

## Some Physical-Chemical Properties of Reduced-Alkylated and Sulphitolysed Human Serum Transferrins and Hen's-Egg Conalbumin

BY ANATOLY BEZKOROVAINY, DIETMAR GROHLICH AND CLAIRE M. GERBECK  
*Department of Biochemistry, Presbyterian-St Luke's Hospital, and Department of Biological Chemistry, University of Illinois College of Medicine, Chicago, Ill. 60612, U.S.A.*

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1. Apparently all disulphide bridges of transferrin and conalbumin were broken by reduction-alkylation, whereas sulphitolysis resulted in incomplete cleavage of disulphide bonds. 2. The molecular weights of reduced-alkylated and sulphitolysed transferrin and reduced-alkylated conalbumin were identical with those of native proteins in a number of solvents, indicating that these proteins exist as single polypeptide chains. 3. Viscosity studies indicated that reduced-alkylated transferrin possesses a partially ordered structure in 0-4M-urea, assumes a random-coil configuration in 6M-urea with a molecular weight of 84000 and is partially aggregated in 8M-urea.

Human serum transferrin and hen's-egg conalbumin have been extensively characterized in recent years (Feeney & Komatsu, 1966). Of special interest are the physical-chemical properties of denatured forms of these proteins because of the possibility that transferrin and conalbumin may exist as aggregates of two identical sub-units. This supposition is based on the fact that transferrin and conalbumin each possess two apparently identical iron-binding sites (Aasa, Malmström, Saltman & Vånngard, 1963), in the case of transferrin two oligosaccharide chains (Jamieson, 1965), and have relatively high molecular weights of 80000-90000 (Bain & Deutsch, 1948; Schultze, Heide & Müller, 1957).

There is ample evidence to suggest that a dissociation of such possible sub-units cannot be effected by urea and guanidine hydrochloride, reagents used to bring about a dissociation of non-covalent interactions in proteins (Bezkorovainy & Grohlich, 1967). Moreover, investigations by Bron, Blanc & Isliker (1968) suggest that transferrin may form aggregates in the presence of 8M-urea. However, a dissociation of transferrin into sub-units was apparently accomplished by Jeppsson (1967) by using the reduction-alkylation reaction (Crestfield, Moore & Stein, 1963) in the presence of 8M-urea. The sub-units were reported to have a molecular weight of 39000-42000 by the equilibrium-centrifugation method. The transferrin samples used by Jeppsson (1967) produced 34-38 peptides when subjected to tryptic digestion,

half the number expected. This finding, however, is inconsistent with that of Roop & Putnam (1967), who noted some 70 peptides in their tryptic digests of transferrin.

Two other groups have subjected transferrin to the reduction-alkylation procedure. Thus Greene & Feeney (1968) compared the sedimentation properties of reduced-alkylated transferrin with similarly treated single-chain proteins whose molecular weights were close to that of native transferrin (albumin, mol.wt. 67800) and to that of the possible transferrin sub-unit (pepsin, mol.wt. 32700). Reduced-alkylated transferrin behaved like reduced-alkylated serum albumin rather than like pepsin. Reduction-alkylation experiments performed on transferrin in our Laboratory (Bezkorovainy & Grohlich, 1967) also led to the conclusion that transferrin exists as a single polypeptide chain. However, our work may be criticized on the basis of the fact that the reduction experiments were done in 2M- and 6M-urea rather than the 8M-urea used by other authors.

The present paper is an extension of our previous work (Bezkorovainy & Grohlich, 1967) on the properties of denatured human serum transferrin and hen's-egg conalbumin with special emphasis on their possible quaternary structures. The denaturation was brought about by reduction-alkylation with mercaptoethanol in the presence of 8M-urea by the method of Jeppsson (1967) and by sulphitolysis in 8M-urea by the method of Henschen (1964).

## MATERIALS AND METHODS

**Transferrin and conalbumin.** Transferrin prepared from pooled human plasma by the Behringwerke Corp. was purchased from Hoechst Laboratories (Woodbury, N.Y., U.S.A.). Its purity had been earlier ascertained to be high (Bezkorovainy & Grohlich, 1967). Crystallized conalbumin was purchased from Nutritional Biochemicals Corp. (Cleveland, Ohio, U.S.A.). The preparation was, however, inhomogeneous by the zone-electrophoresis method, and was purified by chromatography on DEAE-cellulose at pH 8.3 followed by gel filtration on Sephadex G-200, as described for transferrin preparation by Line, Grohlich & Bezkorovainy (1967). The final product was electro-pheretically and ultracentrifugally homogeneous.

Reduced-alkylated transferrin and conalbumin were prepared by the method of Jeppsson (1967). Dialysis of the modified transferrin against water had to be continued for 5 days before precipitation occurred; modified conalbumin was precipitated after 2 days of dialysis. Sulphitolysis of transferrin was done by the method of Henschen (1964). Dialysis of the reaction product against water did not result in precipitation and the protein had to be recovered by freeze-drying the dialysis residue. All preparations contained some 10% of moisture.

**Other reagents.** All ion-exchangers were purchased from BioRad Laboratories (Richmond, Calif., U.S.A.) and gel-filtration media came from Pharmacia Corp. (New York, N.Y., U.S.A.). Iodoacetic acid was purchased from K & K Laboratories (Plainview, N.Y., U.S.A.). The slight yellow colour sometimes observed in the iodoacetic acid solutions was removed by extraction with  $\text{CCl}_4$ . Deuterium oxide (99.8%  $\text{D}_2\text{O}$ ) was obtained from ICN Laboratories (City of Industry, Calif., U.S.A.). All other chemicals were purchased from Fisher Laboratories (Chicago, Ill., U.S.A.) and were of reagent-grade quality.

**Solvents.** All urea-containing solvents were prepared with 0.1M- $\text{NaHCO}_3$ , the final pH being 8.3 for 2-8M-urea solutions. For  $\text{D}_2\text{O}$  solutions, 0.1M- $\text{NaHCO}_3$  solutions in  $\text{H}_2\text{O}$  and  $\text{D}_2\text{O}$  were premixed in the required ratios (0%, 33%, 66% and 100%  $\text{D}_2\text{O}$ ) and added to the appropriate amount of urea in a volumetric flask. Final urea concentrations were measured in the Technicon AutoAnalyzer programmed for blood urea determinations (Skeggs, 1957). Relative viscosities of urea solutions were measured in a no. 150 Ostwald viscometer at 25.0°. Densities of urea solutions were measured at 25.0° in 10 ml. or 20 ml. pycnometers precalibrated with boiled water. The viscosity values were in general agreement with those of Kawahara & Tanford (1965), and densities were close to those expected for formulae presented by Gucker, Gage & Moser (1938). The physical parameters of solvents used in this work are presented in Table 1.

**Amino acid analyses.** Amino acid analyses were done by the method of Moore, Spackman & Stein (1958) with a Spinco model 120A instrument, with the accelerated system. Protein hydrolysates were prepared by heating 5 mg. of the protein in 3 ml. of constant-boiling HCl (redistilled from a  $\text{FeSO}_4$  solution) for 24 hr. at 110° in a sealed tube thoroughly flushed with  $\text{N}_2$ . The HCl was then removed by repeated freeze-drying.

**Diffusion studies.** A Spinco model H apparatus was used for the diffusion studies, which were performed at 4° in the 2 ml. micro-cells. The protein samples were dialysed

Table 1. *Relative viscosities and densities (25°) of some solvents used in the physical studies of transferrins and conalbumin.*

All urea solutions contained 0.1M- $\text{NaHCO}_3$ .

Concn. of urea (M)	Relative viscosity	Density
0*	1.0160	1.0072
2	1.0991	1.0287
4	1.2170	1.0589
4 ( $\text{D}_2\text{O}$ - $\text{H}_2\text{O}$ , 1:2, v/v)	1.2856	1.0865
4 ( $\text{D}_2\text{O}$ - $\text{H}_2\text{O}$ , 2:1, v/v)	1.3700	1.1237
4 ( $\text{D}_2\text{O}$ )	1.4390	1.1534
6	1.3893	1.0884
8	1.6798	1.1207

\* 0.2M-KCl adjusted to pH 11.5.

against the appropriate solvents for 24 hr. before charging the cells. The diffusion coefficient was evaluated from the Rayleigh interference-fringe pattern by using a Kodak Contour projector. The method of Svensson (1951) was used for the calculations. Technical difficulties with the Rayleigh interference system did not permit the evaluation of diffusion coefficients obtained in 6M- and 8M-urea solutions. Several protein concentrations were examined for each  $D_{90,w}$  determination; however, no significant dependence of the measured diffusion coefficient on protein concentration was noted.

**Viscosity studies.** Viscometry was performed at 25.0° in a no. 150 Ostwald viscometer. Protein solutions were kept standing for at least 1 hr. before the determinations were started. A minimum of six readings were taken for each protein concentration, and all such readings were within 0.2 sec. of each other. At least five concentrations were examined for each protein sample. Intrinsic viscosities were calculated by plotting reduced viscosities against the corresponding protein concentrations and extrapolating to zero concentration by the method of least squares. The slopes of all such curves were positive and linear.

**Ultracentrifugation.** All sedimentation-velocity runs were made in the Spinco model E instrument at 59 780 rev./min., with the regular and the positive-wedge window cells. Temperature was maintained at 20° by the RTIC unit. Occasionally analyses were performed with the bucket-valve-type synthetic-boundary cell.

At the present time there is apparently no universally acceptable way to correct ultracentrifugal parameters to standard conditions if the determinations are done in the presence of a third component such as urea, guanidine hydrochloride or sucrose (e.g., Noelken & Timasheff, 1967). Various methods have been invoked with some success by different authors (e.g., Greene & Feeney, 1968; Ullmann, Goldberg, Perrin & Monod, 1968) to circumvent the problem. It has been our experience (Bezkorovainy & Grohlich, 1967) and the experience of others (Gagen & Holme, 1964; Gagen, 1966) that under such circumstances the method of Schachman & Lauffer (1950) works reasonably well in correcting sedimentation coefficients to standard conditions. This involves the determination of the effective partial specific volume ( $\bar{v}_e$  value) by varying the densities of protein solutions and subjecting them to ultracentrifugal

analyses. Apparent sedimentation coefficients ( $S_{\eta}$  values, defined as the product of the uncorrected  $S$  value and the relative viscosity of the solution) are plotted against solution densities, the curve is extrapolated to zero sedimentation rate, and the reciprocal of the  $x$  intercept is then taken as the  $\bar{v}_e$  value. This parameter is then used in the factor  $(1 - \rho_0\bar{v}_e)/(1 - \rho_1\bar{v}_e)$  to correct  $S_{\eta}$  values to standard conditions.  $D_2O$  is recommended as the density-varying component (Schachman & Edelstein, 1966); however, urea (Gagen & Holme, 1964) and sucrose (Schachman & Lauffer, 1950) have been used for the purpose. The sedimentation-velocity results reported in this paper have been treated by the method of Schachman & Lauffer (1950); however, for reasons discussed below, we were obliged to use urea as the density-varying agent.

**Molecular weights.** Molecular weights were calculated from the  $S_{20,w}^0$  and  $D_{20,w}^0$  values by the Svedberg equation. The partial specific volume of the anhydrous protein (0.723; as in Bezkorovainy, Rafelson & Likhite, 1963) was used in this equation for both the native transferrin and the reduced-alkylated transferrin. For native conalbumin the  $\bar{v}$  value 0.721 was used (calculated from Williams, 1962).

$\beta$ -values were calculated by the method of Scheraga & Mandelkern (1953). In some instances molecular weights were determined from viscosity data as described by Tanford, Kawahara & Lapanje (1966). This, of course, involves the assumption that the protein exists in the random-coil configuration. For the purpose of such calculations it was assumed that the reduced-alkylated transferrin has a molecular weight of 82 000 (Table 3) with a mean residue weight of 113 (calculated from Table 2 and carbohydrate analysis by Bezkorovainy *et al.* 1963).

## RESULTS

**Amino acid analysis.** Table 2 lists the amino acid composition of reduced-alkylated transferrin and conalbumin and of sulphitolyzed transferrin. The reduced-alkylated proteins had approx. 1 half-cystine residue/mol. of protein, whereas the sulphitolyzed material had apparently retained significant amounts of its original half-cystine after the modification reaction.

**Sedimentation of transferrins and conalbumin.** Reduced-alkylated transferrin was partially soluble in 0.1M-tris-hydrochloric acid and 0.1M-sodium barbital buffers at pH 8.6. However, ultracentrifugal analysis showed a considerable degree of aggregation of the protein in these solvents. Single symmetrical boundaries were observed for reduced-alkylated transferrin in 0.2M-potassium chloride at pH 11.5 and in 0.1M-glycine-sodium hydroxide buffer at pH 8.3. Likewise, single symmetrical boundaries were observed with sulphitolyzed transferrin and reduced-alkylated conalbumin in 2-8M-urea at pH 8.3. No heterogeneity of the reduced-alkylated transferrin preparations was observed when the ultracentrifugation was done in 4M-urea in the synthetic-boundary cell.

Apparent sedimentation coefficients ( $S_{\eta}$  values) were plotted against concentrations (in mg./ml.) and extrapolated to zero concentration to give the

Table 2. Amino acid composition of modified and native transferrins and conalbumin

A molecular weight of 82 000 was assumed for the transferrins and 76 600 for conalbumin (Warner & Weber, 1951). The results of single analyses are given in all cases.

Amino acid	Amino acid composition (residues/mol.)				
	Native transferrin	Reduced-alkylated transferrin	Sulphitolyzed transferrin	Native conalbumin	Reduced-alkylated conalbumin
Lys	66	64	63	63	62
His	18	20	20	11	13
Arg	29	27	28	32	34
Asp	84	86	85	76	77
Thr	30	31	29	36	35
Ser	40	41	39	44	44
Pro	32	30	29	29	25
Glu	63	66	64	66	69
Gly	52	53	53	51	51
Ala	59	61	60	53	52
Val	39	41	42	44	47
CyS	36	1	16	28	None
Met	9	8	9	10	9
Ile	15	15	15	23	23
Leu	64	63	64	48	48
Tyr	26	27	25	20	21
Phe	30	30	30	26	26
Trp*	8				

\* From Roop & Putnam (1966).

Table 3. Sedimentation coefficients and diffusion coefficients of modified transferrins and conalbumin

Concn. of urea (M)	$10^7 \times D_{20,w}^0$ (cm. <sup>2</sup> sec. <sup>-1</sup> )	$S\eta$ values (s) at the following protein concentrations (in mg./ml.)							$S_{20,w}^0$ (s)	$-\frac{dS\eta}{dc}$	$10^{-3} \times$ Mol. wt.	$\frac{S\eta_{20}^0 (s)}{10^7 \times D_{20,w}^0}$	
		10	7.5	6	5	3.7	2.5	2					0*
Native transferrin in 0.1 M-cacodylate-0.1 M-NaCl, pH 7.0													
0	5.75	4.72	4.86	—	5.04	5.10	5.16	—	5.35	5.35	0.0639	82	0.94
Reduced-alkylated transferrin													
0†	3.15	—	1.85	1.94	2.07	2.38	2.48	—	2.81	2.92	0.136	82	0.89
2	—	1.76	1.82	—	—	2.20	2.36	—	2.52	2.88	0.0822	—	—
4	3.18	1.39	1.53	—	1.75	1.86	—	2.01	2.15	2.87	0.0786	80	0.68
6	—	—	1.20	—	1.41	1.53	1.58	—	1.80	2.87	0.0786	—	—
8	—	0.90	0.90	—	1.04	1.23	1.37	1.45	1.53	3.09	0.0732	—	—
Sulphitolysed transferrin													
4	3.73	1.80	1.87	—	2.06	2.22	—	—	2.43	—	0.0677	—	0.66
6	—	1.43	—	—	—	—	—	—	—	—	—	—	—
8	—	1.09	—	—	—	—	—	—	—	—	—	—	—
Native conalbumin in 0.1 M-cacodylate-0.1 M-NaCl, pH 7													
0	5.72	5.05	5.20	—	5.24	5.35	—	—	5.49	5.49	0.0428	84	0.96
Reduced-alkylated conalbumin													
4	3.31	1.32	1.45	1.50	1.67	1.74	—	—	2.01	—	0.0733	—	0.61
6	—	—	—	—	1.35	—	—	—	—	—	—	—	—
8	—	—	—	—	0.99	—	—	—	—	—	—	—	—

\*  $S\eta_{20}^0$  value.

† In 0.2 M-KCl, pH 11.5.

$S\eta_{20}^0$  values shown in Table 3. The slopes of these curves ( $dS\eta/dc$ ; Table 3) were very similar for reduced-alkylated transferrin and conalbumin in solutions containing urea.

The  $\bar{v}_e$  value of reduced-alkylated transferrin was obtained by plotting the  $S\eta_{20}^0$  values (Table 3) against their solvent densities (Table 1), and the line obtained with the 0–6M-urea solutions was extrapolated to  $S\eta_{20}^0 = 0$ . The  $S\eta_{20}^0$  value obtained in 8M-urea was omitted because of the possible aggregation phenomenon (see below). The  $x$  intercept gave a value of 1.2401 g./ml., and the reciprocal of this, the  $\bar{v}_e$  value, was 0.806 ml./g. The  $S\eta_{20}^0$  values were then corrected for the factor  $(1 - \rho_0\bar{v}_e)/(1 - \rho_1\bar{v}_e)$  to give the  $S_{20,w}^0$  values shown in Table 3. All  $S_{20,w}^0$  values for reduced-alkylated transferrin, except that obtained in 8M-urea, are practically identical.

Molecular weights calculated by the sedimentation-diffusion method are shown in Table 3. Calculations were made only in those cases where diffusion coefficients (Table 3) were available. It appears that the molecular weights of reduced-alkylated transferrin in 4M-urea and in 0.2M-potassium chloride at pH 11.5 are in the 80000–82000 range, identical with that of the native protein. Although molecular weights for reduced-alkylated transferrin in 6–8M-urea could not be unambiguously calculated from the information

presented, the  $S_{20,w}^0$  values suggest that the molecular weights are identical in 4M- and 6M-urea, whereas in 8M-urea the molecular weight appears to be somewhat higher. Not enough information was available to calculate the  $\bar{v}_e$  values for the reduced-alkylated conalbumin and sulphitolysed transferrin. Although the respective  $S\eta_{20}^0$  and  $D_{20,w}^0$  values of sulphitolysed and reduced-alkylated transferrins were different, their  $S\eta_{20}^0/D_{20,w}^0$  values in 4M-urea were almost identical, indicating a similarity in molecular weights. On the other hand, the  $S\eta_{20}^0/D_{20,w}^0$  value of reduced-alkylated conalbumin was lower than that of transferrin in 4M-urea, indicating a slightly lower molecular weight for conalbumin. The same picture emerges if the  $S\eta$  values of reduced-alkylated transferrin in 6M- and 8M-urea are compared with the corresponding values for reduced-alkylated conalbumin and sulphitolysed transferrin. These differences may, however, not be significant.

An attempt was made to determine the  $\bar{v}_e$  value of reduced-alkylated transferrin by the use of  $D_2O$  as the density-varying component of protein solutions in the presence of constant amounts of urea. For solutions containing 5 mg. of protein/ml. and 4M-urea, the following  $S\eta$  values were obtained: in  $D_2O$ - $H_2O$  (1:2, v/v), 1.70 s; in  $D_2O$ - $H_2O$  (2:1, v/v), 1.55 s; in  $D_2O$ , 1.46 s. It is apparent that these sedimentation coefficients are abnormally

Table 4. *Intrinsic viscosities of modified transferrins*

Concn. of urea (M)	[ $\eta$ ] (ml./g.)		
	Reduced-alkylated transferrin	Sulphitolysed transferrin	Native transferrin
0*	48.3	—	4.4†
4	48.4	38.0	—
6	57.1	—	—
8	64.7	52.7	20.2

\* In 0.1 M-glycine-NaOH buffer, pH 11.5.

† From Bezkorovainy & Grohlich (1967), in 0.05 M-cacodylate-0.1 M-NaCl, pH 7.

high and that a high  $x$  intercept (low  $\bar{v}_e$ ) would be obtained if these values were used for  $\bar{v}_e$  determinations.

*Intrinsic viscosities of modified transferrins.* Intrinsic-viscosity values for reduced-alkylated and sulphitolysed transferrins are shown in Table 4. It appears that the [ $\eta$ ] values for reduced-alkylated transferrin are constant at about 48 ml./g. in the presence of 0.4 M-urea. With higher urea concentrations the viscosity appears to increase until a value of 64.7 ml./g. is reached in 8 M-urea. A similar pattern is apparently followed by sulphitolysed transferrin. Calculation of molecular weight for reduced-alkylated transferrin from the viscosity data assuming random-coil configuration (see the Materials and Methods section) gave values of 65 000 in 0.4 M-urea, 84 000 in 6 M-urea and 101 000 in 8 M-urea.

## DISCUSSION

The amino acid analyses of native transferrin and conalbumin (Table 2) are in excellent agreement with those of Roop & Putnam (1966) and of Williams (1962) respectively. The reduction-alkylation reaction apparently produced no changes, other than in the half-cystine value, in the amino acid composition of transferrin. The half-cystine value indicated that no disulphide bridges were present in our reduced-alkylated transferrin preparations. On the other hand, sulphitolysis apparently did not produce a complete loss of disulphide bonds in transferrin. Viscosity (Table 4), sedimentation-velocity and diffusion (Table 3) data indicated that the sulphitolysed material was indeed more symmetrical or less extended than the reduced-alkylated form of transferrin. However, the same data indicated that the tertiary structure of sulphitolysed transferrin was closer to that of reduced-alkylated transferrin than to its native form.

It appears that Jeppsson's (1967) procedure

would indeed result in the complete disruption of disulphide linkages in transferrin. However, we were unable to reproduce the sedimentation coefficients or the molecular weights claimed by Jeppsson (1967) for the highly denatured form of transferrin: our  $S_{20,w}^0$  values appear to be in the region of 2.9 s, whereas Jeppsson's value was 1.24 s. The diffusion coefficients were, however, close:  $2.80 \times 10^{-7}$  cm.<sup>2</sup>sec.<sup>-1</sup> according to Jeppsson (1967) and  $3.15 \times 10^{-7}$  cm.<sup>2</sup>sec.<sup>-1</sup> in our work.

Molecular-weight values obtained by the sedimentation-diffusion method in this work are about 82 000 for both reduced-alkylated and native transferrin. These were somewhat lower than those previously reported from our Laboratory (87 000; Bezkorovainy, 1966; Bezkorovainy & Grohlich, 1967). The discrepancy can probably be explained on the basis of batch differences. The 82 000 value is now in line with the values obtained by Roberts, Makey & Seal (1966) and by Leibman & Aisen (1967). The molecular weight reported for conalbumin (84 000) is consistent with that observed by Bain & Deutsch (1948), although it is higher than that determined on the basis of iron-binding studies (Warner & Weber, 1951). On the basis of the  $S\eta_{20}^0/D_{20,w}^0$  values (Table 3), it appears that the molecular weight of reduced-alkylated conalbumin is slightly lower than that of transferrin, i.e. lower than 82 000. However, in view of the uncertainties arising from physical measurements in 4 M-urea, these conclusions can be made only on a tentative basis.

As indicated in the Results section, abnormally high  $S\eta$  values were obtained for reduced-alkylated transferrin in the presence of D<sub>2</sub>O. This phenomenon can possibly be explained on the basis of H-D exchange. Such extensive exchange reaction was not observed with native proteins (Hill & Cox, 1965). It thus appears that much caution is in order when attempting to perform ultracentrifugal analysis of denatured proteins in the presence of D<sub>2</sub>O.

Viscosity data indicated that an [ $\eta$ ] value of about 48 ml./g. was the lowest obtainable with samples whose disulphide bridges were completely disrupted. A polypeptide chain in the random-coil configuration having a molecular weight of 41 000 and a mean residue weight of 113 (a possible sub-unit of transferrin) would be expected to have an [ $\eta$ ] value of 35.9 ml./g. In 0.4 M-urea the molecular weight calculated from viscosity data alone (65 000) is inconsistent with the random-coil configuration. It therefore appears that reduced-alkylated transferrin in 0.4 M-urea exists in a partially ordered configuration. In 6 M-urea the protein apparently assumes a nearly random-coil shape. The [ $\eta$ ] value in 8 M-urea was higher than that in 6 M-urea and was quite close to that obtained

in 6M-guanidine hydrochloride (Bezkorovainy & Grohlich, 1967).

Two explanations can be offered for this phenomenon. Thus, in view of the work of Bron *et al.* (1968), one may propose that reduced-alkylated transferrin particles aggregate in 8M-urea and exist in a random-coil configuration in 6M-urea. The  $S_{20,w}^0$  value of 3.09s, which admittedly is within experimental error of the 2.8s value obtained in 6M-urea, tends to support this view. Further, the  $[\eta]$  value of sulphitolyse transferrin in 8M-urea was 52.7 ml./g. (Table 4), a value close to that expected for a random coil with a molecular weight of 80000. Yet, with many of its disulphide bridges intact, the sulphitolyse preparation could not possibly assume a random-coil shape, and thus an alternative explanation for the relatively high  $[\eta]$  value could be aggregation. If the above explanation of the observed viscosity differences is accepted, and since the  $[\eta]$  values of reduced-alkylated transferrin in 8M-urea and 6M-guanidine hydrochloride are identical, one could then conclude that 6M-guanidine hydrochloride is not as potent a dissociating agent as has formerly been believed.

On the other hand, it may be argued that molecular-weight determinations by the viscosity method are at best only approximations. Then, if 6M-guanidine hydrochloride is accepted to be a universal dissociating solvent, reduced-alkylated transferrin in 8M-urea must exist in a random-coil configuration and is probably partially ordered in 6M-urea. The latter explanation is probably more likely in view of its simplicity and the lack of more definitive evidence on the possible aggregation of reduced-alkylated transferrin in 8M-urea and 6M-guanidine hydrochloride.

The Scheraga-Mandelkern  $\beta$ -values for reduced-alkylated transferrin were  $2.6 \times 10^6$  and  $2.78 \times 10^6$  in 4M- and 6M-urea respectively, assuming the  $\bar{v}$  value to be 0.723. These values are probably within experimental error of the  $2.5 \times 10^6$  value that is consistent with random coils.

The viscosity and sedimentation data presented in this paper are inconsistent with the proposal that transferrin and conalbumin exist as aggregates of two identical sub-units, and indicate that these proteins exist as single polypeptide chains. Our present position is thus consistent with our earlier findings (Bezkorovainy & Grohlich, 1967) and the work of Greene & Feeney (1968), and in this respect does not support the findings of Jeppsson (1967).

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