree of nitration.

Nitration of iron-free transferrin has demonstrated that its tyrosine residues can be divided into two "classes" on the basis of their reactivity toward TNM (Figure 4): the rapidly reacting groups (10–14), and the less rapidly reacting residues. The iron binding capacity of iron-free transferrin appeared to decrease in proportion to the number of tyrosyl residues nitrated, reaching a plateau region at one-half the iron binding capacity of the native iron-free transferrin upon the nitration of 8–21 tyrosine residues (Figure 7). Further nitration of iron-free transferrin was again accompanied by a uniform drop in its iron binding ability until the latter became nil at 24 tyrosine residues nitrated. The loss of iron binding activity of iron-free transferrin under the conditions of nitration was probably not due to a non-specific denaturation process (see stability studies, Table II), and was more likely due to the specific inactivation of tyrosine residues required for the binding of iron. It would thus appear that one group of the tyrosine residues involved in the binding of iron is associated with the rapidly reacting tyrosine residue class, whereas the other active tyrosine group is associated with the less rapidly reacting class. Moreover,

within a given reactivity class, the active tyrosine residues do not appear to be distinguishable from those of the tyrosine groups not involved in the binding of iron. The behavior of iron-free transferrin, during the nitration reaction may be contrasted to the carboxymethylation reaction of iron-free transferrin (Figure 2B), where the reactivity of the active histidyl groups was apparently different from that of the "nonactive" residues, and where all four active histidyl side chains had an apparently identical reactivity toward bromoacetic acid.

Experiments involving nitration of iron-saturated transferrin, the removal of iron by dialysis against EDTA, and subsequent reconstitution of the nitrated iron-saturated transferrin (Table IV) can be used as an additional argument in favor of the existence in iron-free transferrin of a definite iron binding site involving tyrosine side chains, and that iron protects these side chains against nitration. The number of tyrosine residues involved in the binding of each iron atom may be evaluated from two sets of data presented in this paper. (1) The removal of iron from nitrated iron-saturated transferrin (maximum number of nitrogroups introduced was 17.2/iron-free transferrin molecule as per Table IV and Figure 6) and subsequent nitration resulted in the introduction of six additional nitro groups into the iron-free transferrin molecule (Table IV). (2) The difference between the nitro group content of iron-free transferrin (23.6) and that of iron-saturated transferrin (17.2) was 6 (Figure 7). It would thus appear that each iron-binding site of iron-free transferrin contains three tyrosine residues.

The loss of iron binding activity by the nitrated iron-free transferrin was most likely due, as expected, to the electron-withdrawing properties of the nitro groups introduced into position 3 of the phenolic ring structures. Conversely, the protection against nitration of the active tyrosines by the iron atoms was probably due to the unavailability of electrons, normally present in the phenolic ring, to the attack by the nitronium ion (Sokolovsky *et al.*, 1966).

The titration curve shown in Figure 4 indicated that the nitration of the less reactive tyrosyl residues could be expedited by higher alcohol concentrations. In fact, when 30% v/v alcohol was used for all TNM values (Figure 4), the reactivity of all tyrosyl side chains toward TNM had apparently become the same. The effect of the alcohol was probably not one of unmasking "buried" groups, since Figure 5 shows that some 24 of the 25 tyrosyl groups present can be nitrated under mild conditions given enough time. However, one may note that we were never able to nitrate all 25 residues under mild conditions, whereas in 30% v/v ethanol (Figure 4) this was possible. Thus only one of the 25 tyrosyl groups present in iron-free transferrin may possibly be of the "buried" type.

The nitration experiments described in this paper may be of value in the further investigation of the active site structure present in the iron-free transferrin molecule. One could conceivably nitrate all the tyrosyl residues other than those involved in the iron binding site of iron-saturated transferrin, and subject the nitrated iron-saturated transferrin to proteolytic or mild acid degradation. Peptides containing nonaltered tyrosine would then, of necessity, originate from the active site portion of the iron-free transferrin molecule.

It has been noted in our previous reports (Bezkorovainy, 1966) that there is a definite discrepancy between the molecular weight of iron-free transferrin as measured in our laboratory and that obtained by Charlwood (1963) and Roberts *et al.* (1966). Iron binding studies reported in this paper (*e.g.*, Table III) indicate that 2.3×10^{-5} mole of iron is bound/mg of transferrin. If 2 moles of iron are assumed to be bound to 1 mole of transferrin, this figure then gives a molecular weight of 87,000, consistent with our previous data (Bezkorovainy, 1966), those of Koechlin (1952), and those of Aisen *et al.* (1966).

In conclusion, this paper shows that there are two classes of active tyrosine residues in iron-free transferrin, which can be distinguished from each other by their reactivity with TNM, and that three tyrosyl and probably two histidyl residues are involved in the binding of each iron atom in the iron-free transferrin molecule. It thus provides partial confirmation, based on purely chemical methods, and using reagents previously untried for iron-free transferrin, of a model, which, on the basis of electron paramagnetic resonance studies, proposed the involvement of three tyrosyl, two histidyl, and one bicarbonate residues in the binding of each iron atom by the iron-free transferrin molecule (Windle *et al.*, 1963).

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Demonstration of Further Differences between *in Vitro* and *in Vivo* Synthesized MS2 Coat Protein*

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ABSTRACT: When MS2 ribonucleic acid (RNA) is used as messenger in the *Escherichia coli* cell-free system, most of the proteinaceous material synthesized is less acidic than the virus coat protein. When the small tryptic peptides of MS2 coat protein obtained from such material are analyzed for their content of *in vitro* produced components, the C-terminal peptide is found present in much lesser amounts than the other peptides.

The ability of viral RNA to stimulate [^{14}C]amino acid incorporation in extracts of *Escherichia coli* has been well documented (Nirenberg and Matthaei, 1961; Tsugita *et al.*, 1962; Nathans *et al.*, 1962). However, the definitive identification of the resulting identification of the resulting proteinaceous products has been more problematical. When TMV–RNA was used, little if any of the product resembled native or denatured TMV coat protein (Aach *et al.*, 1964; Tsung and Fraenkel-Conrat, 1965a). Using the RNA of several closely related coliphages, much more of the ^{14}C-labeled product remained associated with the added coat protein during various fractionation procedures (Nathans *et al.*, 1962; Tsung and Fraenkel-Conrat, 1965a; Nathans, 1965; Yamazaki and Kaesberg, 1966; Ohtaka and Spiegelman, 1963; Viñuela *et al.*, 1967). However, the realization that all products are believed to carry N-terminal formylmethionyl groups (Adams and Capecchi, 1966; Webster *et al.*, 1966) would lead one to predict that they should differ from the corresponding carrier proteins lacking this group, for instance by showing a slightly lower anionic mobility upon electrophoresis. Actually the present study has shown that most of the synthesized ^{14}C protein, when subjected to polyacrylamide gel electrophoresis in 8 M urea at pH 3.8, moves further toward the anode than the carrier protein.

An alternate method of identifying the products of cell-free protein synthesis resides in comparing the peptides resulting from their trypsin degradation with digests of the viral coat protein (Nathans *et al.*, 1962; Nathans, 1965). The present study has employed column chromatography rather than two-dimensional paper mapping for this purpose. Clear evidence was obtained that most of the incorporated label which remained associated with coat protein upon DEAE Sephadex chromatography, *i.e.*, 20% of the total, remained associated with those characteristic peptides of the MS2 digests which can readily be isolated and further purified. However, the bigger peptides do not lend themselves to easy chromatographic purification. Thus neither the N-terminal peptide, containing 38 residues, nor its formylmethionyl derivative have, as yet, been identified among the products. The C-terminal hexadecapeptide has been detected, but only about 40% as much was found to be synthesized of this peptide than of the small peptides.

On the basis of these two sets of data it is suggested that much or most of the coat protein formed under

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