Chemical Modifications of Amino Groups of Transferrins: Ovotransferrin, Human Serum Transferrin, and Human Lactotransferrin*

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ABSTRACT: Transferrins and their iron complexes were chemically modified with reagents which primarily react with amino groups, and the effects of these modifications on the properties of the transferrins were studied. The metal-free and the iron complexes of chicken ovotransferrin (conalbumin), human serum transferrin, andhum an lactotransferrin were treated with acetic anhydride, potassium cyanate, or succinic anhydride. All three modifications produced more acidic products as measured electrophoretically but did not cause changes in size as measured ultracentrifugally. Modification of over 50% of the amino groups in the iron complexes did not cause any decreases in iron-binding properties. Modification of less than 50% of the amino groups by acetylation or reaction with cyanate caused only slight effects on the iron-binding properties of the metal-free transferrins. More extensive modifications by acetylation or with cyanate or modification by succinylation did not decrease significantly the stoichiometry of iron binding but changed the properties of the complexes. The stabilities of the complexes to reversible dissociation in urea decreased with increasing modification of the amino groups. Upon reaction of metal-free transferrins with succinic anhydride and subsequent formation of the iron complex, the absorption maximum was shifted toward shorter wavelengths (465–430 mμ).

The transferrins are naturally occurring chelating agents. Serum transferrin, lactotransferrin, and ovotransferrin (conalbumin) are found in blood serum, milk, and egg white, respectively. The transferrins combine specifically with two metal ions per protein molecule (Surgenor *et al.*, 1949; Fraenkel-Conrat and Feeney, 1950; Warner and Weber, 1953). These metal-protein complexes have physical and chemical properties different from those of the apoproteins. The iron transferrins, the most highly associated complexes, are red in color with a maximum absorption at 465 mμ. The metal-protein complexes are more stable to physical and chemical conditions which destroy the metal-binding ability of the metal-free proteins (Azari and Feeney, 1958, 1961). Because of the lability of the metal-free transferrins, definitive chemical modification of the metal-free transferrins with retention of their metal-binding properties apparently has not been accomplished previously (Fraenkel-Conrat and Feeney, 1950; Azari and Feeney, 1961).

The nature of the linkage between the transferrins and the metal ions is not understood. Specific amino acid side chains in the protein have been postulated to act as ligands but no direct evidence is available for the participation of the side chain of any amino acid as ligand in the metal-protein complexes. Warner and Weber (1953) and Wishnia *et al.* (1961) have presented titrimetric and spectrophotometric evidence for the participation of two and three tyrosine hydroxyls in the chelation of copper and iron, respectively. The participation of a carbonate or bicarbonate in the chelation of iron was also shown (Fiala and Burk, 1949; Warner and Weber, 1953). More recently, by means of electron spin resonance measurements, evidence for the presence of at least two nitrogen nuclei in the copper complexes of serum transferrins, lactotransferrins, and ovotransferrin has been obtained (Aasa *et al.*, 1963; Windle *et al.*, 1963). Windle *et al.* (1963) suggested that the two nitrogens, three tyrosines, and a bicarbonate ion were involved in iron binding, and that the two nitrogens were probably in either imidazoles or amino groups. In the present studies the transferrins have been modified with reagents reacting with amino groups in an attempt to show whether or not amino groups are involved in the binding of iron.

Materials and Methods

Materials. Chicken ovotransferrin prepared according to Warner (1954) was purified further by DEAE-cellulose chromatography (Clark *et al.*, 1963).

Human serum transferrin was isolated from Cohn

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TABLE I: Acetylation of Ovotransferrin.

	Conditions[a]			Found			
Expt[b]	Protein	Reaction Time (min)	Moles Reagent per Mole Amino	E^c (280/470 mμ)	Amino Groups per Mole	Amount Acetylation (%)	*O*-Acyl Groups per Mole
A[d]	(1) Iron ovotransferrin	0	0	23	62	0	0
	(2) Iron ovotransferrin	5	0.7	24	52	17	0
	(3) Iron ovotransferrin	10	0.7	23	48	21	0.3
	(4) Iron ovotransferrin	20	1.4	24	43	30	0.7
	(5) Ovotransferrin	5	0.7	25	51	17	0
	(6) Ovotransferrin	10	0.7	25	50	19	0
	(7) Ovotransferrin	20	1.4	27	44	28	0
B	(1) Iron ovotransferrin	60	15	24	25	58	6.0
	(2) Ovotransferrin	60	15	55	27	56	7.0

[a] General conditions for reaction are described in text. Reagent was acetic anhydride. [b] Experiments A and B were conducted at *p*H 8.2 and 6.8, respectively. [c] The optical densities of all samples were determined by adding iron after treatment as described in text. [d] Samples of expt A were same as used in Figure 1.

fraction IV-7 which was obtained from Cutter Laboratories, Berkeley, Calif. The transferrin was separated from Cohn fraction IV-7 and purified by chromatography on DEAE-Sephadex A-50 and 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). The metal-free transferrins were prepared from the metal complexes according to the method of Warner and Weber (1951).

Human lactotransferrin was prepared from frozen pooled samples of human milk from a single donor. The method was an adaptation of that described by Montreuil *et al.* (1960), as modified by Windle *et al.* (1963). The procedure involved precipitation of the crude lactotransferrin with ammonium sulfate and repeated chromatography on DEAE-cellulose.

Chemical Modifications. ACETYLATION. Conditions for acetylation were similar to those initially described by Fraenkel-Conrat (1957) and employed by Stevens and Feeney (1963) on ovomucoid. Acetylation of the metal-free and the iron transferrins was carried out with different concentrations of acetic anhydride for different periods of time. Transferrin (90 mg) was dissolved in 2.0 ml of 50% saturated sodium acetate at 0° and *p*H 6.8 or 8.2. From 5 to 180 μl of acetic anhydride was added in increments to the protein solutions over a period of 100 minutes. The samples were dialyzed against several changes of water at 4° for 3 days and lyophilized.

CARBAMYLATION. Proteins were reacted with cyanate according to the procedure of Stark *et al.* (1960), as modified by Stevens and Feeney (1963). Protein (90 mg) was dissolved in 10 ml of 0.05 M Tris-HCl buffer, *p*H 8.1. The temperature of the solution was raised to 40°, and 0.5 ml of freshly prepared 1.0 M KCNO solution was added.

SUCCINYLATION. Reaction of the proteins with succinic anhydride was carried out essentially as described by Habeeb *et al.* (1958). Protein (30 mg) was dissolved in 2.0 ml of 1.0 M bicarbonate buffer at *p*H 8.0. With constant stirring, 1.18, 2.36, or 4.72 mg of solid succinic anhydride was added to different protein solutions. The reaction mixture was readjusted to *p*H 8.0 with 0.1 M NaOH as necessary during 30 minutes at room temperature (25°).

Analysis of Modified Transferrins. Following the chemical modification, the proteins were dialyzed extensively, lyophilized, and redissolved in 0.1 M bicarbonate buffer, *p*H 8.0. Sufficient 0.002 M ferric iron to form the complex was added and the solution was centrifuged to remove small amounts of insoluble material.

Optical Density Ratio at 280 and 465 mμ and Spectra. The optical density of a solution containing about 10 mg of the iron complex of the protein per ml was determined at 465 mμ. A 1:10 or 1:20 dilution of this solution was prepared and its optical density was determined at 280 mμ. Spectra of the modified proteins were obtained with a Beckman Model DB recording spectrophotometer.

Determination of Free Amino Groups. Free amino groups were determined by the ninhydrin method (Fraenkel-Conrat, 1957) as standardized against values obtained by the Van Slyke gasometric method (Stevens and Feeney, 1963). Values of 76,000, 87,000, and 90,000 g were employed for the molecular weights of ovotransferrin, serum transferrin, and lactotransferrin, respectively. The difference between the free amino groups present in the original and the modified proteins was employed to calculate the per cent modification.

Determination of O-Acyl Groups. The number of *O*-acyl groups introduced into the protein was determined by the hydroxylamine method as modified for proteins (Uraki *et al.*, 1957).

Free-Boundary Electrophoresis and Ultracentrifuga-

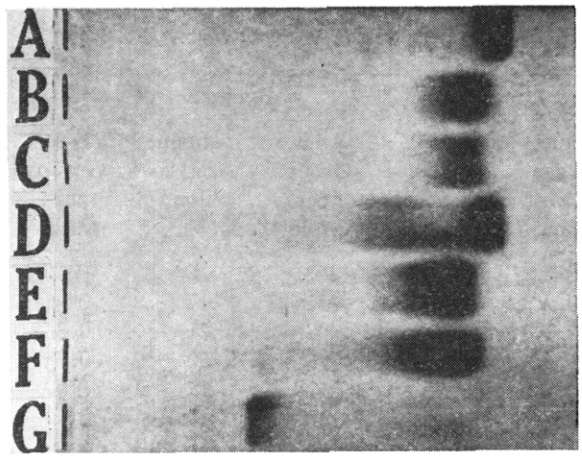

FIGURE 1: Starch-gel electrophoretic patterns of acetylated ovotransferrins. (A, B, and C) Iron ovotransferrin acetylated for 20, 10, and 5 minutes, respectively; (D, E, and F) ovotransferrin acetylated for 20, 10, and 5 minutes, respectively; (G) control ovotransferrin. Samples were the same as employed in expt A, Table I; acetylation with acetic anhydride as described in text.

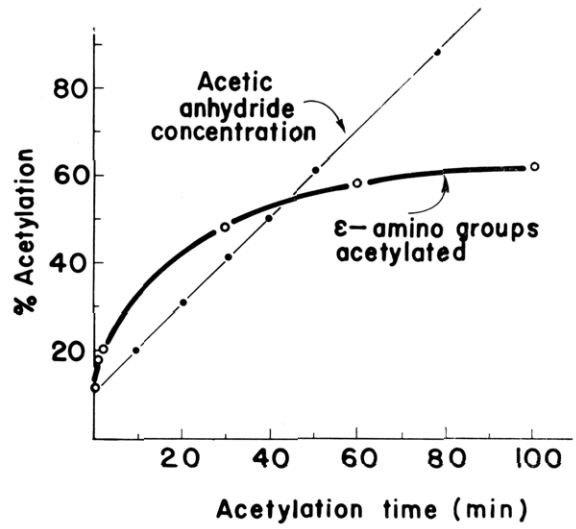

FIGURE 2: Relationship between amount of treatment with acetic anhydride and the percentage of modification. The protein was ovotransferrin. Acetic anhydride was added at the constant rate of 2.5 moles per mole of amino group at 10-minute intervals. General conditions for acetylation described in text.

tion. Electrophoresis was done in a Perkin-Elmer Model 38A Tiselius electrophoresis apparatus at 1°. The protein concentration was 1% in a $\Gamma/2 = 0.1$ barbiturate buffer, *p*H 8.6. Ultracentrifugal analyses were performed in a Beckman Model E analytical ultracentrifuge.

Starch-Gel Electrophoresis. Starch-gel electrophoresis was used to evaluate purity of proteins during the isolation of transferrins and to follow the degree of chemical modification of the proteins. Starch gels for the electrophoresis of ovotransferrins were prepared according to Saifer *et al.* (1961). For serum transferrin the horizontal starch gel described by Smithies (1955) was used. The general conditions and buffers were as recently employed (Clark *et al.*, 1963).

Spectral Titration. Titrations were conducted in a solution of 20 mg of protein dissolved in 2.0 ml of 0.15 M $NaHCO_3$ and 0.375 M NaCl. Micro additions of 1.0 M NaOH or 1.0 M HCl were made. The *p*H and corresponding changes of optical density were recorded for each addition.

Stability of Iron Transferrins in Urea. A 50-mg sample of transferrin was dissolved in 0.55 ml of 0.1 M $NaHCO_3$, 0.02 M sodium citrate, 0.02 M $FeNH_4(SO_4)_2$ at *p*H 8.4. A 0.1-ml portion of the above-mentioned solution was added to 1.9 ml of water or to 1.9 ml of 2–8 M urea solution, *p*H 8.4. The optical densities of these solutions were measured at zero time and again after 60 minutes.

Results

Acetylated Derivatives

Acetylated Ovotransferrins. Acetylation of the transferrins with acetic anhydride formed primarily derivatives of amino groups. At only slightly greater than equimolar ratios of acetic anhydride to amino groups, the degree of acetylation was as high as 30% (Table I). Under these conditions only fractional numbers of *O*-acyls per mole of protein were formed. No change occurred in the optical density ratio *E* 280/465 mμ with the iron complex and only a slight change occurred with the uncomplexed protein, but large increases in electrophoretic mobilities were observed. At higher levels of acetylation, the metal-free protein separated into two main components during starch-gel electrophoresis. These effects are seen in Figure 1, which shows the starch-gel patterns of the samples from experiment A, Table I.

With more extensive acetylation, the chromogenic capacity of the metal-free ovotransferrin was partially destroyed (nearly 50%) but no detectable losses were found with iron ovotransferrin, although 6–7 *O*-acyl groups were formed (expt B, Table I). Some of these *O*-acyl groups may have involved tyrosine hydroxyls, but ultraviolet absorption spectra did not indicate any significant changes in the tyrosines. Starch-gel patterns of these samples showed a preponderance of slower-moving component in the acetylated iron complex. Free-boundary electrophoresis of the acetylated iron complex also showed only a primary fast-moving component, and ultracentrifugal analyses by sedimentation velocity showed only small amounts of more rapidly sedimenting material. The $s_{20,w}$ values were approximately 5.0 S, which was similar to the value for untreated protein. With extensive acetylation of the iron complex, however, partial losses of chromogenic capacity and other changes also were observed. There was no general correlation between the appearance of minor components in starch-gel electrophoresis and loss of metal binding. The slower-moving components were primarily observed with low and high treatments, and were

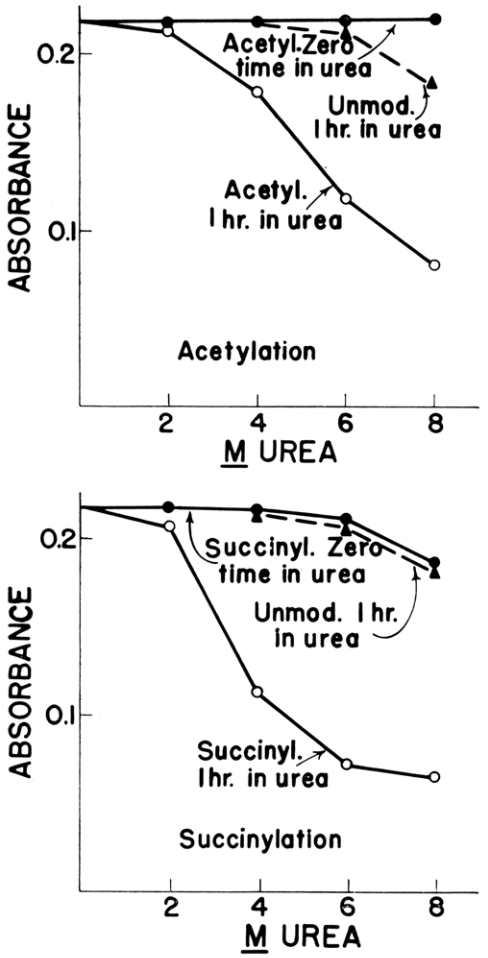

FIGURE 3: Effects of acetylation or succinylation of iron ovotransferrin on stabilities of complexes in urea. Acetylated was 40% modified; succinylated was 60–70% modified. General conditions are described in text. Reactions were performed with 0.5% protein in 0.005 M $NaHCO_3$, 0.001 M sodium citrate, 0.001 M $FeNH_4(SO_4)_2$ at *p*H 8.4, and 0–8 M urea. Optical densities were determined at 470 mμ: ▲- -▲, unmodified (control) iron ovotransferrin in 0–8 M urea for 1 hour; ●—●, acetylated or succinylated ovotransferrin in 0–8 M urea immediately after mixing (zero time); O—O, acetylated or succinylated ovotransferrin in 0–8 M urea for 1 hour.

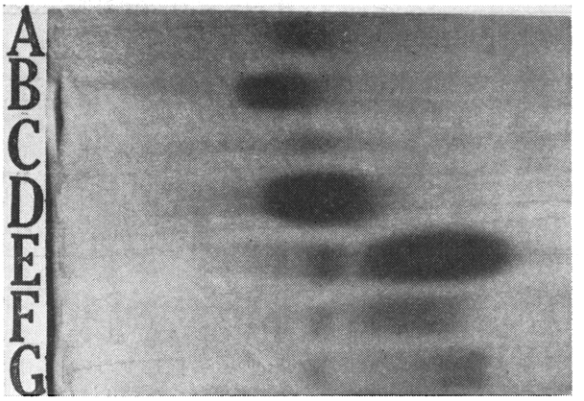

FIGURE 4: Starch-gel electrophoretic patterns of acetylated and carbamylated human serum transferrin. (A) Fraction I of preparation of human serum transferrin; (B) fraction II of preparation of human serum transferrin; (C) fraction I diluted in two volumes of 50% saturated sodium acetate; (D) fraction II treated with KCNO for 30 minutes; (E) fraction II treated with KCNO for 360 minutes; (F) fraction I treated with 1.0 mole of acetic anhydride per mole of amino groups (3-minute treatment); (G) fraction I treated with 2.0 moles of acetic anhydride per mole of amino groups (20-minute treatment). General conditions described in text. B, D, and E samples were samples described as 1, 2, and 3, respectively, of Table IV.

interpreted as representing less-substituted varieties with low treatments and aggregated varieties with high treatments. Intermediate treatments with acetic anhydride or even higher treatments with KCNO (*vide infra*) caused essentially fast-moving components of ovotransferrin.

Acetylation of ovotransferrin was initially rapid but the rate became slow before approximately 50% of the amino groups had been modified (Figure 2). The upper limits of acetylation appeared to be caused, at least in part, by decreased solubilities of the products. In addition to the greater negative charge of the highly (>60%) acetylated iron complexes of ovotransferrin which maintained their chromogenic capacities, other properties were also different from those of the original protein. A major difference was a decreased stability of the complexes in solutions of urea (Figure 3). Although no large difference was found in the absorption spectra, there was a small decrease (approximately 5%) in the ratio of the optical densities at 465 and 400 mμ (see under Succinylated Derivatives).

Acetylated Human Transferrins. Acetylation of human serum transferrin and its iron complex gave results similar to those obtained with ovotransferrin and its iron complex. As compared to acetylation of ovotransferrins, however, greater losses of the chromogenic capacity were consistently observed (Table II and Figure 4). Acetylation of human lactotransferrin and its iron complex gave results similar to those found with human serum transferrin.

Carbamylation Experiments

Carbamylated Ovotransferrin. Treatment of ovotransferrin or its iron complex with KCNO caused changes similar to those caused by acetylation, but less extensive side reactions were apparent (Table III, Figure 5). Low levels of substitution of amino groups (<30%) gave partially and variously modified products as evidenced by the presence of both slow- and fast-moving components on electrophoresis. More extensive modification gave only fast-moving components without the appear-

TABLE II: Acetylation of Human Serum Transferrin.

Protein	Conditions[a]		Found		
	Acetylation Time (min)	Moles Reagent per Mole Amino	E (280/470 mμ)	Amino Groups per Mole	Amount Acetylation (%)
(1) Iron human serum transferrin	0	0	22	52	0
(2) Iron human serum transferrin	3	1.0	26	44	14.5
(3) Iron human serum transferrin	20	2.0	27	30	42

[a] Conditions for reaction are described in text. The reagent was acetic anhydride and the experiment was performed at *p*H 8.0. Samples 1, 2, and 3 are same as B, F, and G, respectively, in Figure 4.

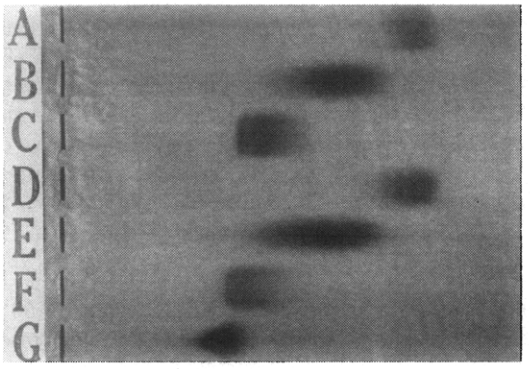

FIGURE 5: Starch-gel electrophoretic patterns of carbamylated ovotransferrin. (A) Iron ovotransferrin in KCNO for 480 minutes; (B) iron ovotransferrin in KCNO for 120 minutes; (C) iron ovotransferrin in KCNO for 30 minutes; (D) ovotransferrin in KCNO for 480 minutes; (E) ovotransferrin in KCNO for 120 minutes; (F) ovotransferrin in KCNO for 30 minutes; (G) ovotransferrin control. Conditions described in text. Samples for patterns A, B, and C are same as for 3, 2, and 1, respectively, of Table III. Samples for D, E, F, and G are 7, 6, 5, and 4, respectively.

ance of the slow and smearing components as obtained with more extensive modification by acetylation.

Carbamylated Human Serum Transferrin. The reaction of human serum transferrin with KCNO caused changes similar to those caused by treatment of ovotransferrin (Table IV and Figure 4). As was also the case with acetylation, however, serum transferrin was less stable than ovotransferrin, as evidenced by some insolubilization and losses in chromogenic capacity.

Succinylated Derivatives

Succinylated Ovotransferrin. Succinylation of ovotransferrin produced more extensive changes than those obtained either by acetylation or carbamylation. The succinylated products were more acidic and showed a higher degree of heterogeneity on starch-gel electrophoretic analysis. Levels of succinic anhydride equivalent to 2 and 3 moles of reagent per residue of amino groups gave values for modifications of amino groups of 42 and 45%. No absolute loss of chromogenic capacity appeared to occur with these relatively high substitutions of the iron complex. However, there was a change in the stability of its iron complex to dissociation in urea. The stability was decreased approximately twice as much as that of the acetylated derivative (Figure 3).

Succinylation of metal-free ovotransferrin caused significant changes in the properties of its iron complex. A distinctive change was in the absorption spectra (Figure 6). There was a shift of the maximum from approximately 465 mμ to shorter wavelengths and an increase in absorption at approximately 400 mμ. In the case of the more extensively modified material (42% of amino groups), the original pink color changed to a golden yellow. The golden-yellow color of the iron complex disappeared upon mixing in 8 M urea.

Succinylated Human Serum Transferrin. Succinylation of human serum transferrin caused changes similar to those produced by acetylation, plus the more extensive effects found with succinylation of the metal-free or the iron complexes of ovotransferrin. The products were more acidic and heterogeneous, and their iron complexes were yellow. An amount of succinic anhydride equivalent to 3 moles of reagent per residue of amino group gave a value of 43% for modification of amino groups.

Succinylated Human Lactotransferrin. Succinylation of the iron complex of human lactotransferrin gave effects similar to those obtained with iron ovotransferrin and iron human serum transferrin, with the exception that higher levels of substitution were observed. An amount of reagent equivalent to 2 moles per amino group caused

TABLE III: Carbamylation of Ovotransferrin.

Protein[b]	Conditions[a]		Found		
	Reaction Time (min)	Moles Reagent per Mole Amino	E^c (280/470 mμ)	Amino Groups per Mole	Amount Carbamylation (%)
(1) Iron ovotransferrin	30	9	23	58	5
(2) Iron ovotransferrin	120	9	25	56	8.3
(3) Iron ovotransferrin	480	9	26	44	28
(4) Ovotransferrin	0	0	24	61	0
(5) Ovotransferrin	30	9	24	59	3.3
(6) Ovotransferrin	120	9	26	57	6.7
(7) Ovotransferrin	480	9	26	45	26

[a] General conditions for reaction described in text. The reagent was KCNO. Experiment was performed at 40° and *p*H 8.1. [b] Samples 1, 2, and 3 are the same as C, B, and A, respectively, and 4, 5, 6, and 7 are the same as G, F, E, and D, respectively, in Figure 5. [c] The optical densities of all samples were determined by adding iron after treatment as described in text.

TABLE IV: Carbamylation of Human Serum Transferrin.

Protein[b]	Conditions[a]		Found		
	Reaction Time (min)	Moles per Mole Amino	E (280/470 mμ)	Amino Groups per Mole	Amount Carbamylation (%)
(1) Iron human serum transferrin	0	0	23	54	0
(2) Iron human serum transferrin	30	11	25	48	11.0
(3) Iron human serum transferrin	360	11	28	32	40.0

[a] General conditions for reaction described in text. Experiment was performed at 40° and *p*H 8.1. [b] Samples 1, 2, and 3 are same as B, D, and E, respectively, in Figure 4.

60–70% modification. Succinylation of the metal-free lactotransferrin was not attempted because of its greater lability.

Discussion

In earlier studies, Fraenkel-Conrat and Feeney (1950) reported that the metal-binding capacity of ovotransferrin was easily destroyed by treatment with chemical reagents. In later studies, Azari and Feeney (1958, 1961) showed that the iron complexes of transferrins were relatively stable to chemical treatments, proteolysis, and denaturation, while the metal-free transferrins were relatively labile. The latter authors believed that the losses in metal-binding capacity of the metal-free transferrins caused by many chemical treatments were indirect results of the labilities to physical treatments, i.e., to denaturation. Successful modification of amino groups of iron-transferrin without loss of iron-binding was recently indicated, however, by the treatment of ovotransferrin with glucose, xylose, or arabinose in alkaline solution (Feeney *et al.*, 1964).

In the present studies it was found that the more readily available amino groups in metal-free and in iron transferrin could be reacted with acetic anhydride, succinic anhydride, and cyanate. Substitution of 20–30% of the amino groups by treatment with acetic anhydride or KCNO did not significantly alter the iron-binding characteristics of these proteins. With further modification of amino groups a gradual loss of the iron-binding capacity of the transferrins occurred. Loss of the metal-binding properties was first noted in the metal-free and

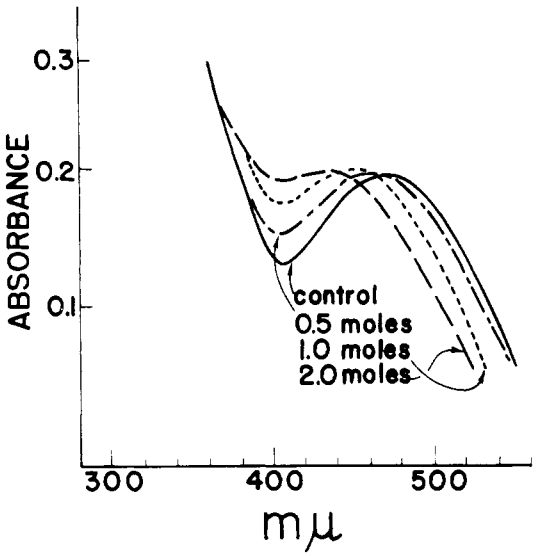

FIGURE 6: Spectral shifts of iron complexes caused by succinylating ovotransferrin to varying degrees. The protein was succinylated in the iron-free state. Following dialysis and lyophilization, the modified transferrins were redissolved and saturated with iron, and the visible spectrum was scanned with a Beckman DB recording spectrophotometer. ———, original ovotransferrin (λ_{max} 465 mμ); -·-·-·-, succinylated ovotransferrin (λ_{max} 460 mμ) (succinylation: 0.5 mole succinic anhydride per mole amino group); ·····, succinylated ovotransferrin (λ_{max} 445 mμ) (succinylation: 1.0 mole succinic anhydride per mole amino group); ---------, succinylated ovotransferrin (λ_{max} 430 mμ) (succinylation: 2.0 moles succinic anhydride per mole amino group). Amount of succinylation for 2.0 moles of reagent per mole of amino group was 45%.

then in the iron-protein complex, in agreement with earlier work showing that the iron-transferrin complex has a greater stability to physical and chemical treatment (Azari and Feeney, 1958, 1961; Glazer and McKenzie, 1963). In the chemical modifications of this study, reagents reacted readily with the more available amino groups of the uncomplexed transferrins without specific destruction of the metal-binding capacity. Unless important amino groups became exposed by chelation or were some other way available for chelation but less available to the modifying reagents, the results indicated that the reagents did not react specifically with ligands at a metal-binding site. The stabilities of the complexes of the iron transferrins to dissociation in urea decreased, however, at the higher levels of modification, and even at lower levels with succinic anhydride. Such changes may have been caused by more extensive reaction with groups other than amino, such as hydroxy groups, and to nonspecific denaturation owing to accumulation of like charges in certain regions of the molecule. Higher levels of acetylation formed 6–7 *O*-acyl groups in ovotransferrin, and higher treatments with KCNO have been reported to introduce much more reagent in ovomucoid than could be accounted for by substitution of amino groups (Stevens and Feeney, 1963).

The specific metal-binding properties of the transferrins must depend upon the fundamental properties of the protein as well as upon the positioning and reactivity of the particular ligands. As the proper orientation of the metal-binding site is most probably stabilized by hydrogen bonds and charge interactions, the entire conformation of the molecule is important (Fraenkel-Conrat and Feeney, 1950). Upon chemical modification, these intramolecular interactions are altered, and the resulting changes may lead to variations in the stability of the chromogenic complex, spectral shifts, and eventually to loss of the metal-binding capacity of the protein. When spectral changes such as those brought about by succinylation are observed, spatial and charge alterations in the vicinity of the iron-binding site must be considered as well as the possibilities that the newly introduced carboxyl groups may be involved as participating ligands, and that there may even be nonspecific binding unrelated to the original binding sites. Conclusions based on partial modification, partial loss of metal-binding capacity, and a relative decrease in stability cannot be definitive, and the exact nature of the ligands involved is still unrecognized. Nevertheless, although high levels of these modifications may exert certain undefined changes in the iron complexes, the present data suggest that, if two nitrogen ligands are directly involved in metal-binding, these nitrogens are probably not contributed by ε-amino groups of lysines. This would leave the guanidyl groups of arginines and the imidazole groups of histidines. From consideration of the data from titration curves, spectrophotometric determinations, and electron-spin resonance studies (Aasa *et al.*, 1963; Windle *et al.*, 1963; Wishnia *et al.*, 1961), the imidazole groups of histidines appear to be involved in the metal-binding sites.

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References

Aasa, R., Malmstrom, B. G., Saltman, P., and Vanngard, T. (1963), *Biochim. Biophys. Acta 75*, 203.

Azari, P. R., and Feeney, R. E. (1958), *J. Biol. Chem. 232*, 293.

Azari, P. R., and Feeney, R. E. (1961), *Arch. Biochem. Biophys. 92*, 44.

Charlwood, P. A. (1963), *Biochem. J. 88*, 394.

Clark, J. R., Osuga, D. T., and Feeney, R. E. (1963), *J. Biol. Chem. 238*, 3621.

Feeney, R. E., Clary, J. J., and Clark, J. R. (1964), *Nature 201*, 192.

Fiala, S., and Burk, D. (1949), *Arch. Biochem.* (now *Arch. Biochem. Biophys.*) *20*, 172.

Fraenkel-Conrat, H. (1957), *Methods Enzymol. 4*, 247.
Fraenkel-Conrat, H., and Feeney, R. E. (1950), *Arch. Biochem.* (now *Arch. Biochem. Biophys.*) *29*, 101.
Glazer, A. N., and McKenzie, H. A. (1963), *Biochim. Biophys. Acta 71*, 109.
Gordon, A. H., and Louis, L. N. (1963), *Biochem. J. 88*, 409.
Habeeb, A. F. S. A., Cassidy, H. G., and Singer, S. J. (1958), *Biochim. Biophys. Acta 29*, 587.
Montreuil, J., Tonnelat, J., and Mullet, S. (1960), *Biochim. Biophys. Acta 45*, 413.
Saifer, A., Robin, M., and Ventrice, M. (1961), *Arch. Biochem. Biophys. 92*, 409.
Smithies, O. (1955), *Biochem. J. 61*, 629.
Stark, G. R., Stein, W. H., and Moore, S. (1960), *J. Biol. Chem. 235*, 3177.
Stevens, F. C., and Feeney, R. E. (1963), *Biochemistry 2*, 1346.
Surgenor, D. M., Strong, L. E., Taylor, H. L., Gorden, R. S., Jr., and Gibson, D. M. (1949), *J. Am. Chem. Soc. 71*, 1223.
Uraki, Z., Terminiello, L., Bier, M., and Nord, F. F. (1957), *Arch. Biochem. Biophys. 69*, 644.
Warner, R. C. (1954), *Proteins 2* (A), 435.
Warner, R. C., and Weber, I. (1951), *J. Biol. Chem. 191*, 173.
Warner, R. C., and Weber, I. (1953), *J. Am. Chem. Soc. 75*, 5094.
Windle, J. J., Wiersema, A. K., Clark, J. R., and Feeney, R. E. (1963), *Biochemistry 2*, 1341.
Wishnia, A., Weber, I., and Warner, R. C. (1961), *J. Am. Chem. Soc. 83*, 2071.

Fundamental Mass-Transport Equations for Zone Sedimentation Velocity*

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ABSTRACT: A theory is presented for the determination of sedimentation coefficients from observation of the migration of zones of sedimenting macromolecules. In developing this theory, it is assumed that mass transport is caused by sedimentation and diffusion alone. With these assumptions it may be shown that (1) for a centrifuge cell of uniform cross-sectional area in a gravitational field, the velocity of the center of gravity of the migrating zone is equal to the average sedimentation velocity of the individual particles; (2) for a radially shaped cell in a centrifugal field, the migration of the "center of sedimentation," defined as the mass averaged value of the logarithm of the radius, is determined by the average sedimentation coefficients of the individual particles; (3) for a uniform cell in a centrifugal field the movement of the center of gravity is equal to the average sedimentation velocity of the individual particles. The effects of concentration dependence of both the sedimentation and the diffusion coefficients are treated in detail as are the effects of a superimposed viscosity and density gradient. Both single-component and polydisperse systems are considered.

During the last decade a new technique has been developed which has been called "rate zonal sedimentation" (Brakke, 1956), or "gradient differential sedimentation" (Anderson, 1956). It has proved to be a tool of great power for the fractionation and characterization of nuclei, mitochondria, and microsomes (Anderson, 1956), polyribosomes (Warner *et al.*, 1963), ribosomes (Britten *et al.*, 1962), virus (Brakke, 1960), nucleic acids (Vinograd *et al.*, 1963), and proteins (Martin and Ames, 1961). In using this technique, a solution containing the particles to be studied is layered as a thin zone on top (or on the bottom for zone flotation) of a second solution which contains or develops a density gradient to stabilize the system against convection. During the subsequent centrifugation, the particles sediment as zones from the top to the bottom of the tube. During the course of this migration, separation of zones of particles possessing different sedimentation coefficients occurs, making possible characterization and fractionation of different types of particles according to *sedimentation coefficient*.

The technique of zone sedimentation velocity should be distinguished from isopycnic density gradient centrifugation (Anderson, 1956), and zone sedimentation equilibrium in a buoyant density gradient (Vinograd, 1963), where particles are characterized and fractionated according to their *densities*.

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