Flaschka, H. (1952), *Mikrochim. Acta 39*, 38.
Heinrikson, R., Stein, W. M., Crestfield, A. M., and Moore, S. (1965), *J. Biol. Chem. 240*, 2921.
Morgan, C. R., Sorenson, R. L., and Lazarow, A. (1964), *Diabetes 13*, 579.
Pastan, I., Roth, J., and Macchia, V. (1966), *Proc. Nat. Acad. Sci. U. S. 56*, 1802.
Sluyterman, L. A. AE. (1955), *Biochim. Biophys. Acta 17*, 169.
Spackman, D. H., Stein, W. H., and Moore, S. (1958), *Anal. Biochem. 30*, 1190.
Stark, G. R., Stein, W. H., and Moore, S. (1960), *J. Biol. Chem. 235*, 3177.
Wolff, J., and Covelli, I. (1969), *Eur. J. Biochem. 9*, 371.

Chemical and Physical Properties of Serum Transferrins from Several Species†

Billy G. Hudson,* Mikio Ohno, William J. Brockway, and Francis J. Castellino

ABSTRACT: Bovine, rabbit, equine, and porcine serum transferrins have molecular weights of 77,300, 76,700, 79,100, and 76,400, respectively, in dilute aqueous solution as determined by sedimentation equilibrium centrifugation. The mercaptoethanol reduced proteins in 6 M guanidine hydrochloride possess molecular weights of 76,800, 77,400, 75,400, and 75,900, respectively. These results indicate that transferrin from all four species consists of a single polypeptide chain. Each transferrin binds two atoms of iron per molecule. Amino acid compositions of the four species of transferrins are similar but some significant differences exist. Comparison of the carbohydrate composition of these glycoproteins reveals similarities in the monosaccharides present but striking differences in their amounts. Each species contains mannose, galactose, glucosamine, sialic acid, and fucose, except equine in which fucose is absent. Bovine, rabbit, equine, and porcine transferrins contain a total number of 10–11, 18–19, 23, and 41–43 monosaccharide residues per molecule, respectively. It is postulated that this large variation in monosaccharide content is principally a result of differing numbers of heteropolysaccharide units per polypeptide chain: bovine contains one unit, rabbit and equine two, and porcine four, in analogy to chicken and human transferrins which have been shown to contain one and two units, respectively.

The transferrins represent a group of homologous nonheme iron binding glycoproteins found in blood serum of vertebrate animals, mammalian milk, and avian egg whites (Feeney and Komatsu, 1966). The function of serum transferrin is one of efficient transport of iron from the intestinal sites of absorption and the sites of hemoglobin breakdown to specific iron-requiring cells and to the various sites of storage (Fletcher and Huehns, 1968).

Serum transferrin from several species has been extensively studied with regard to amino acid composition, molecular weight, subunit structure, and iron binding capacity (Mann *et al.*, 1970; Palmour and Sutton, 1971; Greene and Feeney, 1968; Parker and Bearn, 1962). The amino acid compositions of human, monkey, rabbit, frog, turtle, and hagfish transferrin are quite similar but have significant differences. The molecular weight of human, rabbit, and frog transferrin is about 77,000, whereas turtle and hagfish have molecular weights of 92,000 and 44,000, respectively. Each of these transferrins consist of a single polypeptide chain as determined from molecular weight measurements in 6 M guanidine hydrochloride or 8 M urea on reduced and carboxymethylated proteins.

It is generally agreed that transferrin from different species possesses two iron binding sites, with the exception of hagfish transferrin which has one site (Palmour and Sutton, 1971). The relative affinity of the two sites has not been established. Warner and Weber (1953) and Davis *et al.* (1962) reported that the second ion bound to human transferrin had a greater affinity than the first. Contrary to these studies, Aasa *et al.* (1963) and Aisen *et al.* (1966) have reported that no difference exists in the affinity.

The carbohydrate structure of serum transferrins has been investigated in detail for two species, human and chicken. Striking differences exist between their carbohydrate moieties. Human transferrin has two identical heteropolysaccharide units composed of two residues of sialic acid, two residues of galactose, four residues of mannose, and four residues of *N*-acetylglucosamine (Jamieson, 1965; Jamieson *et al.*, 1971). In contrast chicken transferrin has most of its carbohydrate in a single heteropolysaccharide unit composed of either one or two residues of sialic acid, two residues of galactose, two residues of mannose, and three residues of *N*-acetylglucosamine (Williams, 1968). The carbohydrate units of both human and chicken transferrin are connected to the polypeptide chain through an *N*-glycosidic linkage to the amide of asparagine. Reports on the carbohydrate of transferrin from other species are limited to the sialic acid and hexosamine

† From the Department of Biochemistry, Agricultural Experiment Station, Oklahoma State University, Stillwater, Oklahoma 74074, and from the Department of Chemistry, Programs in Biochemistry and Biophysics, University of Notre Dame, Notre Dame, Indiana 46556. *Received July 14, 1972.* Journal Article 2341 of the Agricultural Experiment Station, Oklahoma State University, Stillwater, Oklahoma. This investigation was supported by grants from the National Institute of Arthritis and Metabolic Diseases (AM 16072) and Herman Frasch Foundation to B. G. H., and from the National Science Foundation (GP-24444) to F. J. C. A preliminary account of this work was presented at the Southwest Regional Meeting of the American Chemical Society at San Antonio, Texas, Dec 1–3, 1971.

* To whom correspondence should be addressed at Oklahoma State University.

composition (Parker and Bearn, 1962; Palmour and Sutton, 1971); however, these data also suggest that substantial differences exist in their carbohydrate portion.

The purpose of this investigation was to determine the amino acid and carbohydrate composition, molecular weight, subunit structure, and iron binding capacity of several transferrins which have not previously been examined. It was of particular interest to determine the species variation in the carbohydrate moiety as evinced from the complete carbohydrate composition. The results of these studies show that a large species variation occurs in the carbohydrate moiety of the transferrin molecule; moreover, these data suggest that the major variation is in the number of heteropolysaccharide units per polypeptide chain.

Experimental Section

Materials

Serum transferrins were purchased from Miles Laboratories, Inc. Guanidine hydrochloride was purchased from Heico, Inc., and used without further purification. All other chemicals were the best commercially available and used without further purification.

Methods

Purification of Transferrins. Each commercial transferrin consisted of several minor components when examined by polyacrylamide gel electrophoresis in 6.25 M urea (pH 3.2) (Panyim and Chalkley, 1969). Each transferrin was purified by chromatography on DEAE-Sephadex A-50 (Pharmacia) essentially by the method of Baker *et al.* (1968). A 1.1 × 25 cm column was equilibrated with 0.05 M Tris·HCl (pH 8.0) at room temperature. Serum transferrin, dissolved in the same buffer, was applied and the column developed with a gradient consisting of 500 ml of 0.05 M Tris·HCl (pH 8.0) as starting solvent and 500 ml of 0.20 M Tris·HCl (pH 8.0) as the limit solvent. The red peak emerging from the column was collected, dialyzed against H_2O, and lyophilized.

Molecular Weight Measurements. Molecular weights of transferrins in dilute salt solutions and in 6 M guanidine hydrochloride in the presence of a reducing agent were obtained by sedimentation equilibrium analysis utilizing the high-speed method of Yphantis (1964). For native molecular weights, each protein (0.1–0.2 mg/ml) was dissolved in 0.1 M KCl (pH 7.0) and dialyzed against the same solvent. For subunit molecular weights, each protein was dissolved in 6.0 M guanidine hydrochloride–0.1 M 2-mercaptoethanol–0.1 M Tris·HCl at a final pH of 8.6 and dialyzed against the same solvent. In each case, the final dialysate was used as the reference solution. Ultracentrifugation was performed at 25° with a 12-mm double-sector cell equipped with sapphire windows using 3-mm column heights. Fluorocarbon FC-43 (0.02 ml, Beckman Instruments) was added to each sector to provide a flat, transparent cell bottom. Rotor speeds were selected so that at equilibrium the meniscus concentration would be essentially zero and the reduced molecular weight, σ, would be approximately 5 cm^{-2} (Yphantis, 1964). Each run was allowed to proceed until there was no further increase in fringe displacement with time. Following each experiment, a water blank was run without disassembling the cell to correct for cell window distortions.

Interference patterns were measured in a Gaertner two-dimensional microcomparator. The fringe displacement of any three fringes was measured as a function of the radial distance and the results were averaged. The blank was evaluated in the same way to obtain the results of an average fringe. The natural logarithms of the differences between the blank-corrected fringe displacements and the base line ($\ln x$) were plotted against the corresponding radial positions in the cell (r^2). Molecular weights (M_w) were calculated from the equation

$$M_w = \frac{2RT}{\omega^2(1 - \phi'\rho)} \frac{d \ln x}{dr^2} \quad (1)$$

in which ϕ' is the effective partial specific volume of the protein, ω is the angular velocity of the rotor, and ρ is the solvent density. The values of $d \ln x/dr^2$ were calculated by linear least-squares analysis of the slopes of plots of $\ln x$ *vs.* r^2. The partial specific volumes ($\bar{v}$) of bovine, rabbit, horse, and porcine serum transferrins calculated from the amino acid compositions of each protein were found to be 0.725, 0.723, 0.725, and 0.728, respectively. In dilute aqueous salt solutions, preferential interactions between solvent components and the protein are minimal and thus, $\bar{v}$ can be used in place of ϕ' in eq 1. However, the choice of ϕ' for a protein in 6 M guanidine hydrochloride is somewhat uncertain. Hade and Tanford (1967) and Castellino and Barker (1968) have shown that ϕ' for several proteins may be decreased by as much as 0.01 cm^3/g due to preferential interactions between guanidine hydrochloride and the protein. However, for most proteins examined the ϕ' in guanidine hydrochloride could be substituted by the $\bar{v}$ without serious error and we have done this in our calculations.

Molecular weights in 6.0 M guanidine hydrochloride were also carried out on reduced, carboxymethylated horse and porcine serum transferrins by the gel filtration technique of Fish *et al.* (1969) using a 4% agarose column.

Sedimentation Velocities. Sedimentation coefficients were determined for Fe^{3+}-saturated horse and porcine serum transferrins in 0.1 M KCl (pH 7.0) and sedimentation coefficients for horse and porcine apotransferrins were determined in 0.1 M acetate–0.1 M citrate (pH 4.5). Rotor speeds of 59,780 rpm were used and the procedure of Schachman (1959), corrected to standard conditions, was employed to calculate the results.

Fe^{3+} Binding Studies. The Fe^{3+} binding capacities of each transferrin were determined by preparing the Fe^{3+}-free apotransferrins and adding back Fe^{3+} in the form of $FeNH_4(SO_4)_2$ in the presence of $NaHCO_3$ according to the procedure of Aisen *et al.* (1966). The increase in absorbance at 470 mμ accompanying the binding of Fe^{3+} was used to monitor the incorporation of Fe^{3+} into each serum transferrin. The apotransferrins were prepared by dissolving the Fe^{3+}-saturated protein in a buffer consisting of 0.1 M acetate–0.1 M citrate (pH 4.0) and dialyzing for several days against frequent changes of the same buffer. Only small residual absorbance at 470 mμ remained in each protein after this treatment.

Amino Acid Compositions. Amino acid analyses were determined by hydrolyzing samples of transferrin 6 N HCl containing 4% thioglycollic acid under reduced pressure for 24 48, and 72 hr at 110° (Matsubara and Sasaki, 1969) and performing the analysis on a Beckman Model 120C amino acid analyzer (Spackman *et al.*, 1958).

The total half-cystine contents of each transferrin were determined as cysteic acid after oxidation of protein with performic acid and hydrolysis in 6 N HCl for 24 hr (Schram *et al.*, 1954).

Tryptophan determinations were performed by the method

of Edelhoch (1967). The total amide content (glutamine and asparagine) was performed by the method of Hoare and Koshland (1967), correcting for the contribution of sialic acid.

Paper Chromatography. The neutral sugars were identified by paper chromatography following hydrolysis of protein samples in 2 N sulfuric acid for 4 hr at 100°. The neutral sugars were separated from the amino sugars, amino acids, and peptides by the method of Boas (1953). Descending chromatography on Whatman No. 1 paper was run in pyridine–ethyl acetate–acetic acid–water (5:5:3:1) for 20 hr (Fisher and Nebel, 1955). Sugars were located on the chromatogram by the silver nitrate method (Trevelyan *et al.*, 1950; Benson *et al.*, 1952).

Identification of the sialic acids was performed by paper chromatography after release by acid hydrolysis and separation by ion-exchange chromatography (Spiro, 1966).

Quantitative Analyses of Carbohydrate Components. The total neutral sugars were determined on the intact protein with the anthrone reagent of Roe (1955). A mixture of galactose and mannose in a ratio of 1:1 was used as a standard.

For release of the neutral sugars and hexosamines the proteins were hydrolyzed in 1 and 2 N sulfuric acid for 4 hr at 100° in sealed tubes. The hydrolysate was passed through a column of Dowex 50-X4 (H^+) (200–400 mesh) coupled to a column of Dowex 1-X8 (formate) (200–400 mesh) (Spiro, 1966). The effluent and wash were taken to dryness by lyophilization. The amount of neutral sugars present in the effluent was determined by the Technicon automatic sugar chromatography system as described by Lee *et al.* (1969).

The hexosamines were determined by the Elson–Morgan reaction (Boas, 1953) and on the short column of the amino acid analyzer after elution from the Dowex 50 with 2 N HCl. Total hexosamine was determined by this procedure after hydrolysis of protein in 4 N HCl at 100° for 6 hr (Spiro, 1966).

Sialic acid was determined by the thiobarbituric assay method of Warren (1959) after hydrolysis of protein with 0.1 N HCl at 80° for 1 hr. *N*-Acetylneuraminic acid was used as a standard.

Results

Purity. Each transferrin, further purified by Sephadex A-50 chromatography, was examined by polyacrylamide gel electrophoresis and shown to consist of a single component (Figure 1). In addition, a single symmetrical peak was obtained for each transferrin when examined by sedimentation velocity in the analytical ultracentrifuge.

Molecular Weights. The high-speed meniscus depletion sedimentation equilibrium technique was used to determine the serum transferrins. Linear plots of the natural logarithm of the Rayleigh fringe desplacement, x, *vs.* the square of the distance from the center of rotation, r^2, for each condition were obtained for each transferrin. Native molecular weights of 77,300, 76,700, 79,100, and 76,400 ± 2000 were calculated for Fe^{3+}-saturated bovine, rabbit, equine, and porcine serum transferrins, respectively.

The same technique was used to examine the molecular weights of the transferrins in 6 M guanidine hydrochloride under reducing conditions. Clearly, a monodisperse system is obtained and 6.0 M guanidine hydrochloride–0.1 M 2-mercaptoethanol is without influence on the molecular weight. Values of 76,800, 77,400, 75,400, and 75,900 ± 2000 are obtained for bovine, rabbit, equine, and porcine serum transferrins, respectively.

The molecular weights of *S*-carboxymethyl equine and porcine serum transferrin were also conducted in 6 M guanidine hydrochloride by the gel filtration technique using *S*-carboxymethyl human serum transferrin, bovine serum albumin, ovalbumin, chymotrypsinogen, and β-lactoglobulin as standards. Values of 77,000 and 75,000 ± 3000 were obtained for horse and porcine serum transferrins, respectively. In addition when each protein was mixed with human serum transferrin of mol wt 76,600 and chromatographed by this method, no significant differences in elution positions were detected.

The molecular weights are summarized in Table I.

Sedimentation Velocities. The sedimentation coefficients ($s_{20,w}$) of Fe^{3+}-saturated transferrins were 5.21 ± 0.14, 5.28, ± 0.1, 5.25 ± 0.1, and 5.20 ± 0.5 S for bovine, rabbit, equine, and porcine, respectively. In each case a single, apparently symmetrical, peak was obtained.

Fe^{3+} Binding Studies. Figure 2 presents a plot of the moles of Fe^{3+} added as $FeNH_4(SO_4)_2$ per mole of transferrin in the presence of HCO_3^- *vs.* the absorbance change at 470 mμ as

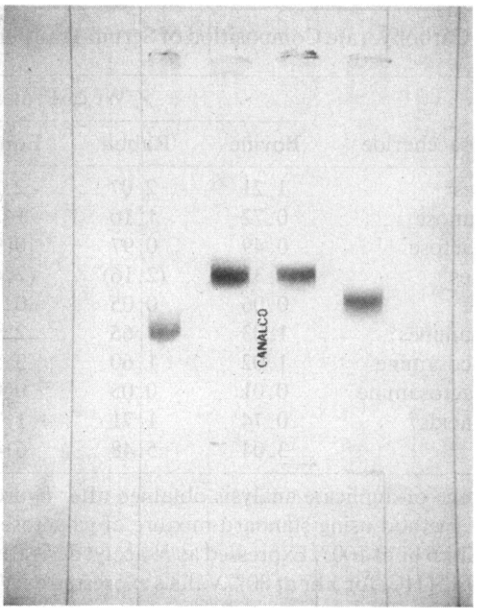

FIGURE 1: Polyacrylamide gel electrophoresis of serum transferrins. From left to right, porcine, equine, rabbit, and bovine. Gels were run in separate experiments.

TABLE I: Physical Properties of Serum Transferrins.

Property	Bovine	Rabbit	Equine	Porcine
Native molecular weight[a]	77,300	76,700	79,100	76,400
Subunit molecular weight[a]	76,800[b]	77,400[b]	75,400[b]	75,900[b]
			77,000[c]	75,000[c]
Sedimentation coefficient ($s_{20,w}$), (S)	5.21	5.28	5.25	5.20
Mol of Fe^{3+} bound/mol of protein	1.90	1.92	1.80	1.95

[a] Values ±2000. [b] Determined by sedimentation equilibrium in 6.0 M guanidine hydrochloride–0.1 M 2-mercaptoethanol. [c] Determined on reduced and carboxymethylated transferrin by gel filtration in 6.0 M guanidine hydrochloride.

TABLE II: Carbohydrate Composition of Serum Transferrins.

Monosaccharide	% Wt of Protein				μmol/100 mg of Protein			
	Bovine	Rabbit	Equine	Porcine	Bovine	Rabbit	Equine	Porcine
Hexoses[a]	1.21	2.07	2.23	3.89	6.72	11.5	12.4	21.6
Mannose	0.72	1.10	1.31	2.07	4.00	6.09	7.30	11.5
Galactose	0.49	0.97	0.92	1.82	2.72	5.41	5.15	10.1
Hexoses[b]	(1.32)	(2.16)	(2.40)	(4.38)	(7.35)	(12.0)	(13.3)	(24.3)
Fucose	0.06	0.05	0	0.74	0.38	0.30	0	4.50
Hexosamines[c]	1.03	1.65	2.53	4.16	4.66	7.43	11.4	18.8
Glucosamine	1.02	1.60	2.52	4.15	4.60	7.20	11.3	18.7
Galactosamine	0.01	0.05	0.01	0.01	0.06	0.23	0.06	0.06
Sialic acids[d]	0.74	1.71	1.73	3.01	2.39	5.52	5.60	9.73
Total	3.04	5.48	6.49	11.8	14.2	24.8	29.4	54.6

[a] Average of duplicate analysis obtained after hydrolysis with 2 N H_2SO_4 for 4 hr at 100°. [b] Average of triplicate analysis by anthrone method using standard mixture of galactose and mannose (1:1). [c] Average of duplicate analysis after hydrolysis with 4 N HCl for 6 hr at 100°. Expressed as *N*-acetyl derivatives. [d] Average of triplicate analysis by thiobarbituric method after hydrolysis with 0.1 N HCl for 1 hr at 80°. Values expressed as *N*-acetylneuraminic acid.

exemplified by equine and porcine serum apotransferrins. There was little absorbancy at 470 mμ prior to addition of Fe^{3+} which indicated that all Fe^{3+} had been previously removed from the proteins. A linear relationship was obtained between the absorbance at 470 mμ and the amount of Fe^{3+} added until saturation was reached, as shown in Figure 2. Saturation of the Fe^{3+} binding sites for each transferrin occurred at 1.8–1.95 mol of Fe^{3+}/mol of protein. The binding data are summarized in Table I.

Identification and Quantitation of the Carbohydrate. Identification of neutral sugars by paper chromatography and from elution profiles on the autoanalyzer revealed the presence of galactose and mannose in bovine, rabbit, equine, and porcine transferrin. Fucose was detected in each species except equine. Glucosamine was identified in each species from the elution time on the amino acid analyzer. Only trace amounts of galactosamine were found.

The total carbohydrate composition of transferrin from all four species is given in Table II. The total carbohydrate ranges from 3% in the bovine to 11.8 in porcine. The amount of hexose released with 2 N sulfuric acid for 4 hr at 100° was not increased on extending the hydrolysis time to 6 hr. The total hexose content, as measured by the anthrone reaction on the unhydrolyzed protein and corrected for the contribution of fucose and sialic acid, is in good agreement with the sum of galactose and mannose determined after hydrolysis of the proteins.

The sialic acid content was further analyzed as reported in Methods. All sialic acid found in each transferrin was determined to be *N*-acetylneuraminic acid. No *N*-glycolylneuraminic acid was detected.

Amino Acid Compositions. The amino acid composition of bovine, rabbit, equine, and porcine transferrin is given in Table III. All values reported represent the average of the 24-, 48- and 72-hr hydrolysis times except for valine, leucine, and isoleucine which are given by the 72-hr hydrolysis times, and serine and threonine which have been extrapolated to zero hydrolysis time. The number of amide nitrogen and tryptophan was determined as described in Methods. Titration of each protein with Ellman's reagent in 6 M guanidine hydrochloride revealed that no free SH groups were present.

A summation of the amino acid, monosaccharide, and amide residue weight indicates that essentially all of the protein weight can be accounted for by these constituents, 97.7, 97.0, 96.3, and 97.1% for bovine, rabbit, equine, and porcine, respectively.

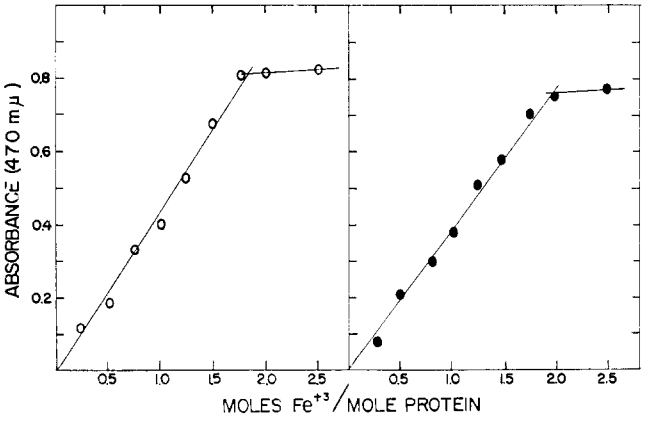

FIGURE 2: Fe^{3+} binding of equine and porcine serum transferrins. (○) Binding of Fe^{3+} by equine serum transferrin. (●) Binding of Fe^{3+} by porcine serum transferrin. The ordinate represents the increase in absorbance at 470 mμ accompanying the binding of Fe^{3+} by each apotransferrin and the abscissa represents the molar ratio of Fe^{3+} to protein which was added to produce the given increase in absorbance.

Discussion

The studies reported here indicate that the transferrins from bovine, rabbit, equine, and porcine have molecular weights of about 77,000. This value is in agreement with that reported by Palmour and Sutton (1971), for rabbit transferrin, but is in disagreement with the value of 88,000 reported earlier by Laurell and Ingelman (1947) for porcine transferrin. Furthermore, these molecular weights are not lowered by treatment of each protein with 6.0 M guanidine hydrochloride–0.1 M 2-mercaptoethanol, a solvent which has been previously shown by a number of investigators to yield the molecular

weight of the maximum subunit. The molecular weights of the reduced horse and porcine serum transferrins in 6.0 M guanidine hydrochloride were determined by two methods which rely on two different principles in order to establish that the molecular weights which we obtained were indeed the true subunit molecular weights. Sedimentation equilibrium measures only the mass of the molecule and from sedimentation equilibrium data alone ambiguity exists as to whether the protein with a given mass is fully dissociated, since an incompletely dissociated molecule can have the same cumulative mass and the results obtained would be identical. Therefore, the gel filtration technique was used in the same solvent. The calculation of molecular weights by this method relies on the molecules assuming the behavior of a linear random coil. Since the molecular weights obtained by these two methods were virtually identical, both horse and porcine serum transferrin must exist as random coils in 6 M guanidine hydrochloride and must in fact consist of a single polypeptide chain.

It is quite clear from this study that all four species of serum transferrin contain two Fe^{3+} binding sites, analogous to other transferrins with the exception of hagfish (Palmour and Sutton, 1971). In Figure 2 the total Fe^{3+} added per mole of protein appears on the abscissa and correctly the free Fe^{3+} per mole of protein should be plotted. Therefore, to obtain the number of binding sites by extrapolation to the abscissa, as we have done, requires that at all points before saturation is reached essentially all the Fe^{3+} added should be bound to the protein. Since transferrins have dissociation constants of approximately 10^{-30} (Aasa *et al.*, 1963; Davis *et al.*, 1962), calculations show that no measurable amount of free Fe^{3+} should be present at concentrations at molar ratios less than or slightly greater than saturation. Therefore, calculations of the number of Fe^{3+} binding sites by this method are justified.

The fact that the amount of Fe^{3+} bound to serum transferrin is a linear function of the amount of Fe^{3+} added, as shown in Figure 2, can be interpreted in two ways: (1) that both Fe^{3+} binding sites are equivalent and noninteracting or (2) that the dissociation constant for the second Fe^{3+} bound, K_2, is greater than K_1 ($K_2 \gg K_1$). In either case linearity would be observed. However, in case 1, before saturation is reached transferrin molecules consisting of 0, 1, and 2 mol of Fe^{3+} per mol of protein should exist in the total transferrin population. On the other hand, case 2 requires that Fe^{3+} is bound only in pairs and that, before saturation is reached, transferrin molecules consisting of only 0 and 2 mol of Fe^{3+} bound per mol of protein should exist in the total transferrin population. Since Aisen *et al.* (1966) have obtained evidence by free electrophoresis that molecules containing 0, 1, and 2 mol of Fe^{3+} bound per mol of protein, for both human serum transferrin and chicken egg-white transferrin, existed in the total transferrin population at concentrations of Fe^{3+} less than those required to saturate the binding sites, we feel that the two Fe^{3+} binding sites on horse and porcine serum transferrin are noninteracting and equivalent.

For comparison purposes, the amino acid and carbohydrate composition of the serum transferrins determined in this study and of human and chicken transferrin determined by Mann *et al.* (1970) Jamieson (1965), and Williams (1962, 1968) are expressed in residues per molecule and presented in Table IV. The amino acid compositions are similar but some significant differences exist. It appears that transferrins can be generally characterized by having very low methionine contents and no free sulfhydryl residue. If the mode of Fe^{3+} binding is the same for chicken, bovine, rabbit, equine, and porcine transferrins as for human transferrin, then approximately 30% of the histidine and tyrosine residues in these proteins play a functional role.

TABLE III: Composition of Serum Transferrins.

Component	Residue Wt, g/100 g of Protein			
	Bovine	Rabbit	Equine	Porcine
Amino Acids[a]				
Lysine	8.66	7.58	7.39	6.82
Histidine	2.64	3.47	2.48	2.06
Arginine	4.90	4.98	4.64	4.57
Aspartic acid	11.00	9.86	10.9	11.70
Threonine[b]	5.81	4.40	4.25	3.26
Serine[b]	5.49	4.55	4.40	4.00
Glutamic acid	10.6	10.1	10.1	9.86
Proline	4.74	4.86	5.63	4.81
Glycine	3.37	3.43	3.12	2.69
Alanine	4.55	4.45	4.60	4.43
Valine[c]	5.71	6.07	5.63	5.23
Methionine	1.12	0.84	0.60	0.91
Isoleucine[c]	2.43	2.47	2.32	2.55
Leucine[c]	6.57	7.36	7.97	7.75
Tyrosine	4.24	4.68	4.51	3.46
Phenylalanine	4.86	4.84	3.81	4.32
Half-cystine[d]	4.53	4.50	4.42	4.34
Tryptophan[d]	2.42	2.28	2.27	2.21
Amide nitrogen[d]	1.33	1.25	1.27	1.27
Monosaccharides[e]				
Mannose	0.65	0.99	1.18	1.86
Galactose	0.44	0.88	0.84	1.64
Fucose	0.06	0.04	0.00	0.66
Glucosamine	0.95	1.47	2.31	3.84
Galactosamine	0.01	0.05	0.01	0.01
Sialic acids	0.70	1.61	1.63	2.83
Total	97.7	97.0	96.3	97.1

[a] Unless otherwise indicated, the values represent an average of the analysis obtained at 24, 48, and 72 hr. [b] These values were extrapolated to zero-time hydrolysis by method of least squares. [c] These values were taken from the 72-hr hydrolysis time. [d] Measured separately as described in text. [e] Calculated from monosaccharide analysis given in Table II. Hexosamine values expressed as *N*-acetyl derivative.

Comparison of the carbohydrate composition of the various transferrins reveals similarities in the monosaccharides present but striking differences in their amounts. Each species contains mannose, galactose, glucosamine, and sialic acid. Fucose is present only in porcine transferrin in integral amounts; bovine and rabbit contain fractional quantities (Table II). Bovine and chicken contain about one-half the total number of monosaccharides per mole of transferrin as human, rabbit, and equine; in contrast porcine transferrin contains twice the number.

The large difference between the number of monosaccharide residues in chicken and human transferrin is mainly due to a difference in the number of heteropolysaccharide units per polypeptide chain. Chicken transferrin contains a single heteropolysaccharide unit (Williams, 1968) whereas human contains two identical units (Jamieson, 1965). The heteropolysaccharide units from both chicken and human are

TABLE IV: Comparison of Amino Acid and Monosaccharide Residues per Mole of Transferrin.

Component	Chicken[a]	Residues/mol of Protein[d]				
		Bovine[b]	Rabbit[b]	Human[c]	Equine[b]	Porcine[b]
Lysine	62	52	46	51	44	41
Histidine	12	15	19	19	14	12
Arginine	34	24	25	24	23	23
Aspartic acid	72	74	66	81	73	78
Threonine	34	44	33	30	32	25
Serine	42	49	40	40	39	35
Glutamic acid	75	63	60	58	60	59
Proline	29	35	36	30	42	36
Glycine		45	46	54	42	35
Alanine	53	49	48	59	50	48
Valine	48	44	47	43	44	41
Methionine	11	7	5	4	4	5
Isoleucine	23	17	17	14	16	17
Leucine	50	45	50	57	54	53
Tyrosine	20	20	22	24	21	16
Phenylalanine	24	25	25	28	20	23
Half-cystine	20	34	34	35	33	33
Tryptophan	20	10	9	9	9	9
Mannose	3	3	5	8	6	9
Galactose	2	2	4	3	4	8
Fucose	0	0	0	0	0	3–4
Glucosamine	5	3–4	5–6	7	9	14
Sialic Acids	2	2	4	3	4	7–8
Total monosaccharide	12	10–11	18–19	21	23	41–43

[a] Values calculated from data from Williams (1962, 1968). [b] Values calculated from data in Table III. [c] Amino acid data from Mann *et al.* (1970) and carbohydrate data from Jamieson (1965). [d] All values based on molecular weight of 77,000 and expressed to nearest integer.

TABLE V: Comparison of Monosaccharide Ratios and Number of Carbohydrate Units in Transferrins.

Monosaccharide	Residues/Galactose Residue					
	Chicken[a]	Bovine[b]	Rabbit[b]	Human[c]	Equine[b]	Porcine[b]
Galactose	1.0	1.0	1.0	1.0	1.0	1.0
Sialic acid + fucose	1.0	1.0	1.1	1.0	1.1	1.4
Mannose	2.0	1.5	1.1	2.5	1.4	1.1
Glucosamine	3.2	1.7	1.3	2.2	2.2	1.9
Number of carbohydrate units/molecule[d]	0.9 (1)	1.0 (1)	2.1 (2)	1.6 (2)	2.0 (2)	3.9 (4)

[a] Values calculated from data of Williams (1968). [b] Values calculated from data in Table II. [c] Values calculated from data of Jamieson (1965). [d] Values were calculated from number of galactose residues per mole of transferrin based on two galactose residues per carbohydrate unit. Galactose residues per mole of transferrin were calculated from data in Table II based on molecular weight of 77,000; these are: 2.1, 4.2, 4.0, and 7.8 for bovine, rabbit, equine, and porcine, respectively. Values of 3.2 for human and 1.73 for chicken were calculated from data of Jamieson (1965) and Williams (1968), respectively, based on molecular weight of 77,000.

identical in their sialic acid and galactose contents, two residues of each, but differ in their mannose and glucosamine contents; chicken contains two mannose and three glucosamine residues, whereas human contains four mannose and four glucosamine residues.

To further evaluate the variation in the carbohydrate compositions determined in this study for bovine, rabbit, equine, and porcine transferrins, a comparison was made of their molar ratios of monosaccharides to those of chicken and human as presented in Table V. Galactose was chosen as reference since it is present in the same amounts in the heteropolysaccharide units of both chicken and human transferrin.

It is evident that galactose occurs in a 1:1 ratio to sialic acid plus fucose for each species except porcine which contains a comparatively large amount of fucose. The mannose to galactose ratio varies from 1.1 in rabbit to 2.5 in human. The glucosamine to galactose ratio is approximately 2 for each species except rabbit and chicken. This comparison shows that sialic acid and galactose are present in the same relative amounts in each species but that the amounts of mannose and glucosamine varies.

These results lead to the proposal that the heteropolysaccharide units of bovine, rabbit, equine, and porcine are similar in composition to those of chicken and human with some differences in the mannose and glucosamine content and that the large variation in the total number of monosaccharide residues per molecule of transferrin is principally a result of differing numbers of heteropolysaccharide units of identical composition. On this basis the number of heteropolysaccharide units can be calculated as given in Table V. Thus, chicken and bovine transferrins contain a single heteropolysaccharide unit, rabbit, human, and equine contain two, and porcine contains four. In this connection, a single unit has been suggested by Bearn and Parker (1966) to occur in cynomologus monkey based on the evidence that it contains half the amount of sialic acid and glucosamine as human. In support of this proposal, preliminary studies on the carbohydrate structure of porcine transferrin show that the glycopeptides obtained from a pronase digest chromatograph as a single peak on Bio-Gel P-10 and P-6 which is indicative of a single type unit (R. A. Munsinger and B. G. Hudson, unpublished data). Unequivocal evidence for a single unit awaits characterization of purified glycopeptides.

Species variations in the carbohydrate moiety of other glycoproteins is known to occur with respect to number of identical units per polypeptide chain and to units with large differences in composition, as exemplified by thyroglobulin and ribonuclease, respectively. Human thyroglobulin contains a greater number of units composed of mannose and glucosamine than bovine, porcine, and ovine (Spiro and Spiro, 1965). In contrast, porcine ribonuclease contains three different units which differ widely in size and composition and only one is common to the single unit found in the bovine species (Jackson and Hirs, 1970).

Acknowledgments

We thank Dr. Kenneth G. Mann for obtaining the molecular weights by gel filtration and Mrs. Peggy Goodwin for skillful technical assistance.

References

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

Aisen, P., Liebman, A., and Reich, H. A. (1966), *J. Biol. Chem. 241*, 1666.

Baker, E., Shaw, D. C., and Morgan, E. H. (1968), *Biochemistry 7*, 1371.

Bearn, A. G., and Parker, W. C. (1966), *in* The Glycoproteins, Gottschalk, A., Ed., New York, N. Y., Elsevier, p 420.

Benson, A. A., Bassham, J. A., Calvin, M., Hall, A. G., Hirsch, H. E., Kuwaguchi, S., Lynch, V., and Tolbert, N. E. (1952), *J. Biol. Chem. 196*, 703.

Boas, N. F. (1953), *J. Biol. Chem. 204*, 553.

Castellino, F. J., and Barker, R. (1968), *Biochemistry 7*, 2207.

Davis, B., Saltman, P., and Benson, S. (1962), *Biochem. Biophys. Res. Commun. 8*, 56.

Edelhoch, H. (1967), *Biochemistry 6*, 1948.

Feeney, R. E., and Komatsu, S. K. (1966), *in* Structure and Bonding, Jorgensen, C. K., Ed., West Berlin and Heidelberg, Germany, Springer-Verlag, p 149.

Fish, W. W., Mann, K. G., and Tanford, C. (1969), *J. Biol. Chem. 244*, 4898.

Fisher, F. G., and Nebel, H. J. (1955), *Hoppe-Seyler's Z. Physiol. Chem. 302*, 10.

Fletcher, J., and Huehns, E. R. (1968), *Nature (London) 218*, 1211.

Greene, F. C., and Feeney, R. F. (1968), *Biochemistry 7*, 1366.

Hade, E. P. K., and Tanford, C. (1967), *J. Amer. Chem. Soc. 89*, 5034.

Hoare, D. G., and Koshland, D. E., Jr. (1967), *J. Biol. Chem. 242*, 2447.

Jackson, R. L., and Hirs, C. H. W. (1970), *J. Biol. Chem. 245*, 624.

Jamieson, G. A. (1965), *J. Biol. Chem. 240*, 2914.

Jamieson, G. A., Jett, M., and DeBernardo, S. L. (1971), *J. Biol. Chem. 246*, 3686.

Laurell, C. B., and Ingelman, B. (1947), *Acta Chem. Scand. 1*, 770.

Lee, Y. C., McKelvy, J. F., and Lang, D. (1969), *Anal. Biochem. 27*, 567.

Mann, K. G., Fish, W. W., Cox, A. C., and Tanford, C. (1970), *Biochemistry 9*, 1348.

Matsubara, H., and Sasaki, R. M. (1969), *Biochem. Biophys. Res. Commun. 35*, 175.

Palmour, R. M., and Sutton, H. E. (1971), *Biochemistry 10*, 4026.

Panyim, S., and Chalkley, R. (1969), *Arch. Biochem. Biophys. 130*, 337.

Parker, C. W., and Bearn, A. G. (1962), *J. Exptl. Med. 114*, 83

Roe, J. H. (1955), *J. Biol. Chem. 212*, 335.

Schachman, H. K. (1959), Ultracentrifugation in Biochemistry, New York, N. Y., Academic, p 82.

Schram, E., Moore, S., and Bigwood, E. J. (1954), *Biochem. J. 57*, 33.

Spackman, D. H., Stein, W. H., and Moore, S. (1958), *Anal. Chem. 30*, 1190.

Spiro, R. G. (1966), *Methods Enzymol. 8*, 3.

Spiro, R. G., and Spiro, M. J. (1965), *J. Biol. Chem. 240*, 997.

Trevelyan, W. E., Procter, D. P., and Harrison, J. S. (1950), *Nature (London) 166*, 444.

Warner, R. C., and Weber, I. (1953), *J. Amer. Chem. Soc. 75*, 5094.

Warren, L. (1959), *J. Biol. Chem. 234*, 1971.

Williams, J. (1962), *Biochem. J. 83*, 355.

Williams, J. (1968), *Biochem. J. 108*, 57.

Yphantis, D. A. (1964), *Biochemistry 3*, 297.