Denaturation of Globular Proteins. Interaction of Guanidinium Salts with Three Proteins†

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ABSTRACT: The interaction of guanidinium thiocyanate and guanidinium chloride with bovine serum albumin, ovalbumin, and lysozyme has been measured by an equilibrium dialysis technique. The extent of salt–protein interaction was observed to correlate with the completeness of denaturation, as followed by changes in optical rotatory parameters of the proteins. The data obtained with these three proteins indicates that a ratio of around one salt molecule interacts per four amino acid residues of a maximally denatured protein molecule; the three proteins were found to be somewhat *less* unfolded in Gdm·SCN when compared to Gdm·Cl. In contrast, each protein demonstrated a preferential interaction with water (*i.e.*, a negative salt interaction) in aqueous solutions of guanidinium sulfate and they exhibited near-native optical rotatory parameters. Additional studies revealed that sodium sulfate not only inhibits the denaturing action of 3 M Gdm·Cl or 2 M Gdm·SCN toward serum albumin (reversion of the optical rotatory parameters toward native values), it also diminishes the interaction of each denaturant with albumin. The identity of the site(s) of guanidinium ion interaction with denatured protein molecules is not clear. However, a repeating protein unit such as the peptide bond remains a strong possibility as we found (1) a similar magnitude of guanidine–protein interaction for the thiocyanate and chloride salts with three different proteins, and (2) a correlation between denaturing effectiveness and ability to solubilize a model peptide (*N*-acetyltetraglycine ethyl ester) for all three guanidinium salts.

The preceding papers in this series (Gordon and Warren, 1968; Warren and Gordon, 1970, 1971) dealt with the interaction of urea, thiourea, and simple amides with bovine serum albumin, lysozyme, β-lactoglobulin, and ovalbumin. It was suggested that any proposed mechanism of urea denaturation should include interaction between urea and the denatured form(s) of these proteins.

A similarity in physicochemical parameters of many proteins in concentrated urea and in concentrated guanidinium hydrochloride (Gdm·Cl) as well as the solubility behavior of various protein models in these two solvents (Nozaki and Tanford, 1970; Gordon, unpublished) has been generally interpreted to mean that these agents share similar, if not identical, mechanism(s) of denaturation (Tanford, 1968, 1970), although some exceptions have been pointed out (Wetlaufer *et al.*, 1964; Riddiford, 1966; Martin and Bhatnagar, 1967; Woodlock and Harrap, 1968).

In this paper we report that the interaction of Gdm·Cl with three globular proteins parallels the extent of unfolding measured by optical rotation. This study provides data for comparison with the results of our urea studies and with other studies of guanidine hydrochloride interaction using different techniques (Hade and Tanford, 1967; Noelken and Timasheff, 1967; Reisler and Eisenberg, 1969; Aune and Tanford, 1969a,b, and references therein). Furthermore, we report that the interaction of Gdm·SCN with proteins also parallels its denaturing effectiveness, while $(Gdm)_2 \cdot SO_4$ *neither* interacts *nor* causes protein denaturation.

The apparent similarity in the ratios of salt interaction with the three proteins and preliminary solubility studies on a model polypeptide, $Ac(Gly)_4OEt$,[1] are consistent with the repeating peptide bond units as sites of interaction for denaturing guanidine salts.

Materials and Methods

Proteins. Bovine serum albumin (Armour Pharmaceutical, lots numbered D71209, E71503), ovalbumin (salt-free and crystalline product from three commercial sources: Immunology, Inc., lot 567; Nutritional Biochemicals, lot 6687; Mann Research Laboratories, lot T5166), and lysozyme (Worthington Biochemical Corp., lots 8AA, 8DB, 8BE) were routinely dialyzed in Visking tubing against 0.1 M NaCl and then distilled H_2O for several days to remove any dialyzable contaminants. The solutions were centrifuged and the clear supernatant was carefully lyophilized to dryness. Residual water in the lyophilized preparations tested less than 2.5% as determined by weight loss following the drying of an aliquot at 105° for 24 hr.

Guanidine Salts. Reagent grade hydrochloride, thiocyanate, and sulfate salts of guanidine were purchased from Eastman Organic Chemicals (Rochester, N. Y.). Ultra-Pure Gdm·Cl (Mann Research Laboratories, New York, N. Y.) was free of optically absorbing impurities, having an absorbance near 0.11 unit at 230 mμ for a 6 M solution. As it has been recently reported that the optical criteria of purity is not entirely sufficient (Wong *et al.*, 1971), purified Gdm·Cl was prepared from guanidine carbonate by the method of Nozaki and Tanford (1967). Our reported results utilizing this material (mp 187–188°) were experimentally identical with those obtained with the Ultra-Pure commercial preparation at 2 and 6 M

Stringent criteria for the purity of Gdm·SCN are not so clear. Concentrated solutions of reagent grade material were usually somewhat pink and slightly hazy. Purification over charcoal and subsequent filtration gave a clear and colorless solution which was lyophilized. Purposeful contamination by small amounts of unclarified material was without effect on the reported results.

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[1] Abbreviations used are: Gdm·Cl, guanidinium chloride; Gdm·SCN, guanidinium thiocyanate; $(Gdm)_2 \cdot SO_4$, guanidinium sulfate; $Ac(Gly)_4OEt$, acetyltetraglycine ethyl ester; ORD, optical rotatory dispersion.

Preparation of Solvents. All solvents were made up fresh on a molar basis in distilled water and filtered through sintered glass. The final concentration was determined by refractive index measurement and referral to a standard concentration–refractive index curve.

Equilibrium Dialysis. The technique of ultrafiltration has been described previously in some detail (Gordon and Warren, 1968). Small cylindrical equilibrium dialysis cells were manufactured from glass-reinforced Teflon. The chambers were faced with Visking tubing which had been boiled in distilled water and then placed in the appropriate solvent for at least 2 days. Four to seven cells, each containing aliquots of solvent (capacity 0.2–0.3 ml), were placed in 40 ml of the same solvent; the jar was tightly sealed and the solution vigorously agitated for 120 min at room temperature. One of the cells was then removed and the refractive index of the contents compared to the external solution, to again verify solvent equilibration inside and outside. Now dry protein (<2% water) was added to the outside solvent to generally give a final concentration of 4% protein. The solution was agitated rapidly and the equilibrium dialysis cells were removed one at a time and the contents quickly analyzed by refractometry in the third and fourth hours. It was shown by simple aqueous dilution of the external (outside) solution that changes of the magnitude twice that reported here were reflected *inside* the cells within 30 min. Furthermore, the identical refractive indices of the contents from the serially removed equilibrium dialysis cells (over 1 to 2 hr) insured that equilibrium conditions had been met. For comparison purposes some experiments were performed utilizing LSG-60 membranes (Schleicher and Schuell) and no difference in results was seen.

This technique then allows us to obtain a relatively small amount of "bulk" solvent free from a much larger volume of solvent containing protein in a manner compatible with quantitative determination of solvent concentration, as only the two nonprotein components (water and salt) penetrated the membrane. As a precaution, biuret determinations for protein were made, where possible, on the cell contents in the presence of the guanidine salts. Results were always negative in the absence of unusual increases in the refractive index of the cell contents, indicating a major protein leak into the contents of the cell. In any event the conditions of the experiments are such that protein penetration into the cell would lead to results (see below) which indicate less, and not more, apparent protein–ligand interaction.

A slight penetration of the untreated Visking membranes by lysozyme was completely prevented by pretreatment of the membranes in 20% acetic anhydride in pyridine for 20 hr before use. This treatment was also shown to be without effect on the data obtained with bovine serum albumin and ovalbumin when the results with untreated membranes served as controls.

Measurements on the solutions containing protein ranged between the apparent pH 5 and 7 in all solvents.

Analyses. Refractive indices relative to air at the sodium D line were determined to an accuracy of ±0.00002 unit on a Bellingham and Stanley (Abbe 60 High Accuracy) refractometer thermostated to 20° ± 0.05°. The refractometer was calibrated daily with water. Concentration curves for each reagent were constructed and the validity and reproducibility of the refractive index readings and experiments were subject to stringent criteria as previously outlined (Gordon and Warren, 1968).

ORD. Optical rotatory dispersion measurements were made at 9 wavelengths from 340 to 600 mμ on a modified Perkin-Elmer Model 141 spectropolarimeter. Thermostated polarimeter cells (10 cm) were used. The Moffit–Yang parameters, a_0 and b_0, were calculated in the usual fashion by inserting the appropriate values of the mean residue rotation (Gordon, 1968), adjusting for the refractive index of water, and utilizing a λ_0 value of 212 mμ. For comparison, some calculations of the parameters included the adjustment made for the refractive index of the Gdm·Cl solution.

The refractive indices of Gdm·Cl were obtained at all wavelengths by a modified Sellmier approximation of known data for a 6 M solution (Hooker, 1966). The refractive indices at lower Gdm·Cl concentrations were obtained by drawing a series of curves through the determined experimental values at 589 and 540 mμ parallel with the behavior of the curves for water and 6 M guanidinium chloride.

Ac(Gly)$_4$OEt Solubility. *N*-Acetyltetraglycine ethyl ester (Ac(Gly)$_4$OEt) was prepared by the technique outlined by Robinson and Jencks (1965). The product, which was obtained in 20% yield after two recrystallizations, decomposed between 264 and 266°, in good agreement with reported values.

We also found that the presence of small amounts of these guanidine salts interfered with a quantitative biuret assay for Ac(Gly)$_4$OEt (Robinson and Jencks, 1965a,b). Therefore to obtain a relative order of solubility, known amounts of solid Ac(Gly)$_4$OEt were added to a series of vials containing either the thiocyanate, chloride, or sulfate salt of guanidine at concentrations of 1, 2, and 3 M. The vials were then capped, sealed in paraffin, and powerfully agitated for 72 hr at 20°. The tubes were then visually inspected for remaining solid and the relative solubility of Ac(Gly)$_4$OEt in the salts was ordered. The same series was then heated to 50° for 30 min and reagitated at 20° for 72 hr to insure equilibrium had been previously obtained. Microscopic examination of the remaining solid indicated no visible change.

Dissociation. A preliminary isopiestic study examined the relative dissociation of these salts in solution. Quantitatively prepared 3 *m* solutions of Gdm·Cl and/or Gdm·SCN in beakers were weighed and placed in a vacuum desiccator together with a 2 *m* solution of (Gdm)$_2$·SO$_4$. The dessicator was evacuated; the solutions were agitated over a period of 4 weeks with weekly beaker weighings. Equilibrium, as determined by stability in weight, was reached before the third week at room temperature (23 ± 2°). The experiments were repeated once with identical results.

Viscometry. Viscosities were determined in Cannon semimicrodilution viscometers having water flow times of approximately 130 sec at 20 ± 0.05°. Viscosities were performed immediately and 3 hr after mixing. No disulfide reagent was used and the acidity of the original protein solution ranged between pH 5 and 6.5. Prior to use, all solutions were filtered by gravity through Millipore filters. Results are given as the intrinsic viscosity determined by extrapolating the reduced viscosities obtained at five protein concentrations ranging from 0.4% to 4% bovine serum albumin. The determinations were made in triplicate.

Results

Equilibrium Dialysis. The solution external to the small equilibrium dialysis cells contains three components. Two of the components, water and the salt, freely pass into the dialysis cells with zero reflection; the external protein was shown to have 100% reflection at the membrane surface (*i.e.*, impenetrable). Experimentally, the refractive index of the internal

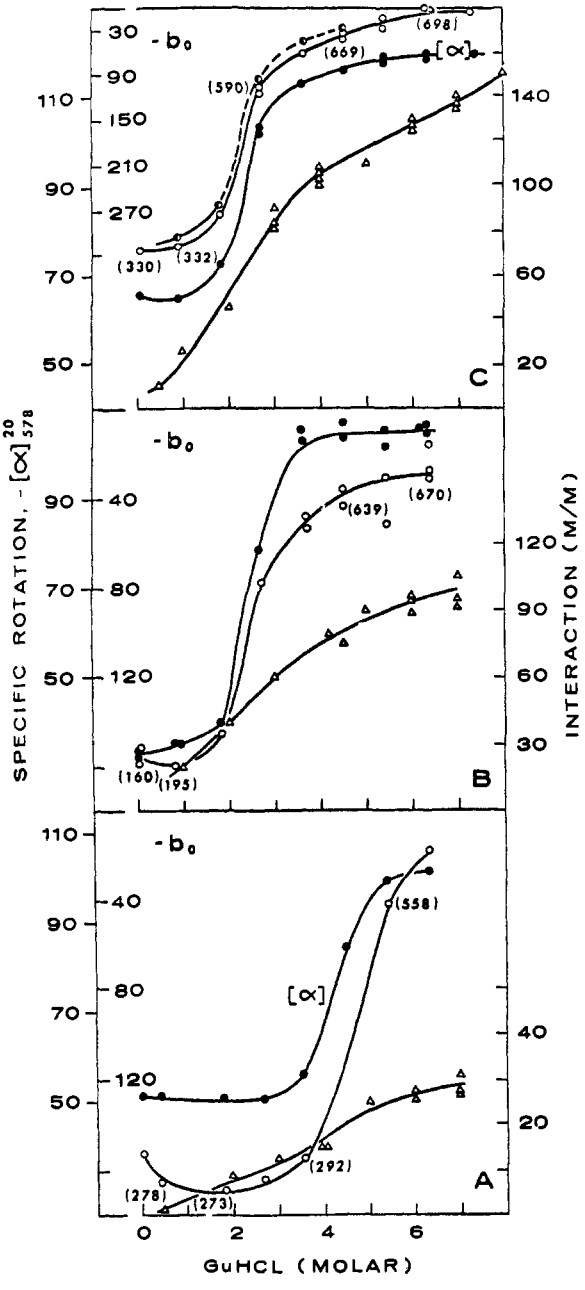

FIGURE 1: The extent of guanidinium hydrochloride interaction (△, right ordinate) with lysozyme (A), ovalbumin (B), and bovine serum albumin (C) is given as a function of guanidinium hydrochloride concentration. The interaction is expressed in the terms of moles of Gdm·Cl interacting per mole of protein assumed to be hydrated (0.20 g of water per g of protein; see text). The optical rotatory parameters of each protein are expressed on the left ordinate in terms of specific rotation (●) and b_0 (○). Representative values of the companion Moffitt–Yang parameter a_0 are placed near the symbols for b_0. The half-shaded symbol (◐) indicates the value of b_0 adjusted for the refractive index increment added by the solute.

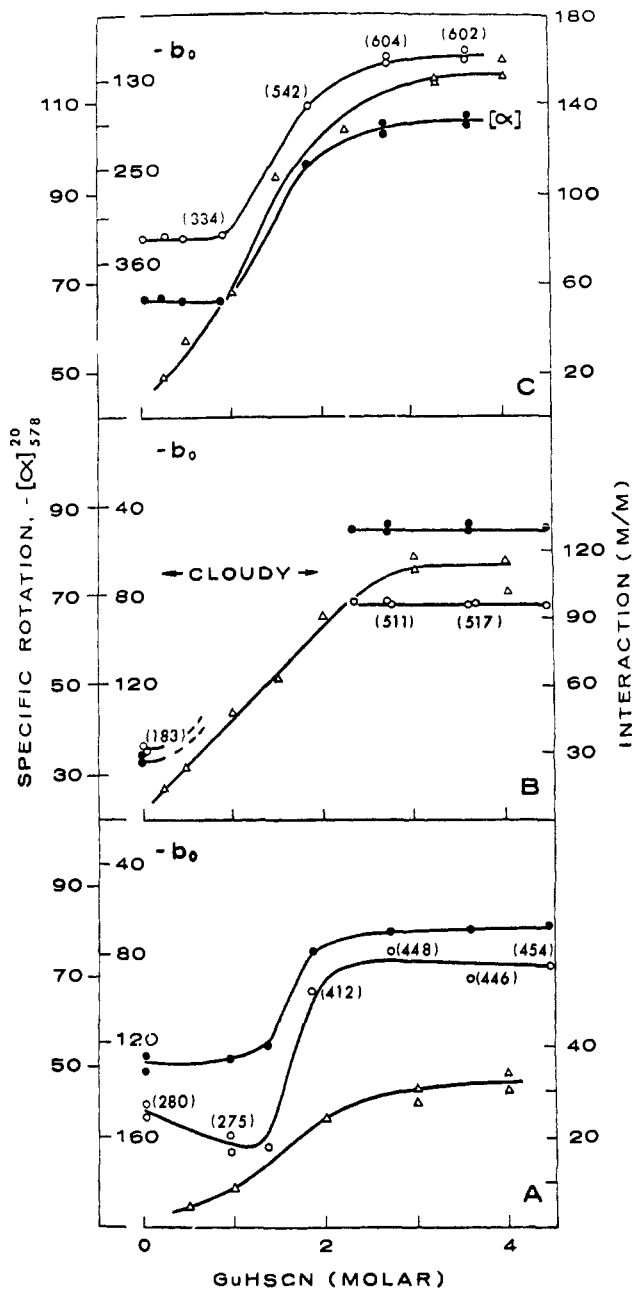

FIGURE 2: The extent of guanidinium thiocyanate interaction (right ordinate) with lysozyme (A), ovalbumin (B), and bovine serum albumin (C) plotted as a function of guanidinium thiocyanate concentration. All symbols are identical with those of Figure 1.

protein-free dialysate can be greater, lesser, or equal to the experimentally known refractive index of the dialysate before the addition of the small amount of protein to the external solution. A *decrease* in refractive index of the dialysate (internal solution) indicates preferential interaction of the guanidine salt with the added protein outside the cell while conversely an *increase* in refractive index indicates that the concentration of the guanidine salt in the immediate domain of the protein in the external solution is less than the original bulk solvent.

The latter finding is most easily explained by preferential water–protein interaction with salt exclusion. *No* change in the refractive index of the internal solution, then, indicates there is no preferential interaction of the protein with either water or salt in the external solution following the addition of protein. The observed refractive indices (and the difference, if any, seen after protein addition) of the protein-free dialysate can be expressed on a molar scale. We emphasize that the use of the molarity scale to express the results is used in the following fashion. To a given volume of completely diffusible components whose refractive index is very accurately known, a small amount of salt-free protein (containing less than 2% water) is added to one side of the membrane(s). The change in volume of the original solvent is generally 3% when 4% protein (w/v) is added. The results to be described, when

TABLE I: Preferential Interaction of Three Proteins with Water (Hydration) in the Presence of Concentrated $(Gdm)_2 \cdot SO_4$.

Protein	$(Gdm)_2 \cdot SO_4$ (M)	Optical Rotatory Parameters[a]			Equilibrium Dialysis	
		$-[\alpha]_{578}^{20°}$	$-b_0$	$-a_0$	Change in Filtrate[b] (ΔM)	Hydration[c] of Protein (g/g of Protein)
Serum albumin	2.5	62.0	264	320	+0.017	0.17
	2.0	64.0	278	330	+0.023	0.28
	1.5	64.7	299	334	+0.014	0.23
	0	66.6	327	338	NA	NA
Ovalbumin	2.5				+0.020	0.20
	2.0	37.1	157	206	+0.019	0.23
	1.5	36.7	154	205	+0.014	0.23
	0	33.1	148	183	NA	NA
Lysozyme	2.5				+0.010	0.10
	2.0	54.0	147	284	+0.009	0.11
	0	52.8	141	277	NA	NA

[a] Obtained from solutions 1% in protein. [b] The values given are the average of three or four experiments. The observed change in refractive index following the addition of protein (generally to 4%) to the external solution expressed as the increase (+) in molarity (ΔM) of the contents of the dialysis cell. [c] The values of the preceding column expressed in terms of the amount of water apparently made unavailable to $(Gdm)_2 \cdot SO_4$ per gram of protein added, *i.e.*, the amount of water required to return a protein-free solution to its original concentration divided by the grams of protein added.

expressed on the basis of added protein, were independent of protein from 1 to 6%. In the usual experiment, the refractive index of the protein-free dialysate (less than 2% of the total volume and contained inside the dialysis cells) was compared to the refractive index of the original solvent, *i.e.*, before the addition of protein. The comparison is expressed in the tables in terms of molarity. The reference state of zero or no preferential interaction between protein and solvent components is defined as no change in the refractive index translated into molarity of the bulk solvent after protein addition. Alternatively, we could have expressed the refractive indices in terms of weight molality or mole fraction. It is true that the molal scale permits an easier association with certain expressions of physical laws, but the molal scale is not intrinsically more fundamental than the molar scale. Therefore we elected to express the results of these experiments on the molar scale as it is more common in biochemistry. It is reemphasized that the molarity of the diffusible components is recorded in the table. In other words, if the protein was completely inert (*i.e.*, did not interact with water or salt), the addition of the small amount of protein should lead to no change in refractive index of the diffusible components as it would be equivalent to changing the shape of the vessel holding the solvent by the addition, for example, of a small amount of glass balls. This latter prediction was experimentally verified many times.

In the experimental system under consideration, an observed preferential interaction of a guanidine salt must represent a minimum value of interaction as the protein was added dry (less than 2% water) to the bulk solvent. This follows from the consideration that at least some water (at concentrations never less than 30 M) is also bound to sites on the protein which are independent of any interaction of the guanidine salt with the protein. This assumption is supported by the small amount of available data for denatured proteins (Timasheff and Inoue, 1968; Privalov and Mrevlishvili, 1967; Glasel, 1968) and the known continued availability of groups to the solvent which normally interact with water in the native state of the protein. Calculation, then, of the *total* interaction of guanidine salt to the protein from the experimentally determined refractive indices, representing *preferential* interaction, should include adjustment for the quantity of water simultaneously interacting with the dry protein (Timasheff and Inoue, 1968). In the absence of a reliable estimate of protein hydration under our conditions, we assume a hydration value for each protein of 0.20 g of water per g of protein. This assumption for the unfolded protein considers only the total amount of water unavailable to the bulk solvent irrespective of any changes in the state of water (Timasheff, 1970). The results obtained with Gdm·Cl and Gdm·SCN are shown in Figures 1 and 2 in terms of moles of salt bound per mole of protein, as a function of salt concentration (abscissa).

If the hydration of the denatured protein had been assumed to be zero, the interaction values (now corresponding to only the observed or "preferential" interaction) for the guanidine salts would only have been suppressed by 32% in 4 M salt and 50% in 6 M salt; this drastic possibility seems very unlikely. On the other hand, protein unfolding could lead to an increased interaction of water and protein, as for example with freshly exposed peptide units. In the absence of definite information we elected to pursue a conservative assumption of 20% hydration for the unfolded molecule. In any case, the major conclusion(s) of this paper would not be seriously altered if the real hydration turns out to be overestimated or underestimated.

The results in various concentrations of $(Gdm)_2 \cdot SO_4$ is shown in Table I. Here the protein-free dialysate (internal solution) was found to be *more* concentrated after the addition of protein to the external solution, giving a *negative* preferential interaction of the salt with the protein. We have elected to express these results in terms of the grams of water seemingly removed from the bulk solvent, *i.e.*, unavailable to $(Gdm)_2 \cdot SO_4$, per gram of added protein (Gordon and Warren, 1968).

Optical Rotation. The ORD measurements in all cases were

TABLE II: ORD Parameters of Bovine Serum Albumin in Gdm·SCN under Modified Conditions.[a]

Gdm·SCN (M)	Modified Condition(s)	ORD Parameters		
		$-[\alpha]_{578}$	$-b_0$	$-a_0$
2.7	None	101.6	90	580
2.7	Gdm·Cl[b]	106.1	22	615
2.7	NaSCN[c]	95.2	105	542
3.2	None	101.2	80	585
3.2	50°	97.2	85	558
3.2	Gdm·Cl[b]	107.1	15	620
3.2	Gdm·Cl,[b] 50°	98.5	0	580

[a] Bovine serum albumin present at 0.82% (w/v). Temperature 25° unless otherwise noted. [b] 2.7 M. [c] 1.8 M.

routinely made 3 and 24 hr after addition of protein. This procedure assured that the observations represented equilibrium values as the unfolding of lysozyme and ovalbumin was time dependent up to 3 hr at 20°.

The results are summarized (Figures 1 and 2) as plots of $[\alpha]^{20°}_{578}$ (left ordinate, outside) and b_0 (left ordinate, inside) *vs.* the molarity of Gdm·Cl or Gdm·SCN on the abscissa. In general, increasing concentrations of the chloride and thiocyanate salts of guanidine lead to increasingly levorotatory values of the specific rotation and decreasingly negative values of b_0, parallel to solute interaction. In addition, the protein transitions begin at a lower concentration of the thiocyanate salt when compared to the chloride salt.

Following the major transition of all three proteins, the specific rotation and the value of b_0 remained considerably less altered in Gdm·SCN than in Gdm·Cl. Adjustment for the addition of Gdm·SCN or Gdm·Cl to the refractive index correction would only accentuate this difference, as the refractive index of very concentrated Gdm·Cl is equal to or less than that of Gdm·SCN. That the relatively large final values of $-b_0$ in concentrated Gdm·SCN indicate remaining protein structure is shown as follows: (1) the specific rotation becomes more levorotatory and the value of $-b_0$ of bovine serum albumin approaches closer to zero when 2.7 M Gdm·Cl is added to solutions of either 2.7 or 3.2 M in Gdm·SCN, (2) in contrast, the addition of the denaturant NaSCN leads to a small *increase* in the value of $-b_0$ accompanied by a decrease in the specific rotation, (3) and in addition (Table II), heating of a bovine serum albumin solution containing 3.2 M Gdm·SCN leads to a much smaller dextrorotatory change in the specific rotation compared to a solution in which Gdm·Cl is also present (Tanford, 1970). This conclusion is supported by viscosity data (see below).

The values of the specific rotation and b_0 for all proteins in the presence of increasing concentrations of $(Gdm)_2\cdot SO_4$ (Table I) essentially remained constant, except for bovine serum albumin.[2]

Dialysis and ORD Studies in Two Salts. It seemed of interest to see if both the ORD and equilibrium dialysis experiments in the presence of two salts might approximate the additive results of the individual salt experiments (summarized in Table III). The addition of $(Gdm)_2\cdot SO_4$ to a solution containing 3 M Gdm·Cl leads to both a reduction in the unfolding of bovine serum albumin as well as less of a decrease than expected in the refractive index sum. The last two columns express (1) the observed change in refractive index and (2) the expected sum if the separate values of the individual salts were completely additive. Of course the contribution of each ionic species to the change in refractive index cannot be untwined. However, it can be concluded that the refolding of the protein in the presence of added $(Gdm)_2\cdot SO_4$ necessarily represents some lessening of Gdm·Cl interaction. This is so because the observed changes (*i.e.*, difference between experimental and expected) in the sum of the refractive indices can only be offset somewhat by the unlikely positive interaction of the sulfate anion, for example, with the refolding protein in the presence of a denaturing salt.

Na_2SO_4 is found to be even more effective in inhibiting the action of Gdm·Cl (Table III) (von Hippel and Schleich, 1969). Similar experiments replacing Gdm·Cl by Gdm·SCN are also shown.

Other Results. We had difficulties in quantitating the absolute solubility of $Ac(Gly)_4OEt$ in the three salt solutions for technical reasons (see Methods). However, it was clear by the simple serial-dilution inspection technique that the solubility of $Ac(Gly)_4OEt$ was increased in water when Gdm·Cl or Gdm·SCN was present (Castellino and Barker, 1969). The solubility of $Ac(Gly)_4OEt$ in water was progressively decreased by the addition of $(Gdm)_2\cdot SO_4$.

A preliminary isopiestic study gave the following results from duplicate isoionic solutions: (1) a solution of 3 *m* Gdm·Cl or 3 *m* Gdm·SCN gained 13–14% while the accompanying 2 *m* $(Gdm)_2\cdot SO_4$ solution lost 14% by weight. The indication is that $(Gdm)_2\cdot SO_4$ at these concentrations is the least osmotically active guanidine salt. Nevertheless, the association of $(Gdm)_2\cdot SO_4$ must be weak as the refractive index increment was found to be constant to 3 M compared to Na_2SO_4 where the association constant is only 5 M^{-1} (Denny and Monk, 1951) and the refractive index increment is known to slightly decrease with concentration. The possibility of chemical changes in our experiments has not been completely ruled out; however reconstitution of the original $(Gdm)_2\ SO_4$ solution by the addition of the water (equal to the lost weight) restored the original refractive index and spectral behavior in the near-ultraviolet.

The ORD studies (Table II) suggested that the proteins were less unfolded in concentrated Gdm·SCN than in concentrated Gdm·Cl. Viscometric measurements were done between pH 5 and 6.5 in the absence of buffer and disulfide reducing agents, at 20°. The intrinsic viscosity, $[\eta]$, of bovine serum albumin in 3.5 M Gdm·SCN was found to be 24.1 ± 1.0 ml/g compared to 29.9 ± 0.5 ml/g in 6 M Gdm·Cl, all after 3 hr so as to temporally coincide with the other experiments. The values obtained soon after mixing were in the same relative

[2] The behavior of bovine serum albumin in $(Gdm)_2\cdot SO_4$ as determined by ORD parameters is somewhat different. The values of b_0 indicate some loss of protein structure (probably α-helix) with increasing salt concentration although the specific rotation values are less levorotatory. Adjustment for changes in the refractive indices (as discussed) would only further decrease the values of $-b_0$ (*i.e.*, indicating even further loss of α-helix) with increasing concentrations of $(Gdm)_2\cdot SO_4$. The observation is not apparently a nonspecific solvent effect as supported by the relatively small changes in the values of a_0; the change might represent a more specific type of ion effect on bovine serum albumin (Markus *et al.*, (1964) although Na_2SO_3 gives no change in the Moffitt–Yang parameters (Table III). Sulfate salts have been shown to *lower* the thermal stability of collagen compared to the absence of salt (Woodlock and Harrap, 1968), but this appears not to be the general case.

TABLE III: Some Effects of Two Salts on Bovine Serum Albumin.[a]

Solvent Composition (M)				ORD Parameters			Change in Dialysate (Units of Refractive Index)	
Gdm·Cl	Gdm·SCN	$(Gdm)_2·SO_4$	Na_2SO_4	$-[\alpha]_{578}^{20°}$	$-a_0$	$-b_0$	Experimental[b]	Expected[c]
2.7				108	625	118	−0.00047	N.A.
2.7		0.09		107	620	125	−0.00043	−0.00045
2.7		0.45		106	605	134	−0.00022	−0.00038
2.7		0.90		101	575	141	0	−0.00025
2.7		2.25		91	515	150	+0.00015	−0.00001
2.7			0.09				−0.00044	−0.00044
2.7			0.45	67	333	333	−0.00014	−0.00033
2.7			0.90	66	335	334	0	−0.00018
2.7			1.35		Turbid		Turbid	
	1.8			97	542	156	−0.00163	N.A.
	1.8	0.09					−0.00158	−0.00160
	1.8	0.45		96	540	175	−0.00130	−0.00154
	1.8	0.90		87	495	195	−0.00100	−0.00141
	1.8	1.35			Turbid		−0.00066	−0.00121
	1.8	2.25			Turbid		Turbid	
	1.8		0.09	97	543	151	−0.00141	−0.00160
	1.8		0.45	87	480	186	−0.00105	−0.00150
	1.8		0.90	77	419	211		
	1.8		1.35		Turbid		Turbid	
			0.09	67	344	329	+0.00003	N.A.
			0.45	67	343	330	+0.00013	N.A.
			0.90	67	342	331	+0.00029	N.A.
			1.35	66	340	335	+0.00042	N.A.
		0.09		67	338	327	+0.00002	N.A.
		0.45		68	344	320	+0.00009	N.A.
		0.90		65	334	311	+0.00022	N.A.
		2.25		64	315	268	+0.00048	N.A.

[a] The equilibrium dialysis and ORD experiments were carried out in a fashion identical with the single salt experiments. Routinely, the ORD values were obtained from 1% solutions of bovine serum albumin while the equilibrium dialysis experiments were 4% in protein. [b] The data only expressed as the loss (−) or gain (+) in units of refractive index when compared to the solvent prior to the addition of bovine serum albumin. No adjustment made for the simultaneous uptake of water, a factor which would only undergo minor variations upon the addition of the second salt and would not be expressed in terms of differences seen between the last two columns. [c] The change in the refractive index expected if the changes seen in the experiments with the single salts were summed. For example, the *expected* results from the addition of 2.25 M $(Gdm)_2·SO_4$ (a gain of 0.00048 unit) to 2.7 M Gdm·Cl (a loss of 0.00047 unit) does not result in a net (or expected) gain of 0.00001 unit, indicating a change in interaction of Gdm·Cl. In the absence of added protein, the total refractive index of these solutions equalled the sum of the refractive indices of the single salts.

order but approximately 10% less. Most interesting was the finding that the addition of Gdm·Cl (3.2 M) to 3.5 M Gdm·SCN raised the intrinsic viscosity to 29.7 ml/g, the same value as found in 6 M Gdm·Cl, soon after mixing.

Discussion

ORD Studies in Gdm·SCN and Gdm·Cl. From the optical rotatory dispersion data shown (Figures 1 and 2) the following not unexpected observations are drawn: (1) both Gdm·Cl and Gdm·SCN are very effective protein denaturants; (2) the conformational (optical rotatory) transitions begin at lower concentrations of Gdm·SCN and are somewhat sharper when compared to transitions in Gdm·Cl (von Hippel and Schleich, 1969; Castellino and Barker, 1968a,b).

Unexpectedly we found that in concentrated solutions of Gdm·SCN the values of b_0 and the specific rotation of these proteins do not approach those parameter values suggestive of complete loss of α-helical structure (*i.e.*, attaining values of $-b_0$ near zero) and therefore this suggests more protein structure remaining in Gdm·SCN compared to Gdm·Cl. This conclusion would not be altered by the application of any known adjustment factor (Cassim and Taylor, 1965) for the change in refractive index of both salts. Reinforcement of our observation is found in the following experimental results: (1) the intrinsic viscosity of bovine serum albumin in 3.5 M Gdm·SCN (24.1 ml/g) is significantly less than in 6.0 M Gdm·Cl (29.9 ml/g) and (2) the ability of Gdm·Cl to cause additional change in both optical rotatory parameters *and* viscosity values of bovine serum albumin in concentrated Gdm·SCN (Table II and Results) both of which indicate loss of protein structure. It appears that the greater denaturing effectiveness of Gdm·SCN has generally been experimentally based on the midpoint of a transition not generally shown

TABLE IV: Apparent Maximum Ratios of Guanidine Salt Interaction with Three Proteins in Posttransition Regions[a]

Protein	Gdm·Cl (moles/mole of Protein)	Gdm·SCN[b] (moles/mole of Protein)	Gdm·Cl (moles/mole of Amino Acid)	Gdm·SCN[b] (moles/mole of Amino Acid)
Bovine serum albumin (570)[c]	~150[d]	150	~0.26[d]	0.26
Ovalbumin (390)[c]	105	115	0.26	0.30
Lysozyme (129)[c]	30	32	0.23	0.25

[a] Derived from the maximum number of molecules interacting with the protein shown in Figures 1 and 2, generally the value of the relative plateau in interaction observed in the posttransition region of the protein. [b] Average of the values in the posttransition plateau. [c] Number of amino acids per monomer used in calculation shown in last column. [d] Subject to some uncertainty as a definite plateau in the interaction of bovine serum albumin with 5 through 7 M Gdm·Cl is not seen. Note also that bovine serum albumin (Figure 1) demonstrated experimentally significant changes in the values of the ORD parameters under these conditions.

(Stefanye *et al.*, 1964), the optical rotation at one wavelength (Castellino and Barker, 1968b), or the ability of Gdm·SCN to *initiate* denaturation at concentrations lower than Gdm·Cl (von Hippel and Schleich, 1969).

It was shown by von Hippel and coworkers that the transition of ribonuclease as a function of temperature decreased in size with increasing concentrations of the strong denaturant KSCN although the transition size increases with Gdm·Cl (von Hippel and Wong, 1965). From this and isothermal examples (Noelken, 1970) it appears that the posttransition state in the powerful neutral salt denaturants like LiSCN differ from those in Gdm·Cl by not completely unfolding (von Hippel and Schleich, 1969). This difference in solvent behavior can be attributed to the salting-in effect by Gdm·Cl on nonpolar compounds not seen with other neutral salts capable of denaturation such as LiSCN (Wetlaufer *et al.*, 1964; Robinson and Jencks, 1965a,b; Schrier and Schrier, 1967). With this in mind, it is possible that in solutions of Gdm·SCN some nonpolar residues of the protein remain (or become) clustered even in the presence of the guanidinium cation (Noelken, 1970). The initiation of the denaturation transitions at concentrations of Gdm·SCN lower than Gdm·Cl is not paradoxical in view of the ability of the thiocyanate anion to salt-in amide groups and polar residues and thereby complement the ability of the guanidium cation in this regard, chloride anion being relatively ineffective (Robinson and Jencks, 1965a,b; Jencks, 1969).

Studies in $(Gdm)_2SO_4$ and Na_2SO_4. The data (Table I) indicate that these native proteins[2] are preferentially *hydrated* in aqueous $(Gdm)_2SO_4$. The values of hydration are within the range of reported values using other techniques and under other conditions (Fisher, 1965; Privalov and Mrevlishvili, 1967). The somewhat smaller value of hydration obtained with native lysozyme seems an open question and is beyond the scope of this paper; however the result might be secondary to some extraordinary binding of $(Gdm)_2SO_4$ to native lysozyme due to a large net charge below neutral pH.

Most importantly, the guanidinium ion causes no observable denaturation (Greenstein, 1939) by ORD in the presence of the sulfate counterion and we find no detectable interaction with proteins. Our isopiestic distillation results indicate that concentrated $(Gdm)_2SO_4$ is only somewhat more associated than either Gdm·Cl or Gdm·SCN in solution as might be expected from other sulfate salts (Denny and Monk, 1951). This small association cannot account for the observation differences in denaturing ability *even* if the associated form of $(Gdm)_2SO_4$ was assumed to be inactive as a denaturant.

Interaction with Gdm·Cl and with Gdm·SCN. A correspondence is seen between a positive interaction of the salt component with protein and the change in conformation (denaturation) as followed by optical rotation. A positive salt interaction seen here (see Results) cannot simply be interpreted as the binding of less water to the denatured when compared to the native protein (Aune and Tanford, 1969a,b; Reisler and Eisenberg, 1969) as the protein was added dry. A semiquantitative comparison of both salts made at salt concentrations which are beyond the regions of the major transitions of the proteins (Figures 1 and 2) suggests that the ratios of interaction (extent of binding) of Gdm·Cl and Gdm·SCN with the three proteins are similar when expressed per amino acid (see Table IV). This finding is compatible with sites of interaction on the protein molecule for Gdm·Cl and Gdm·SCN which are independent of amino acid composition and thus supports our previous work with urea (Warren and Gordon, 1970, 1971).

Our experimental methods do not easily allow the experimental data to be separated into the denaturing ability of the cation and the anion and their respective binding affinities. Thiocyanate anion (as the Li^+ or K^+ salt) has been shown to have a preferential affinity for the denatured state (von Hippel and Schleich, 1969; Bull and Breese, 1970a; Cifferri *et al.*, 1967). In this report, the influence of the thiocyanate anion is seen in (1) the finding of only a partially disordered protein in Gdm·SCN as discussed above and (2) in the observed effect of NaSCN addition (Table II) on the ORD parameters. Yet the similarities in denaturing effects of these two guanidinium salts outweigh the dissimilarities; the similarities seem best interpreted in this report on the basis of the common cation (also see Aune and Tanford, 1969a,b).

Experiments containing $(Gdm)_2SO_4$ or Na_2SO_4 in solution with 3 M Gdm·Cl and 4% bovine serum albumin (Table III) were performed to more completely understand the role of denaturant–protein interaction on conformational changes. It is known that certain salts with urea (Burk, 1943; Simpson and Kauzmann, 1953), detergents with salts, urea with detergents (Meyer and Kauzmann, 1962), and so forth lead to additive, nonadditive, or inhibitory effects (Joly, 1965). Studies such as these usually have to be interpreted with some caution since the addition of a second agent can possibly (qualitatively) perturb the equilibrium being studied in the original reaction, which leads to misinterpretations (Tanford, 1968). We can conclude that the inclusion of Na_2SO_4 and $(Gdm)_2SO_4$ leads to an inhibitory effect in solutions containing denaturing concentrations of Gdm·Cl or Gdm·SCN. The inhibition of denaturation is expressed *not* only in the reversal of the optical rotatory parameters of bovine serum albumin toward more native values in these solutions *but* also the addition definitely leads to some loss in observed guanidine interaction when compared to a simple additivity effect (last column in Table III), an observation *necessary* to the premise that the protein interactions of Gdm·SCN and Gdm·Cl can be correlated with change in conformation.

On the Mechanism. The mechanism(s) by which Gdm·Cl, other neutral salts, and urea alter the conformation of proteins is not clear (von Hippel and Schleich, 1969; Tanford, 1970).

The theories which have been proposed to explain protein denaturation by these denaturants can be separated as follows: (1) those mechanisms which require, more or less, interaction of the denaturing agent with groups on the protein; (2) those mechanisms whose effects are generally expressed through some alteration in the bulk solvent; (3) and mechanisms involving features of both. The available evidence and the detailed discussions of theories concerning the effects of a wide variety of salts and urea on macromolecules have been summarized (von Hippel and Schleich, 1969; Jencks, 1969).

Various objections to a mechanism of *protein* denaturation incorporating some sort of ligand interaction would be largely negated if (1) most of the sites of interaction were on the interior of the protein and not normally exposed to the solvent and (2) the sites of interaction would be essentially independent of chemical differences between proteins so that the general effectiveness of a denaturant for many proteins may be explained (von Hippel and Wong, 1965). Other objections, which do not concern us here, involve the generality of a denaturant's effectiveness, such as Gdm·Cl, toward both nucleic acid and protein.

The work of Bello *et al.* (1956) refocused attention on the possible interaction of denaturing salts with peptide groups (of gelatin) as a mechanism to lower the stability of proteins. Other evidence has since accumulated for the interaction of salts with polypeptides and proteins (Kurtz and Harrington, 1966; Bello, 1965; Cifferri *et al.*, 1967), to crystalline amides (Bello *et al.*, 1966), to simple amides (Schrier and Schrier, 1967), to polyacrylamide gel (St. Pierre and Jencks, 1969), and to a tetrapeptide (Robinson and Jencks, 1965a,b) at molar concentrations of salts, sometimes including the guanidinium cation and urea. These studies implicating the peptide and amide groups as sites of interaction have been supported by a study of the effect of salt denaturants on the visible spectrum of a vinylogous amide (Davidson and Jencks, 1969).

Evidence directly concerned with the interaction of Gdm·Cl with proteins in solution is also available. The magnitude of the interaction in our report (about 1 Gdm·Cl:4 amino acid residues) in concentrated Gdm·Cl solutions containing bovine serum albumin reasonably agrees with the values of Noelken and Timasheff (1967) and Woods *et al.* (1963) with myosin, and possibly the work of Bull and Breese (1970b). Partial specific volume studies (Kielley and Harrington, 1960) and isopiestic studies (Hade and Tanford, 1967) have indicated a somewhat smaller interaction of Gdm·Cl.

A comparison of the data of this report at 6 M Gdm·Cl with the other report involving a study of several proteins uncovers a difference. Hade and Tanford (1967) conclude that the extent of binding of Gdm·Cl to bovine serum albumin, lysozyme, and ovalbumin, normalized for molecular weight, is protein *dependent* although the observed differences seem small with the exception of ribonuclease (their Table IV). In contrast, we conclude that the extent of interaction is apparently protein *independent* when so expressed although ribonuclease was not included in this limited study (as it penetrates the dialysis membranes). We can offer no explanation of this apparent discrepancy in results except to say that the inclusion of small quantities of unsuspected salt would have more effect on isopiestic measurements than in our studies.

By means of the experimental method used here, it seems an inescapable conclusion that the number of guanidinium ions *associated* with the protein increases with the observed extent of protein unfolding, thus nullifying one of two objections raised above against any mechanism of protein denaturation which involves some sort of ligand to protein interaction. The second objection is also met, in a limited fashion, by showing that the stoichiometry of maximum interaction is at least relatively independent of protein composition so that the known ability of these denaturants toward many proteins may be eventually explained (Table IV).

Work under way in this laboratory utilizing several *homopolymers* provides prospects for assurance as to the involvement of the peptide unit and/or specific amino acid residues or nonstoichiometric (domain) interactions (Aune and Tanford, 1969a,b; Tanford, 1968) with urea and the three guanidine salts. A detailed discussion as to the detailed mechanism(s) of interaction between protein and guanidine salt will follow the conclusion of these studies.

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Solubility of Amino Acids in Aqueous Guanidinium Thiocyanate Solutions†

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ABSTRACT: The solubilities of amino acids and a model polypeptide, acetyltetraglycine ethyl ester, have been determined in H_2O and 1–3 M aqueous solutions of guanidinium thiocyanate. From these data, free energies of transfer of amino acid side chains and peptide-bond units from water to this potent protein denaturant were calculated. The results demonstrate that aqueous solutions of guanidinium thiocyanate are more effective than corresponding concentrations of urea, guanidinium chloride, ethanol, or dioxane in decreasing the free energies of transfer of hydrophobic amino acid side chains and the peptide bond from water to these solvents. The results account for the previously demonstrated fact that guanidinium thiocyanate is approximately twice as effective a protein denaturant as is guanidinium chloride.

We have previously shown that the denaturing effectiveness of salts of guanidinium, carbamoylguanidinium, and guanylguanidinium cations toward several proteins increases according to the Hofmeister anion series: $Cl^- < Br^- < I^- < CNS^-$ (Castellino and Barker, 1968). Further, when the anion is held constant, the denaturing effectiveness of the cation increases according to the series: guanidinium < carbamoylguanidinium < guanylguanidinium (Castellino and Barker, 1968). In another article, we have demonstrated that this same anion and cation series occurs in increasing the solubility of a model hydrophobic compound, benzoyl-L-tyrosine ethyl ester, and a model peptide compound, acetyltetraglycine ethyl ester (Castellino and Barker, 1969). In many previous studies it has been shown that guanidinium chloride is a more effective denaturant than compounds such as urea, dioxane, ethylene glycol, etc., and this difference in denaturing effectiveness has been explained in terms of the free energies of transfer, calculated from solubility measurements, of constituent parts of the protein from water to these solvents (Nozaki and Tanford, 1963, 1970, 1971; Tanford, 1968, 1969). Since protein denaturation is accompanied by a transfer of the side chains which exist in the interior of the native protein molecule, *i.e.*, hydrophobic residues and peptide bonds, to the denaturing solvent, it would be expected that solvents which interact more favorably with these newly exposed residues would promote denaturation more readily. This has been shown to be the case in a semiquantitative sense with the above model compounds.

The purpose of this manuscript is to describe studies on the solubility of various amino acids and a model for the peptide bond in aqueous GdmCNS[1] solutions in order to quantitatively account for the increased denaturing potency of this

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[1] Abbreviations used are: GdmCNS, guanidinium thiocyanate; GdmCl, guanidinium chloride.