Vertebrate Transferrins. Molecular Weights, Chemical Compositions, and Iron-Binding Studies*

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ABSTRACT: Transferrins were identified by ^{59}Fe autoradiography in the sera of several vertebrate species. Iron-binding proteins were also found in the hemolymph of three moth species (*Anatheraea polyphemus*, *Hyalophora cecropia*, and *Actias luna*). The transferrins from human plasma, rabbit plasma, frog (*Rana catesbiana*) serum, turtle (*Pseudemys scripta*) serum, and hagfish (*Eptatretus stoutii*) serum were isolated. The molecular weight of each purified transferrin was assessed by equilibrium sedimentation, gel filtration, and sodium dodecyl sulfate electrophoresis. Human, rabbit, and frog transferrins appear to have molecular weights of 76,000; turtle transferrin has a molecular weight of about 92,000, while hagfish transferrin has a molecular weight value of approximately 44,000. Sedimentation of reduced, carboxymethylated transferrins in 8 M urea indicates a possible subunit structure for turtle transferrin; no subunits were detected in any of the other transferrins studied. The amino acid composition of each purified protein was determined. Human and turtle transferrins contain approximately four sialic acid residues per molecule; the other purified transferrins contain an average of two residues per molecule. Hagfish transferrin binds one atom of iron per molecule, whereas the other four transferrins bind two atoms of iron per molecule. The visible absorbance spectra of human and rabbit transferrins show a single peak at 465 nm, while frog, turtle, and hagfish transferrins show a main peak around 410 nm. Turtle, frog, and hagfish transferrins show a secondary absorbance peak at 455–465 nm. Both chemical and physical studies strongly indicate that a partial gene duplication took place early in vertebrate evolution, yielding an elongated protein with some repeating sequences.

The structure of human transferrin (Tf)[1] has been investigated in several laboratories. Two identical carbohydrate chains (Jamieson, 1966) and two iron-binding sites (Surgenor *et al.*, 1949) suggest that a gene duplication may have occurred at some time during the evolution of the molecule (Greene and Feeney, 1968). Early reports gave a molecular weight for human Tf of 88,000–90,000, but more recent analyses of sedimentation and diffusion, sedimentation equilibrium, osmotic pressure, iron content, and gel filtration yield values of 68,000–78,000 for human Tf (Charlwood, 1963; Roberts *et al.*, 1966). Intensive studies of the sedimentation velocities of RCM Tf in 8 M urea and 6 M Gdn·HCl (Greene and Feeney, 1968) and of the sedimentation equilibrium values for RCM Tf in 8 M urea and 6 M Gdn·HCl (Mann *et al.*, 1970) have failed to demonstrate the presence of disulfide-linked subunit chains. However, the possibility of a complete or partial duplication of amino acid sequence within a single polypeptide chain exists. We have surveyed iron-binding proteins in lower vertebrates and invertebrates in the search for forms of transferrin that would correspond to a primordial gene prior to duplication.

Boffa *et al.* (1966) reported an iron-binding protein in lampreys with a sedimentation value of $s_{20,w}$ = 4.8–5.1 S, corresponding to a molecular weight of 70,000–80,000. Manwell (1963) reported iron-binding proteins in both marine lampreys and hagfish but did not determine the molecular weight of hagfish Tf. As lampreys and hagfish are members of the most primitive vertebrate class, Cyclostomata, we examined the serum of the California hagfish, *Eptatretus stoutii*, for iron-binding proteins. The Tf's from the New Zealand white rabbit, the bullfrog (*Rana catesbiana*), and the red-eared slider turtle (*Pseudemys scripta*) were studied for comparative evolutionary purposes.

Materials

Human Tf was purchased from Behringwerke Co. (Certified Blood Donor Service) and further purified by starch block electrophoresis in pH 8.6 barbital buffer (Sutton and Karp, 1965). Frogs and turtles were purchased from Carolina Biological Supply and were bled by heart puncture. Hagfish serum was obtained from Pacific Bio-Marine Co. BSA, ovalbumin, cytochrome *c*, and iodoacetamide were purchased from Sigma Chemical Co., and pepsin and trypsin from Worthington Biochemical Corp. β-Mercaptoethanol was obtained from Eastman Organic Chemicals and $^{59}FeCl_3$ from International Nuclear Corp. Urea (Baker analyzed reagent) was deionized before use.

Methods

Identification of Tf's. Serum samples containing 0.236 μg of ^{59}Fe/ml of serum (a final concentration of 6–7 μCi of ^{59}Fe/ml) were subjected to polyacrylamide gel electrophoresis (0.1 M Tris–0.05 M glycine buffer, pH 8.8) or to horizontal starch gel electrophoresis (0.3 M LiOH, pH 8.2). The gel was sliced longitudinally, one-half was stained with Amido-Schwarz 10B, and autoradiography was performed with the second slice (Giblett *et al.*, 1959).

Purification of Tf's. Rabbit Tf was purified by a combination

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[1] Abbreviations used are: Tf, transferrin; SDS, sodium dodecyl sulfate; RCM, reduced carboxymethylated; BSA, bovine serum albumin; IgG, immunoglobulin G; Gdn·HCl, guanidine hydrochloride.

of rivanol (2-ethoxy-6,9-diaminoacridine lactate) precipitation and starch block electrophoresis (Sutton and Karp, 1965). Frog and turtle Tf's were purified by rivanol precipitation, followed by preparative acrylamide slab electrophoresis (pH 8.9 Tris·HCl buffer) and subsequent electrophoretic elution of the Tf band (Chen, 1968). Alternatively, the rivanol precipitates from frog or turtle serum were chromatographed on Bio-Gel A-5 m agarose columns (2.5 × 70 cm) equilibrated with 0.05 M Tris–0.1 M KCl buffer (pH 7.6). Hagfish serum was fractionated by gel filtration on Bio-Gel A-5 m agarose columns, followed by ion-exchange chromatography on a 1.5 × 30 cm DEAE-Cellex (Bio-Rad) column equilibrated with 0.01 M Tris·HCl (pH 7.8). Proteins were eluted by a linear concentration gradient from 0.01 to 0.45 M Tris·HCl (pH 7.8). All Tf's were saturated with iron (ferric ammonium citrate) prior to purification.

Protein Concentration. The protein concentration of most samples was determined by weighing the dried samples or by reading optical density at 280 nm, assuming $E_{1\,cm}^{1\%} = 13.8$ for Fe-Tf or 11.2 for apoTf (Laurell, 1960). For quantitative iron-binding determinations and sialic acid assays, protein concentration was determined by the method of Lowry *et al.* (1951).

Ultracentrifugal Studies. Sedimentation velocities of human, rabbit, turtle, frog, and hagfish Tf's were determined in the Spinco Model E analytical ultracentrifuge at protein concentrations of 1.0, 0.6, 0.4, and 0.2% in 0.1 M sodium phosphate buffer (pH 7.5). Sedimentation coefficients for all Tf's were calculated from the slopes of plots of log x *vs.* t (Svedberg and Pedersen, 1940).

The molecular weights of the purified Tf's were determined at a protein concentration of 0.15% in 0.1 M sodium phosphate (pH 7.5) buffer by sedimentation equilibrium at 20° and 10,589 rpm. Samples were dialyzed against the solvent for at least 24 hr prior to analysis. The molecular weight was calculated from the slope of plots of ln $j(r)$ *vs.* r^2 (where j, the fringe number, is proportional to c, the concentration) according to the equation $d \ln c/dr = w^2M(1 - \bar{v}\rho)r/2RT$ (Svedberg and Pedersen, 1940). The partial specific volumes ($\bar{v}$) of native Tf's were calculated from the amino acid composition, after the example of Schachman (1967). For human and rabbit Tf's, $\bar{v} = 0.725$; for turtle Tf, $\bar{v} = 0.726$; for frog Tf, $\bar{v} = 0.733$; and for hagfish Tf, $\bar{v} = 0.721$.

Gel Filtration Studies. The molecular weights of purified Tf's were studied by gel filtration on a 1.5 × 60 cm Sephadex G-100 column (Andrews, 1966) using 0.05 M Tris–0.1 M KCl buffer (pH 7.7). The elution volumes for appropriate protein standards were measured and plotted against the log of the molecular weight of each standard; the elution volume of each Tf was then located on the standard curve.

SDS Electrophoresis. Acrylamide gel electrophoresis using SDS was performed according to the technique of Weber and Osborne (1969). Purified proteins (3 mg/ml) were dissolved in 0.01 M sodium phosphate buffer (pH 7.0) containing 1% SDS and 1% β-mercaptoethanol and incubated at 37° for 3 hr. After electrophoresis of 75-μg samples on 10% acrylamide gels, the migration distance of each protein was measured in millimeters and divided by the migration distance of a standard (cytochrome *c*) to yield a relative mobility which was plotted against the log of the molecular weight of the protein being studied. A standard curve for similarly treated proteins of known molecular weights was prepared, and unknown proteins were located on that curve.

RCM Tf's. Purified Tf's were reduced and alkylated following the procedure of Crestfield *et al.* (1963), as modified by Greene and Feeney (1968). Tf in 8 M urea–0.3 M Tris·HCl (pH 8.6) was reduced with a 100-fold molar excess of β-mercaptoethanol (based on the potentially available sulfhydryl groups in each protein) for 16 hr at 50°, then alkylated with a 10% excess of iodoacetamide (based on the amount of β-mercaptoethanol used). These solutions were then dialyzed for 96 hr against several changes of 8 M urea–0.1 M Tris·HCl buffer (pH 8.6) to remove the reagents. BSA and pepsin were prepared in this fashion for use as standards. Sedimentation coefficients for RCM proteins were determined in a synthetic boundary cell, using 8 M urea–0.1 M Tris·HCl buffer in the reference side. Samples were centrifuged at 42,040 rpm and 20° using a protein concentration of 0.2%. The solvent density value for the 8 M urea system was taken as 1.116 g/cm³ (Greene and Feeney, 1968), and the values used for $\bar{v}$ were 0.723 for human and rabbit Tf's, 0.725 for turtle Tf, 0.730 for frog Tf, 0.721 for hagfish Tf, 0.725 for pepsin, and 0.729 for BSA (after Greene and Feeney, 1968).

Amino Acid Analysis. Total amino acid content of each purified Tf was determined on a Spinco Model 120B automatic amino acid analyzer (modified to the sensitivity of a Model 120C), using duplicate samples of 18-, 36-, and 72-hr 6 N HCl hydrolysates of both performic acid oxidized (Hirs, 1956) and untreated Tf's. Tryptophan was determined in triplicate by the thioglycolic acid method of Matsubara and Sasaki (1969).

Sialic Acid Content. Sialic acid was assayed by the thiobarbituric acid method (Warren, 1959). A known standard containing 0.15 μmole of sialic acid/ml was included for calibration.

Absorption spectra in the visible region were determined for 1% solutions of both apoTf's and iron-saturated Tf's using a Cary Model 15 recording spectrophotometer with a 1-cm light path. Samples were dissolved in 0.1 M Tris·HCl (pH 7.5), containing 0.015 M $NaHCO_3$, then dialyzed extensively against several changes of the same buffer to remove any excess iron. An aliquot of the final dialysis fluid was used as a blank. Extinction coefficients were determined at the wavelengths of maximum absorbance.

Quantitative Iron-Binding Analysis. Quantitative iron-binding determinations were carried out on each purified Tf (Schade and Reinhart, 1966; Masson and Heremans, 1968; Spik, 1968). The Tf's were deprived of iron by dialysis against 1.0 M citric acid, followed by extensive dialysis against deionized water. The protein was lyophilized and dissolved in 0.1 M Tris·HCl–0.015 M $NaHCO_3$ (pH 7.5). An aliquot was removed for protein determination, and the absorbance of the apoTf solution was read at 465 nm to provide a reference point. Stepwise 2-μl aliquots of a standard iron solution (0.00179 M ferric ammonium sulfate, 0.00179 M citric acid, 8.5×10^{-5} M ascorbic acid) containing 0.1 μg of Fe/μl were added to 1-ml portions of the apoTf solutions. These preparations were incubated at 25° for 1 hr, then optical densities at wavelengths of 465 and 410 nm were read in a Gilford Model 240 spectrophotometer. When absorbance was plotted as a function of microliters of Fe solution added, a plateau was reached at which further addition of iron caused no significant increase in optical density. The inflection point of the curve was located in terms of microliters of Fe solution added, and this value was converted to micrograms of Fe bound per milligram of protein.

Results

Iron-binding proteins were identified by acrylamide electro-

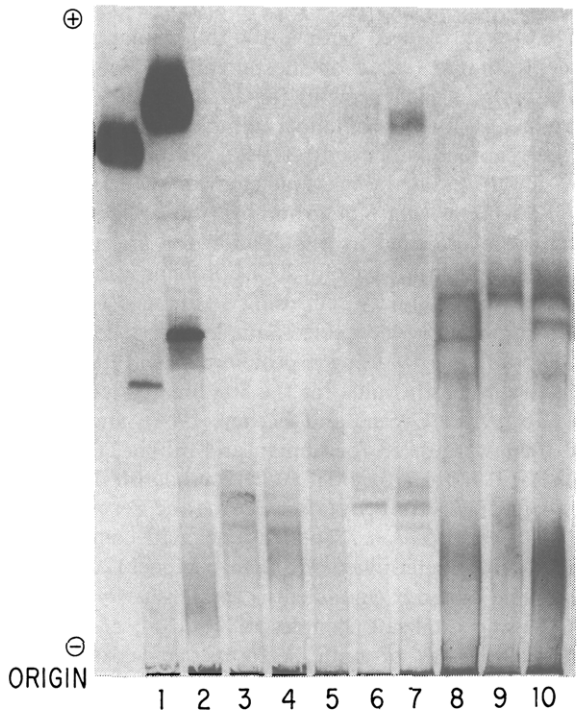

FIGURE 1: Polyacrylamide gel electrophoresis of $^{59}FeCl_3$ labeled human serum (1), rabbit serum (2), turtle sera (3–5), frog sera (6–7), and hagfish sera (8–10).

phoresis in all vertebrate species examined, as seen in Figure 1. The sera or body fluids from several invertebrate animals (*Amphioxus;* three moth species, *Anatherea polyphemus*, *Hyalophora cecropia*, and *Actias luna;* the horseshoe crab, *Limulus; Daphnia*, the water flea; the crab, *Cambarus;* the sea cucumbers, *Cucumaria* and *Malpodia;* the snail, *Helix;* the clam, *Arca;* the limpet, *Megathuria;* the leech, *Haemopis;* two earthworms, *Lumbricus* and *Tubifex;* two marine worms, *Glycera* and *Amphitrite;* a nematode, *Cephalobus;* two sipunculoids, *Dendrostrom* and *Golfingia;* and *Dugesia*, the brown planaria) were screened for the presence of iron-binding proteins. Positive results were obtained from the hemolymph of the moth species only.

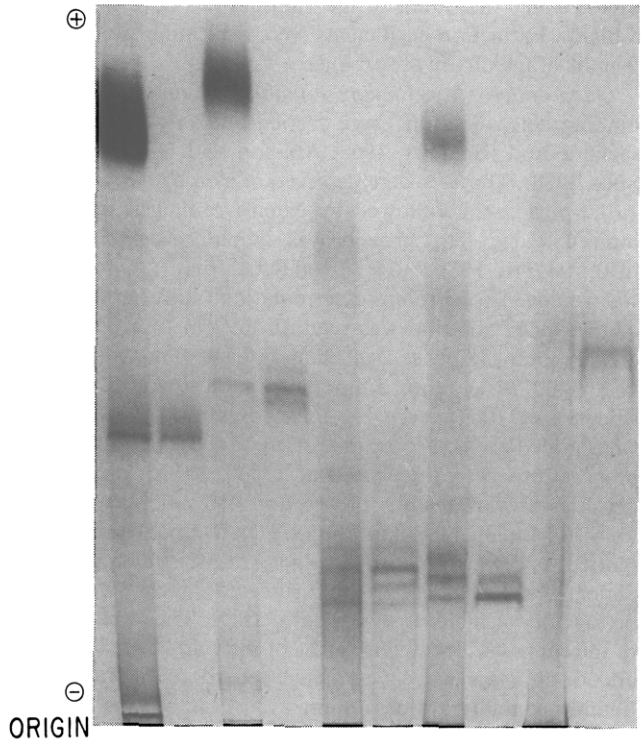

FIGURE 2: Polyacrylamide gel electrophoresis from left to right of human serum, human Tf, rabbit serum, rabbit Tf, turtle serum, turtle Tf, frog serum, frog Tf, hagfish serum, and hagfish Tf.

TABLE I: Comparative Sedimentation Coefficients of Tf's in 0.1 M Na_2HPO_4 (pH 7.5).

Tf	$s^0_{20,w}$ (S)	Comparative s^a
Human	5.32 ± 0.19	1.00000
Rabbit	5.29 ± 0.10	0.99436
Turtle	5.63 ± 0.26	1.05827
Frog	5.16 ± 0.12	0.96992
Hagfish	3.45 ± 0.14	0.64850

[a] Ratio of $s^0_{20,w}$ of each Tf to $s^0_{20,w}$ of human Tf.

Transferrins from human, rabbit, turtle, frog, and hagfish sera were purified for further study. A comparison of the purified Tfs with the serum from which each Tf was derived is shown in Figure 2; the purified Tf's all retained their ability to bind radioactive iron. In addition, each preparation showed a single, symmetrical peak during analytical ultracentrifugation and gel filtration. The human Tf preparations contain less than 5% hemopexin, as estimated from immunoelectrophoresis and immunodiffusion against antihemopexin.

Each purified Tf was examined by several different physical methods in order to determine its molecular weight. The sedimentation coefficient of each Tf was determined at three different protein concentrations and then extrapolated to standard conditions and infinite dilution by the usual methods (Table I). Molecular weight values were determined by sedimentation equilibrium studies. Values for human and rabbit Tf's were determined in duplicate; three independently isolated samples of each of the other Tf's (turtle, frog, and hagfish) were analyzed. An average of the values obtained for each protein yields the following molecular weights: human Tf, 76,200; rabbit Tf, 74,500; turtle Tf, 93,790; frog Tf, 79,370; and hagfish Tf, 44,340. Gel filtration properties of each protein were examined as a further indication of molecular size (Figure 3). This approach indicated molecular weights of 78,000, 76,000, and 72,000, respectively, for human, rabbit, and frog Tf's. Turtle Tf was located at a molecular weight of 96,000, while hagfish Tf appeared to have a molecular weight of 45,500. Finally, electrophoretic migration after treatment with SDS and a reducing agent was studied. Table II summarizes the migration distances of a series of molecular weight standards and of each Tf being studied, while Figure 4 relates the relative mobility to the log of the molecular weight. By this method, human and frog Tf's showed molecular weights of 81,000, rabbit Tf yielded a value of 79,500, and turtle Tf had a molecular weight of 85,000. The main protein band for hagfish Tf indicated a molecular weight of 41,500; however, a faint band corresponding to a molecular weight of 85,000 was observed. The molecular weight values determined by the foregoing methods are summarized for each Tf in Table III. For pur-

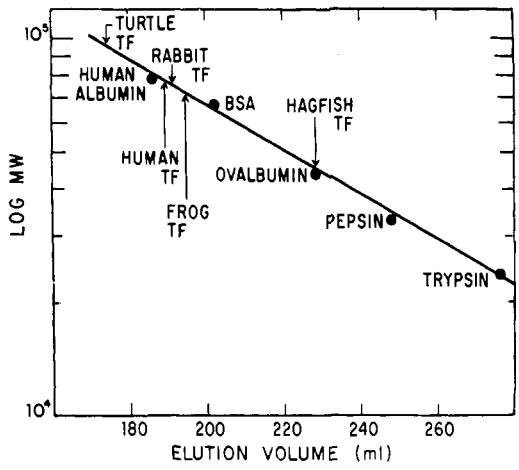

FIGURE 3: Gel filtration analysis of vertebrate Tf's on Sephadex G-100, pH 7.7, Tris-KCl buffer, with the log of the molecular weight of selected standards plotted against their elution volumes.

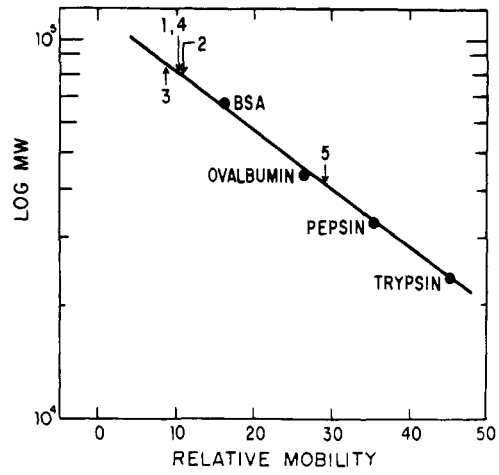

FIGURE 4: The relative electrophoretic mobilities of SDS-treated human Tf (1), rabbit Tf (2), turtle Tf (3), frog Tf (4), and hagfish Tf (5), located on a curve standardized with SDS-treated reference proteins.

poses of calculations, the molecular weight of human, rabbit, and frog Tf's was taken as 76,000; turtle Tf was considered to have a molecular weight of 92,000; while the molecular weight of hagfish Tf was estimated as 44,000.

Each Tf was examined for the presence of disulfide-linked subunit chains. Following the approach of Greene and Feeney (1968), the sedimentation velocity of each RCM Tf was determined in 8 M urea and compared to the sedimentation velocity of RCM BSA and RCM pepsin (Table IV). Amino acid analysis of an aliquot of each RCM protein indicated that conversion of cystine to carboxymethylcysteine was complete. Assuming that RCM proteins assume a random coil form (Tanford *et al.*, 1967), their sedimentation behavior should be solely a function of chain length or particle weight. The sedimentation coefficients of RCM human, rabbit, and frog Tf's are all higher than the sedimentation coefficient for RCM BSA, with a known chain weight of 67,800. The sedimentation coefficient for RCM hagfish Tf is lower than that for RCM BSA, but higher than that of RCM pepsin, with a particle weight of 33,000. RCM turtle Tf has two components, one of which sediments faster than RCM BSA, while the second component sediments slightly slower than RCM BSA.

Tanford *et al.* (1967), in a study of the sedimentation properties of a variety of RCM proteins, found that for proteins the relationship of s_{obsd} to chain length is $\log s/(1 - \bar{v}\rho) \propto 0.5 \log M$. Thus a plot of $\log s/(1 - \bar{v}\rho)$ *vs.* $\log M$ should yield a line with a slope of approximately 0.5. Figure 5 graphically demonstrates that human, rabbit, frog, and hagfish Tf's are single polypeptide chains; turtle Tf may be composed of two polypeptide chains.

Total amino acid content was determined for each isolated Tf after 18-, 36-, and 72-hr hydrolysis. Very few differences were observed in the amino acid yields for different hydrolysis periods. However, some valylvaline, identified by elution on a Beckman automatic amino acid analyzer (J. R. Brown, personal communication), remained in the hydrolysates of hagfish Tf even after 72 hr. Duplicate samples of this protein were hydrolyzed for 102 hr to assure complete cleavage of valine residues; the valine value for these hydrolysates was used in computing molar ratios of amino acids for hagfish Tf. Amino acid content in grams of protein is given for each Tf in Table V. All values are the average of at least three determinations and are corrected to include tryptophan as determined following thioglycolic acid hydrolysis and half-cystine as determined after performic acid oxidation. By analogy to human Tf, 5.8% carbohydrate was allowed for

TABLE II: Electrophoretic Migration of Proteins on SDS Acrylamide Gels.

	Absolute Migration (mm)	Rel Migration (mm)	Mol Wt
Reference Proteins			
IgG half-molecule	8	6.45	90,000
BSA	20	16.12	67,000
Ovalbumin	33	26.60	44,000
Pepsin	44	35.46	33,000
Trypsin	56	45.15	23,500
Purified Tfs[a]			
Human	12.5	10.08	81,000
Rabbit	13.0	10.48	79,000
Turtle	11.0	8.87	85,000
Frog	12.5	10.08	81,000
Hagfish	36.0	29.02	41,500

[a] Molecular weight as determined by this technique.

TABLE III: Molecular Weight Values for Tf's.

	Method of Determination		
Tf's	Sedimentation Equil	Gel Filtration	Migration on SDS Gels
Human	76,200	78,000	81,000
Rabbit	74,590	76,000	79,500
Turtle	93,700	96,000	85,000
Frog	79,370	72,000	81,000
Hagfish	44,340	45,500	41,500

TABLE IV: Sedimentation Behavior of RCM Proteins in 8 M Urea.

RCM Proteins	Particle Wt[a]	$s_{20,obsd}$	$\bar{v}^b$
Human Tf	77,900	0.75	0.723
Rabbit Tf	77,900	0.76	0.723
Turtle Tf	92,900[c]	0.83	0.725
	46,450[d]	0.59	0.725
Frog Tf	77,300	0.73	0.731
Hagfish Tf	44,400	0.53	0.719
BSA	67,800	0.68	0.729
Pepsin	33,000	0.45	0.725

[a] Particle weight of RCM proteins using molecular weights calculated from this study. [b] Calculated from amino acid composition and corrected for CM-cysteine content, after Greene and Feeney (1968). [c] Particle weight for a single-chain structure. [d] Particle weight for a double-chain structure.

each Tf; this allowance was taken into account when calculating the number of residues of each amino acid per molecule of protein.

In addition, amino acid analysis indicated the presence of glucosamine in all samples studied; galactosamine peaks were seen for turtle and hagfish Tf's only. Both amino sugars elute from the basic column immediately following the neutral and acidic amino acid peak. Quantitative assay for the amino sugars was not performed.

The sialic acid content of each protein was determined; the results are summarized in Table VI. Based on determined molecular weights, human and turtle Tf's contain an average of four residues of sialic acid per molecule, while rabbit, frog, and hagfish Tf's each contain an average of two residues per molecule. The sialic acid content of turtle Tf may well account for the multiple bands seen on acrylamide electrophoresis, in a fashion analogous to that described by Chen and Sutton (1967) for bovine Tf.

The visible absorption curve for each of the Fe–Tf complexes is shown in Figure 6. Both human and rabbit Tf's show

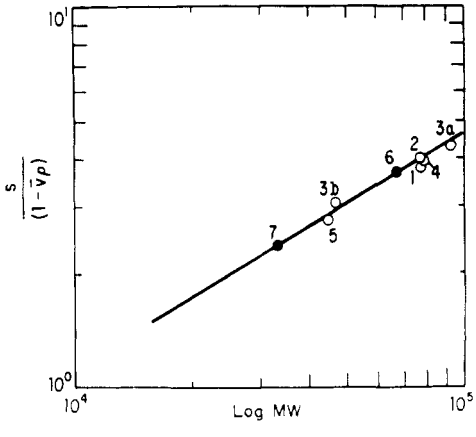

FIGURE 5: The relationship between sedimentation behavior of RCM proteins in 8 M urea and chain length or particle weight. The calculated values for $s/(1 - \bar{v}\rho)$ are plotted against particle weights of RCM human Tf (1), RCM rabbit Tf (2), RCM turtle Tf (3), RCM frog Tf (4), RCM hagfish Tf (5), RCM BSA (6), and RCM pepsin (7) on a log–log scale.

TABLE V: Amino Acid Content of Tfs (g of AA/100 g of Protein).

Amino Acid	Human[a]	Rabbit	Turtle	Frog	Hagfish
Lysine	9.60	8.95	8.40	12.12	7.37
Histidine	3.11	3.10	2.34	2.80	3.48
Arginine	5.29	5.16	4.82	4.15	4.62
Aspartic acid	11.11	11.07	10.10	10.23	10.57
Threonine	3.92	2.81	5.93	4.89	5.52
Serine	3.99	4.03	6.34	4.47	5.30
Glutamic acid	10.24	10.68	11.46	11.11	11.22
Proline	4.30	4.32	3.61	4.68	3.97
Glycine	3.65	3.54	4.13	3.70	3.75
Alanine	5.19	5.06	4.71	5.16	5.09
Half-cystine	4.31	4.40	1.95	3.30	2.61
Valine	5.30	5.73	6.16	4.85	4.85
Methionine	1.25	1.09	1.15	1.30	2.52
Isoleucine	2.08	2.34	3.81	3.41	4.51
Leucine	8.51	9.03	7.61	7.49	7.87
Tyrosine	5.28	5.19	4.30	4.40	3.25
Phenylalanine	5.26	4.96	4.99	5.23	5.42
Tryptophan	1.81	2.23	1.91	1.10	2.28
Total[b]	94.20	93.66	94.20	94.29	94.20

[a] Wang (1966). [b] Calculations are corrected to include 5.8% carbohydrate, by analogy to human Tf.

maximum absorbance at 465 nm. Although all three other vertebrate Tf's have a shoulder on the absorption curve at 465 nm, maximum absorbance is seen at 405 nm for turtle Tf, 407 nm for frog Tf, and 415 nm for hagfish Tf. Extinction coefficients for all Tf's were calculated at wavelengths of 465, 410, and 280 nm; they are summarized in Table VII. Although this region corresponds to that of heme absorption, we have ruled out contamination by a non-Tf component. The absorbance peak at 410 nm disappears when the Tf is deprived of bound iron and returns when the iron content is restored stoichiometrically to that same sample.

The iron-binding capacity of each Tf was quantitatively determined; the values for each experiment were read at wavelengths of 465 and 410 nm. Each experiment was continued until several successive tubes showed no change in optical density. The increase in optical density was plotted

TABLE VI: Sialic Acid Assay of Tf's.

Tf	Protein Concn (mg/ml)[a]	Sialic Acid (μmole/ml)	Sialic Acid (μmole/mg)	Av No. of Residues/Molecule[b]
Human	3.00	0.1503	0.0501	3.81 (76,000)
Rabbit	3.00	0.0822	0.0274	2.08 (76,000)
Turtle	3.08	0.1445	0.0469	4.31 (92,000)
Frog	2.96	0.0687	0.0232	1.76 (76,000)
Hagfish	2.52	0.1230	0.0488	2.15 (44,000)

[a] Protein concentration determined by the Folin reaction. [b] Based on the molecule weight in parentheses.

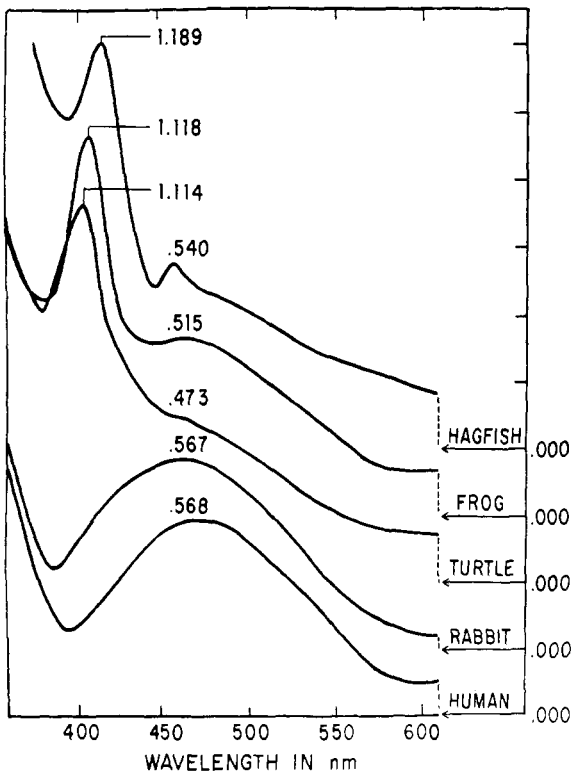

FIGURE 6: Spectral absorbance curves for 1% solutions of purified Tf's, with zero points and extinction coefficients for each curve.

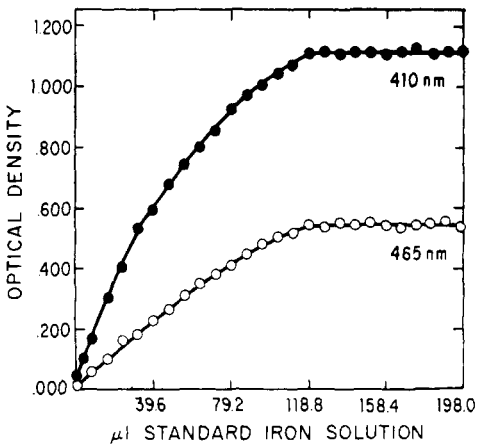

FIGURE 7: Quantitative determination of the iron-binding capacity of hagfish Tf, with the protein concentration corrected to a 1% solution. The optical density at 410 and 465 nm was plotted as a function of the amount of a standard iron solution required to achieve the absorbance indicated.

against the increments of iron solution for each protein (Figure 7). The iron-binding capacity of each protein was calculated in micrograms per milliliter and in atoms per molecule based on the determined molecular weights; these data are compared in Table VIII. Hagfish Tf bound 1.19 μg of Fe/mg of protein, corresponding to one atom per molecule, while the other Tf's being studied bound from 1.23 to 1.43 μg of Fe per mg of protein (two atoms of Fe per molecule).

Discussion

This study examines a series of animal proteins which show the physiologically important capacity for binding iron. Many electrophoretic variants of human Tf have been reported in the literature (Sutton and Jamieson, 1971), and two electrophoretic forms of rabbit Tf have been seen (Jordan *et al.*, 1967). The turtle sera examined in this study each contained multiple iron-binding bands; the variability between samples may be due to subspecies differences. Two electrophoretic variants of frog Tf were found, and several variants of hagfish Tf were observed. An examination of the primary amino acid sequence of each molecule, together with the determination of the functional ability to donate iron to reticulocytes, would be necessary before absolute homology could be established.

The fact that iron-binding proteins are present in moth hemolymph argues that precursors to vertebrate Tf may have existed in some other invertebrate animals. Other arthropods were examined, but iron-binding proteins were not found in the species studied. An analogy between moth iron-binding proteins and vertebrate Tf's may be expected on the basis of their iron-binding capacities; until sequence studies are completed, however, no homology can be assumed.

For many years, the structure of human Tf has been an enigma to investigators; the circumstantial evidences for a gene duplication (two iron-binding sites, two identical carbohydrate side chains) are quite strong, but the most diligent methods have failed to identify any subunit structure. The initial purpose of this study was to locate either a primordial low molecular weight Tf or to locate a Tf which had maintained its subunit structure. From the available evidence, hagfish Tf, with a molecular weight of 44,000 and a binding capacity of one atom of Fe per molecule, fulfills the immediate requirements. Although a comparison of the amino acid content of human and hagfish Tf's clearly indicates that human

TABLE VII: Extinction Coefficients of Iron-Saturated Tf's in 0.1 M Tris–0.015 M $NaHCO_3$ (pH 7.8).

Tf	$E^{1\%}_{1\,cm}$ 280 nm	$E^{1\%}_{1\,cm}$ 410 nm	$E^{1\%}_{1\,cm}$ 465 nm
Human	14.06	0.315	0.568
Rabbit	13.74	0.308	0.567
Turtle	13.33	1.012	0.473
Frog	11.91	1.082	0.515
Hagfish	10.78	1.166	0.499

TABLE VIII: Iron-Binding Capacities of Tf's.

Tf	Protein Content[a] (mg/ml)	μl of Fe Soln Added	μg of Fe Bound/ mg of Protein	Atoms of Fe Bound/Molecule[b]
Human	3.00	42	1.40	1.91 (76,000)
Rabbit	3.00	43	1.43	1.95 (76,000)
Turtle	3.08	38	1.23	2.04 (92,000)
Frog	2.96	40	1.35	1.85 (76,000)
Hagfish	2.52	30	1.19	0.94 (44,000)

[a] Protein concentration determined by the Folin method. [b] Based on the molecular weights indicated in the parentheses.

Tf is not an exact duplicate of hagfish Tf, the theory of a partial gene duplication, followed by multiple point mutations in both human and hagfish Tf's, is plausible.

Furthermore, the data accumulated by Boffa's laboratory on lamprey Tf's indicate that the hagfish may be the only vertebrate now living which has a Tf of low molecular weight. If the hagfish is a degenerate, rather than a primitive, organism, its Tf may be the result of massive gene deletions rather than the precursor for subsequent gene duplication. In most well-studied genetic systems, massive gene deletions tend to be lethal, whereas gene duplication can be advantageous. In addition, preliminary reports (Ohno *et al.*, 1968) indicate that lamprey erythrocytes contain twice as many chromosomes as hagfish erythrocytes do, suggesting that the whole genome may have duplicated early in vertebrate evolution.

The subunit structures of the Tf's from *Pseudemys scripta* and several fish species are under investigation in this laboratory. A recent study suggests that chicken ovoTf may have maintained a duplicate structure, as Phillips and Azari (1971) have found that 2 moles of each of the cyanogen bromide fragments are recovered from each mole of ovoTf. However, Elleman and Williams (1970) find no evidence of duplicate structure in their sequences of 34 unique cysteic acid containing peptides from chicken ovoTf.

In order to establish unequivocally that a gene duplication is responsible for the differences in molecular size and chemical composition of the Tf's studied here, sequence data on the primary amino acid structure of the proteins will be required. Sequence studies on human Tf are well under way at the present time, while sequencing of the hagfish molecule has been initiated.

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