Unfolding of *S*-Cysteinyl Bovine Serum Albumin by Aqueous Lithium Salts*

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ABSTRACT: A study was made of the unfolding of *S*-cysteinyl bovine serum albumin in aqueous solutions of the following salts: guanidine hydrochloride, LiCl, LiBr, LiI, LiSCN, $LiClO_4$, and $LiNO_3$, by means of optical rotatory dispersion and viscosity measurements. The optical rotatory dispersion results with the aqueous lithium salts were complicated by an anomalous solvent effect that caused a decrease in the levorotation. A similar effect was observed in a parallel study on β-casein B which: (1) had a highly disordered conformation; and (2) did not appear to undergo a significant conformation change. In spite of the anomaly, the results indicate that the product(s) of denaturation by lithium salts is more organized than that obtained with 6 M guanidine hydrochloride. The intrinsic viscosity of the linear macromolecule *S*-carboxymethyl bovine serum albumin was measured in concentrated solutions of guanidine hydrochloride, LiBr, LiI, and LiSCN. The values found with the lithium salts were substantially lower than that obtained in 6 M guanidine hydrochloride, a solvent in which the protein derivative is apparently a random coil. The viscosity results, too, indicate that the product of lithium salt denaturation is more highly organized than that obtained with guanidine hydrochloride.

Many neutral salts, when added to an aqueous protein solution, will cause the protein to undergo a change in size and shape. Tanford (1968) has recently reviewed the characterization of the products of denaturation by salts as well as other denaturants. Von Hippel and Wong (1964) and Von Hippel and Schleich (1969) have dealt with the mechanism of unfolding. This study is concerned with the characterization of the denatured state of *S*-cysteinyl-BSA[1] in concentrated aqueous solutions of lithium salts. The role of denaturant binding is the subject of another study (Noelken, 1970).

Optical rotatory dispersion and viscosity measurements were the principal methods used in this study. One of the difficulties encountered in the interpretation of optical rotation data when lithium salts are used is a nonspecific solvent effect that leads to a reduction of the magnitude of the rotation. Bigelow and Geschwind (1961) found, for example, that the levorotation of oxidized ribonuclease, a highly disorganized protein, diminished with increasing concentration of several salts, apparently in the absence of an order–disorder transition. In the present study similar results were obtained with β-casein B, another disorganized protein (Herskovits, 1966; Noelken and Reibstein, 1968). The significance of this effect is discussed.

Viscosity measurements were made on the linear polymer *S*-carboxymethyl-BSA in concentrated guanidine hydrochloride, LiBr, LiI, and LiSCN to permit comparison to the results of Tanford *et al.* (1967). They found that protein in 6 M Gd·HCl could be approximated by random coils, provided that they contained no disulfide cross-links. Their results thus provided a basis for determining whether or not a denatured protein resembles a random coil.

Experimental Section

Proteins. BSA was purchased from Nutritional Biochemicals (lots 8176 and 9385). The free SH group (lot 8176) was blocked by reaction with cystine (King, 1961) to prevent disulfide–SH interchange when the protein was unfolded. The total lipid content was determined by the cold extraction method of Entenman (1957); a value of 0.8% by weight was found.

For the viscosity studies BSA (lot 9385) was reduced and alkylated with iodoacetic acid in order to form the linear polymer *S*-carboxymethyl-BSA. BSA (10 g) was dissolved in 1 l. of 4 M Gd·HCl–0.1 M 2-mercaptoethanol–0.001 M Na-EDTA (pH 8.0, 25°). After about 18 hr 0.25 mole of sodium iodoacetate in 50 ml of water (pH 8.0) was added. The pH was maintained at pH 8.0 for 1 hr. The solution was then dialyzed exhaustively against cold water and subsequently lyophilized.

The β-casein B was very kindly supplied by Dr. Marvin P. Thompson, Eastern Regional Research Laboratory, U. S. Department of Agriculture, Philadelphia, Pa.

Protein Concentration. For the optical rotatory dispersion measurements, a stock *S*-cysteinyl-BSA solution was prepared by dissolving the protein in water and passing the solution through an Amberlite MB-1 ion-exchange resin (Mallinckrodt). The concentration was determined by dry weight measurements at 107–109°.

The concentration of *S*-carboxymethyl-BSA was determined spectrophotometrically using the value 5.96 for $A^{1\%}_{1cm}$ at 278 mμ measured in 6 M Gd·HCl–0.02 M NaEDTA (pH 8). Corrections were made for light scattering using the method described by Reddi (1957); the amount of scattering was determined from the absorbance at 330 mμ. It was found that this value could be used even if the lithium salts were present at a concentration of 0.4 M. Accordingly concentra-

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[1] Abbreviations used that are not listed in *Biochemistry 5*, 1445 (1966), are; BSA, bovine serum albumin; Gd·HCl, guanidine hydrochloride.

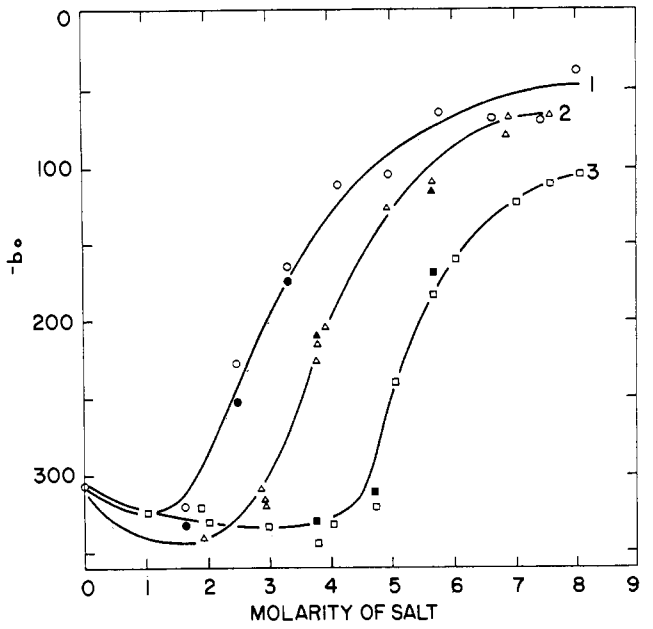

FIGURE 1: The value of the optical rotation parameter b_0 for *S*-cysteinyl-BSA as a function of denaturant concentration. Temperature = 25°. Curve 1: LiI (0.01 M sodium thiosulfate (pH 6.2–6.6); curve 2: LiBr (pH 5.2–5.5); and curve 3: LiCl (pH 5.2–5.5). Unfilled symbols: forward course of transition; filled symbols: reverse points (see Results). The solvent used for zero molar denaturants is 0.14 M KCl (pH 5.3).

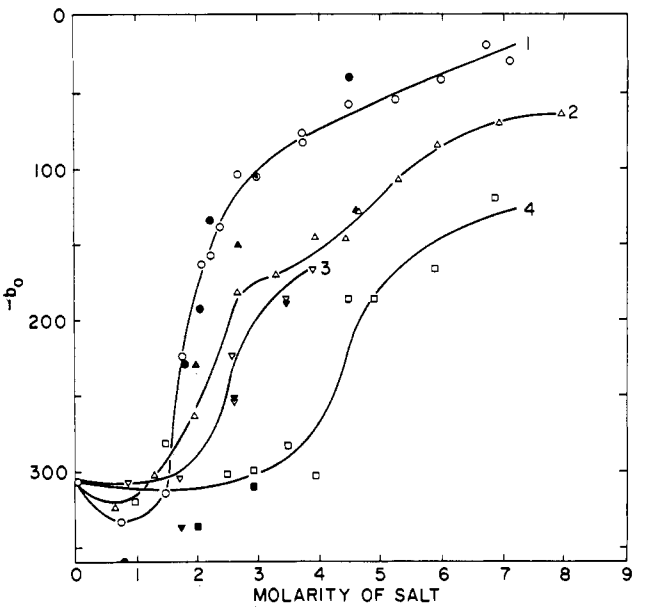

FIGURE 2: The value of the optical rotation parameter b_0 for *S*-cysteinyl-BSA as a function of denaturant concentration. Temperature = 25°, pH 5.2–5.5. Curve 1: Gd·HCl; curve 2: LiSCN; curve 3: $LiClO_4$; and curve 4: $LiNO_3$. Unfilled symbols: forward course of transition; filled symbols: reverse points. The solvent used for zero molar denaturant is 0.14 M KCl (pH 5.3).

tions of *S*-carboxymethyl-BSA in aqueous lithium salts were determined after dilution with concentrated Gd·HCl.

A stock solution of β-casein B in 0.02 M NaEDTA (pH 7.0) was used in the optical rotatory dispersion studies. Its concentration was determined spectrophotometrically using the value 4.7 for $A^{1\%}_{1cm}$ at 280 mμ (Thompson and Pepper, 1964).

Reagents. Gd·HCl was prepared from the carbonate (Eastman Organic Chemicals, and Matheson, Coleman & Bell) by the method of Anson (1941). The other reagents were of the highest quality that could be obtained commercially.

Concentration of Lithium Salts. The concentration of a stock salt solution was determined by a density measurement with the use of density *vs.* composition data. For this purpose least-squares-based polynomial equations were obtained, by use of a computer, that related the density to the weight fraction. The data for aqueous $LiClO_4$ were obtained from Geffcken (1929); for LiSCN in this laboratory (M. Noelken, 1970, unpublished data); and for the other lithium salts from the tables compiled by Beattie and Gillespie (1928). The molarity of a stock salt solution could be determined to within 0.04 M by this method.

Density. Densities were measured at 25.0 ± 0.01° with either a 10- or 20-ml Lipkin-type pycnometer; the estimated error was 2×10^{-4} g/ml.

Optical Rotatory Dispersion. The data were obtained with a Jasco optical rotatory dispersion recorder Model UV-5. The measurements were generally made over the wavelength range 300–450 mμ in a thermostatted fused silica cell of 1-cm path length. The kinetics of unfolding was studied in each case to be sure that a final state had been reached.

Viscosity. Viscosity measurements were made at 25.0 ± 0.01° with Cannon-Ubbelohde viscometers (size 75) that had flow times for water of about 100 sec. Flow times for protein solutions generally ranged from 15 to 130 sec over solvent (diffusate) and were reproducible to at least ±0.15 sec.

Results

Optical Rotatory Dispersion. The optical rotation data were analyzed by use of the Moffit–Yang equation (1956). The value 107 for the mean residue weight of *S*-cysteinyl-BSA was assumed to be the same as for BSA (Spahr and Edsall, 1964). A higher value, 109, was used for *S*-carboxymethyl-BSA to account for the added carboxymethyl groups The value 119 was used for β-casein B (Kalan *et al.*, 1965). The value of $(3/n^2 + 2)$ at 436 mμ was used in the calculations involving a particular solvent. A value of 212 mμ was used for λ_0.

The results for *S*-cysteinyl-BSA in various denaturing solutions are presented in Figures 1 and 2. In 0.14 M KCl (pH 5.3, 25°), the values of $-a_0$ and $-b_0$ were 290 and 305, respectively. These results are in good agreement with those reported for native BSA under similar conditions (Leonard and Foster, 1961; Herskovits and Mescanti, 1965). The sharp decrease in the magnitude of b_0 at intermediate salt concentrations is indicative of a transition to a disorganized, unfolded form. The reversibility of the transition was determined after dilution of samples that had been incubated for at least 24 hr, to obtain a lower salt concentration. The reverse points are represented by filled symbols in Figures 1 and 2. As can be seen in these figures, the unfolding caused by the neutral salts is largely reversible.

The optical rotatory dispersion of *S*-carboxymethyl-BSA was studied at LiSCN concentrations of 4.8, 5.8, 6.8, and 7.7 M. At concentrations below 4.8 M, the solutions were too

TABLE I: Dependence of $[m']_\lambda$ on Salt Concentration for Disorganized Proteins.

Salt	Protein	Percentage Decrease in $[m']_\lambda$ 300 mμ	320 mμ	360 mμ	589 mμ
LiCl	β-Casein B	4.1	4.1	4.3	
LiBr	β-Casein B	4.8	5.0	5.1	
LiBr	Ribonuclease				4.2[a]
LiBr	Ox RNase[b]				4.8[c]
LiBr	Ox RNase				6.4[a]
NaBr	Ox RNase				6.8[a]
LiI	β-Casein B	7.8	8.0	8.1	
KI, NaI	Ox RNase				8.8
Gd·HCl	β-Casein B	1.1	1.2	1.1	
$LiNO_3$	β-Casein B			3.9	
LiSCN	β-Casein B	5.2	5.3	5.5	
LiSCN	Poly-DL-alanine				6.3[d]
LiSCN	S-Carboxymethyl-BSA	2.5	2.3	2.9	2.6 (436 mμ)

[a] From the results of Bigelow and Geschwind (1961). [b] Periodate-oxidized ribonuclease. [c] From the results of Harrington and Schellman (1957). [d] From the results of Downie *et al.* (1957).

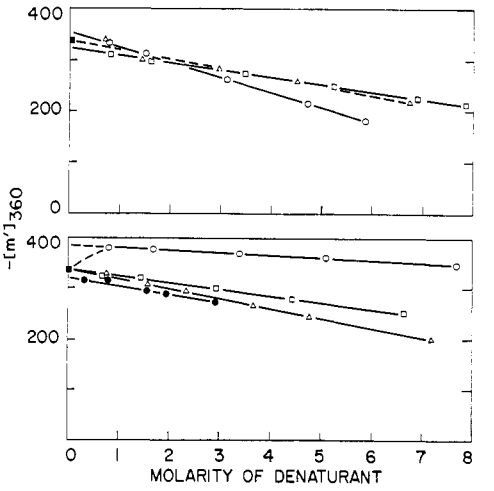

FIGURE 3: The value of the reduced mean residue rotation at 360 mμ of β-casein B in various salt solutions. Temperature = 25°; the solutions, except for LiI and Gd·HCl, contained 0.02 M sodium EDTA. Upper: (■) 0.02 M NaEDTA (pH 7.0), (□) LiCl (pH 6.8), (△) LiBr (pH 7.0), and (○) LiI–0.01 M $Na_2S_2O_3$ (pH 7.2); lower: (○) Gd·HCl (pH 7.4–7.9), (□) $LiNO_3$ (pH 7.0), (△) LiSCN (pH 7.0), and (●) $LiClO_4$ (pH 7.0).

turbid to make accurate measurements. The value of $-b_0$ was found to be essentially independent of the LiSCN molarity with an average value of 74 ± 4. The magnitude of the reduced mean residue rotation $[m']_\lambda$ was found to decrease with increasing LiSCN concentration (Table I).

The effects of salts on the optical rotation of β-casein B are shown in Figure 3. For a given salt there was no significant variation in the value of b_0 with salt concentration, although small differences were noted between the salts; the values were close to zero. In aqueous Gd·HCl the value of $-b_0$ was −15 ± 15, while in the aqueous lithium salts, except the nitrate, it was 30 to 40.[2] A value of −20 ± 20 was observed in aqueous $LiNO_3$.

In contrast to the lack of dependence of the value of b_0 on salt concentration, the value of $-[m']_\lambda$ showed a significant linear decrease. The results at 360 mμ are shown in Figure 3; similar results were obtained at 300 and 320 mμ. This effect has been observed previously. Bigelow and Geschwind (1961) found that the specific rotation (levo) of periodate-oxidized ribonuclease decreased linearly with increasing concentration of NaI, KI, LiBr, or NaBr. In another study Downie *et al.* (1957) found that the value of $[m']_D^{20}$ for a series of randomly coiled copolymers of D- and L-alanine of varying D:L ratio was about 66% lower in saturated LiSCN (10.4 M; M. Noelken, unpublished result) than it was in water.

The results of several optical rotation studies are compared in Table I. The percentage decrease in $[m']_\lambda$ per unit increase in the molarity of salt is given for several proteins in different solvents. This quantity is obtained by dividing the change in $[m']_\lambda$ per molar increase in salt concentration by the value of $[m']_\lambda$ obtained by extrapolation to zero salt concentration, and multiplying by 100.

Viscosity. The viscosity data for S-carboxymethyl-BSA were fitted by the method of least squares to the relation: $\eta_{sp}/c = [\eta] + k[\eta]^2c$, where η_{sp} is the reduced viscosity in milliliters per gram, c is the protein concentration in grams per milliliter, and k is the dimensionless Huggins constant (Huggins, 1942). Values of $[\eta]$ and k are listed in Table II.

TABLE II: Values of the Intrinsic Viscosity and the Huggins Constant for S-Carboxymethyl-BSA in Several Solvents.

Solvent[a] (M)	$[\eta]$	k
Gd·HCl (6)	57.0	0.39
LiSCN (4.9)	29.6	1.04
LiSCN (5.9)	32.3	0.56
LiSCN (6.0)	32.7	0.56
LiSCN (6.8)	32.0	0.58
LiI[b] (6.2)	24.3	1.41
LiI[b] (8.4)	23.5	1.55
LiBr (7.0)	22.0	3.89
LiBr (7.6)	19.0	2.27

[a] The apparent pH was adjusted to 7.6–8.0, except for 4.9 and 6.0 M LiSCN which were at pH 6.1. [b] The solution was 0.01 M in $Na_2S_2O_3$.

[2] These results are similar to those of Herskovits (1966) for β-casein A, a mixture of several variants (Peterson and Kopfler, 1966). The value of $-b_0$ in 8 M urea was the same as that obtained in this study for variant B in concentrated Gd·HCl (−15). Low positive values were found in dilute salt in both cases. Herskovits has suggested that this difference may be due to a small change in α-helix content as well as to aggregation in dilute salt at 25°.

TABLE III: Values of $-b_0$ and $[m']_{360}$ for *S*-Cysteinyl-BSA.

Solvent (M)	% Decrease[a] in $[m']_{360}$	$[m']_{360}$ Obsd	$[m']_{360}$ Cor[b]	$-b_0$
Gd·HCl (7)	8	310	340	25
LiI (8)	64	140	390	50
LiSCN (8)	43	180	310	65
LiBr (7.5)	44	175	320	65
$LiNO_3$ (7)	28	250	340	120
LiCl (8)	34	195	300	105
KCl (0.14)		240	240	305

[a] From Table II. [b] Approximate; see Discussion.

The protein derivative was not sufficiently soluble in concentrated $LiClO_4$ (3.5 M), $LiNO_3$ (6.4 M), or LiCl (5–9.5 M) to make viscosity measurements.

The value of $[\eta]$ in 6 M Gd·HCl was 57 ml/g and that of k was 0.39. These values are in reasonable agreement with the corresponding values of 52 ml/g and 0.29 found by Tanford *et al.* (1967) for BSA in 6 M Gd·HCl with 2-mercaptoethanol present as a reducing agent.

Discussion

The isothermal denaturation curves shown in Figures 1 and 2 indicate that *S*-cysteinyl-BSA is reversibly unfolded by Gd·HCl and the lithium salts used in this study. The order of decreasing effectiveness as a denaturant is Gd·HCl > LiI, $LiClO_4$, LiSCN, > LiBr > $LiNO_3$ > LiCl. With the exception of the reversal of positions of LiBr and $LiNO_3$ this order is the same as that anticipated from the results of Von Hippel and Wong (1964).

Plots of $[m']_\lambda$ *vs.* denaturant concentration showed transition regions in the same salt concentration ranges as in Figures 1 and 2. Superimposed on these transitions, however, were anomalous decreases in the value of $-[m']_\lambda$ with increasing salt concentration. This effect was so serious at high salt concentrations (Table III) that the value of $-[m']_{360}$ was generally lower than that of the native protein rather than higher as would be consistent with unfolding. Bigelow and Geschwind (1961) found with oxidized ribonuclease that this is a solvent effect unaccompanied by a conformation change (see also Tanford, 1968, for a discussion of their results). The effect on the optical rotation of β-casein B is likewise due primarily to a general solvent effect rather than a conformation change. The protein is disorganized even in the absence of a denaturant (Herskovits, 1966; Noelken and Reibstein, 1968) and there is no evidence for an order–disorder transition in concentrated salt solutions (Figure 3). The magnitude of the solvent effect is about the same for several unfolded proteins[3] and one randomly coiled polypeptide with a given salt although it does depend on the type of salt (Table I). These results can be used to make an approximate correction for the effect on the value of $[m']_{360}$ of *S*-cysteinyl-BSA.[4] For this purpose, the results for a given salt were averaged. The corrected values of $[m']_{360}$ for *S*-cysteinyl-BSA in concentrated lithium salts are about the same as that obtained in 7 M Gd·HCl (Table III) and, as do the corresponding values of $-b_0$, indicate extensive unfolding. These corrected values are probably somewhat high, especially in aqueous $LiNO_3$ and LiCl, because the effect on the folded form of a protein is greater than that on the unfolded form (Bigelow and Geschwind, 1961) and the values of $-b_0$ indicate that denatured *S*-cysteinyl-BSA in solvents other than concentrated Gd·HCl has residual folded portions.

The viscosity experiments were performed with *S*-carboxymethyl-BSA rather than *S*-cysteinyl-BSA in part because cleavage of the disulfide bonds would result in high viscosities for the unfolded protein thus making the technique more sensitive. The secondary structure of the *S*-carboxymethyl derivative is apparently less stable than that of *S*-cysteinyl-BSA at intermediate concentrations of LiSCN but is equally stable at higher concentrations. The value of $-b_0$ at 5 M LiSCN was 76 for *S*-carboxymethyl-BSA compared to 120 for *S*-cysteinyl-BSA. In the range 6–8 M LiSCN, the results for the two were very similar; the values of $-b_0$ were in the range 65–85. Thus, the secondary structure is partially stabilized by disulfide bonds. This conclusion is consistent with the results of Harrap and Woods (1965) who found that $-b_0$ for *S*-carboxymethyl-BSA in dilute salt at pH 7 had a value of 90, compared to the value of about 300 characteristic of the native protein.

The value of the intrinsic viscosity and that of the Huggins constant for *S*-carboxymethyl-BSA in 6 M Gd·HCl (Table II) is close to that found by Tanford *et al.* (1967) for reduced BSA in 6 M Gd·HCl. Since Tanford and coworkers found that reduced BSA and other proteins without disulfide bonds can be approximated by random coils in 6 M Gd·HCl, this must also be true for *S*-carboxymethyl-BSA. The values of the intrinsic viscosity in concentrated LiI and LiBr substantiate the conclusion from the optical rotation results for *S*-cysteinyl-BSA that the denaturation product is more highly organized that that obtained in concentrated Gd·HCl. Also, the values of k are above the value 0.35 found for flexible polymers (Huggins, 1942) and the range 0.16–0.95 found for randomly coiled proteins (Tanford *et al.*, 1967). The values of k found in aqueous LiBr are even higher than the maximum value for k of 2.0 for polymers under conditions, such as those used in this study, where charge effects are negligible (Tanford, 1961). This could be due to aggregation, especially in 7 M LiBr, although further evidence would be needed to prove this.

The viscosity results in 4.9–6.0 M LiSCN are consistent with, but do not necessarily support the conclusions drawn from the optical rotation studies. The constancy of $[\eta]$ and k above 4.9 M LiSCN suggests that the maximum possible amount of unfolding has been achieved at a concentration between 4.9 and 5.9 M. The value of k of about 0.6 in 5.9–6.8

[3] The value for *S*-carboxymethyl-BSA in aqueous LiSCN disagrees with those for β-casein B and poly-DL-alanine. This is possibly a result of the more limited LiSCN concentration range used in the former case.

[4] Corrections could not be obtained from the results for *S*-cysteinyl-BSA because the transitions were not complete at sufficiently low salt concentrations.

M LiSCN is consistent with a random coil, but does not exclude other structures. The low value of $[\eta]$ relative to that in 6 M Gd·HCl is consistent with a more ordered, more compact structure than a random coil. An alternative, however, is that solute–solvent interactions are less favored in concentrated LiSCN than they are in 6 M Gd·HCl. The effect of such interactions is to increase the size of the molecular domain and consequently cause an increase in the intrinsic viscosity. Work is in progress to characterize this denaturation product more exactly.

The results of other studies indicate that proteins are incompletely unfolded in concentrated solutions of lithium salts. The results of Hamaguchi *et al.* (1963) for lysozyme, obtained from viscosity measurements and difference spectroscopy, indicate that neither concentrated LiCl or LiBr yields a denaturation product as disorganized as that obtained with Gd·HCl. Sarfare and Bigelow (1967) have found that not all of the tyrosyl groups of unfolded ribonuclease are exposed to the solvent in 10 M LiBr, thus indicating incomplete unfolding.

The difference in the denaturation products formed in aqueous solutions of the lithium salts used in this study and that in aqueous Gd·HCl is perhaps due to differences in the effects on nonpolar residues. Nonpolar compounds are salted in by Gd·HCl (Wetlaufer *et al.*, 1964; Robinson and Jencks, 1965a) while salting out is expected in the aqueous lithium salts (Schrier and Schrier, 1967). All of these denaturants are expected to salt in amide groups according to the results of Robinson and Jencks (1965b) and Schrier and Schrier (1967). A more disorganized denaturation product would be expected with aqueous Gd·HCl than with solvents that would enhance, or at least fail to disrupt, hydrophobic clusters.

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